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RESEARCH ARTICLE

Apelin-13 as a novel target for intervention in secondary injury after traumatic brain injury

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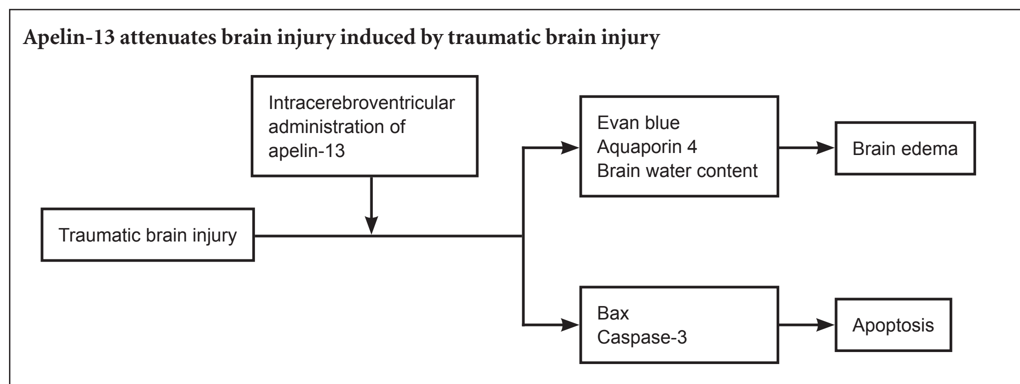
Graphical Abstract



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Abstract

The adipocytokine, apelin-13, is an abundantly expressed peptide in the nervous system. Apelin-13 protects the brain against ischemia/reperfusion injury and attenuates traumatic brain injury by suppressing autophagy. However, secondary apelin-13 effects on traumatic brain injury-induced neural cell death and blood-brain barrier integrity are still not clear. Here, we found that apelin-13 significantly decreases cerebral water content, mitigates blood-brain barrier destruction, reduces aquaporin-4 expression, diminishes caspase-3 and Bax expression in the cerebral cortex and hippocampus, and reduces apoptosis. These results show that apelin-13 attenuates secondary injury after traumatic brain injury and exerts a neuroprotective effect.

Key Words: *nerve regeneration; apoptosis; apelin-13; traumatic brain injury; brain edema; blood-brain barrier; brain water content; aquaporin-4; caspase-3; neural regeneration*

Introduction

Survival of traumatic brain injury (TBI) patients from the primary injury (*e.g.*, development of brain edema or delayed axonal or neuronal degeneration) is mainly dependent on secondary insults (Plesnila et al., 2007). Brain edema is still one of the main causes of death in TBI (Unterberg et al., 2004; Khan et al., 2009). Edema is harmful because it changes metabolite concentration and alters cellular physiology, biochemistry, and function (Unterberg et al., 2004; Khan et al., 2009). Previous research, including our own, has shown that both brain edema and apoptosis participate in neuronal cell death and functional loss after TBI (Liu et al., 2008; Luo et al., 2010; Bao et al., 2012; Zhang et al., 2014).

The peptide, apelin, is an endogenous ligand for the apelin receptor (also known as the APJ receptor). Apelin was first isolated from bovine stomach (Tatemoto et al., 1998). The APJ receptor was first discovered by O'Dowd et al. (1993), and is analogous to the angiotensin-I receptor. There are three biologically active forms of apelin, which are composed of 13, 17, or 36 amino acids. All the peptides are derived from a 77-amino acid prepropeptide precursor (O'Carroll et al., 2013), although apelin-13 has much greater biological potency than apelin-36 (Tatemoto et al., 1998). Accordingly, apelin-13 is regarded as an important neuroprotective reagent in the nervous system (Khaksari et al., 2012; Jiao et al., 2013).

The central nervous system distribution pattern of apelinergic neurons suggests diverse roles of apelin, for example in

circadian rhythms, controlling feeding behavior, body fluid homeostasis, and pituitary hormone release (Hosoya et al., 2000). Additionally, apelin and the APJ receptor are broadly expressed in neurons and oligodendrocytes, but less so in astrocytes (Choe et al., 2000). To the best of our knowledge, no experimental studies have addressed the role of apelin-13 on cerebral water content and neural cell death due to apoptosis after TBI. Thus, based on the above-mentioned findings, we investigated the hypothesis that apelin-13 may ameliorate brain edema and reduce cellular death in mice after TBI.

Materials and Methods

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of Xuzhou Medical College, China, and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

Experimental animals

In total, 108 adult specific-pathogen-free male CD1 mice weighing 20–25 g and aged 8 weeks were purchased from the Experimental Animal Center of Xuzhou Medical College of China (lisence No. SCXK (Su) 2010-0003). Mice were equally and randomly assigned to sham, TBI (TBI only), and apelin-13 (TBI + apelin-13) groups.

Establishment of the TBI model

The procedure for performing TBI in mice has been previously described (Bao et al., 2012). Briefly, mice were anesthetized with 0.4 mg/g chloral hydrate (4%). Then to induce TBI, a 40 g weight was dropped from 20 cm onto a 2-mm-diameter footplate on the left part of the brain, using a controlled depth of 1.0 mm. Mice in the sham group received craniotomy only, without impact injury. Injured cortical (including the impact site and surrounding area; 2 × 2 × 2 mm^3 tissue block) and hippocampal (including the impact site and surrounding area of the entire ipsilateral hippocampus) tissue was dissected for use in several assays.

Apelin-13 administration

Apelin-13 (sc-351718; Santa Cruz Biotechnology, Santa Cruz, Northern CA, USA) was dissolved in normal saline. Apelin-13 was administered ipsilaterally intracerebroventricularly (1 mg/mL; 5 μL) immediately after TBI in the apelin-13 group. The sham group did not receive apelin-13 or normal saline, while the TBI group received vehicle (saline, 5 μL) by intracerebroventricular injection.

Measurement of cerebral water content

Adult male CD1 mice were anesthetized with 4% chloral hydrate (0.4 mg/g), and sacrificed at 24 or 48 hours after apelin-13 injection. The brain was removed and placed in a glass petri-dish. Cerebellar tissue was removed, and the left and right hemispheres divided along the anatomic midline. The wet weight of each hemisphere was weighed. The tissue was completely dried in an oven at 100°C for 5 days, and the dry weight of each hemisphere measured. Brain water content (% water) was calculated using the Elliott formula: brain water content = (wet weight – dry weight) / wet weight × 100% (Xi et al., 2006; Bierbach et al., 2008; Bao et al., 2012).

Evaluation of blood-brain barrier permeability

Evans Blue (4 mL/kg, 2% solution; Sigma-Aldrich, St. Louis, MO, USA) in saline was injected *via* the tail vein (6 mice in each group). The dye was allowed to circulate for 2 hours. Mice were then treated with 50 mL ice-cold phosphate buffered saline (PBS) by transcardiac perfusion. The brain was separated into right and left hemispheres and stored at –80°C. Samples were homogenized in 1.1 mL PBS, sonicated, and centrifuged for 30 minutes at 15,000 r/min, 4°C. Supernatants were collected in aliquots, and trichloroacetic acid (50%) added to 500 μL aliquots. The mixture was incubated at 4°C overnight, and then centrifuged for 30 minutes at 15,000 r/min, 4°C. Evans blue concentration was determined at 620 nm using a spectrophotometer (Thermo Spectronic Genesys 10 UV, Thermo Fisher Scientific Inc., Waltham, MA, USA). Data were quantified from a standard curve. The results are presented as: Evans Blue stain (μg)/tissue (g).

Terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay

Brain cell apoptosis was detected using a TUNEL assay kit, specifically, *In Situ* Cell Death Detection Kit (Roche, Basle, Switzerland; cat. no. 116848179). First, one of eight 12-μm serial sections was collected from the injured cortex of each animal (Bao et al., 2012). Next, paraffin sections were deparaffinized in xylene and rehydrated through a graded ethanol series. After washing with PBS, sections were digested in 20 mg/mL protease at 37°C for 15 minutes, and then washed again with PBS. Prepared sections were incubated in TUNEL reaction mixture at 37°C for 1 hour. After washing in PBS, sections were labeled with converter-POD for 30 minutes at 37°C, and stained with 3,3′-diaminobenzidine. TUNEL-positive cells were counted under a light microscope (BX53; Olympus, Tokyo, Japan) (Bao et al., 2012).

Western blot assay

Injured cortical and hippocampal samples were homogenized using western blot analysis buffer that included 10 mM Tris-HCl (pH 7.4), Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1 mM phenylmethyl sulfonylfluoride, 5 mM ethylenediamine tetraacetic acid, 0.28 kU/L aprotinin, 1 mM benzamidine, 50 mg/L leupeptin, and 7 mg/L pepstain A (all chemicals were from Sigma-Aldrich). Homogenates were centrifuged for 10 minutes at 12,000 r/min, 4°C. Supernatants were stored at –80°C for later use. Protein concentration was determined using a bicinchoninic acid kit (Pierce, Appleton, WI, USA). Protein (30 mg) extracted from each sample underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis using

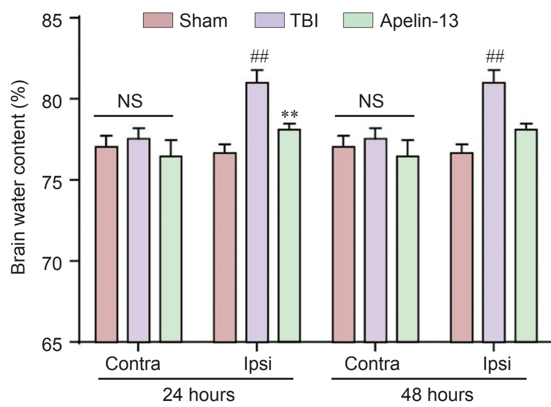

Figure 1 Apelin-13 ameliorates TBI-induced brain edema.
Brain water content in the ipsilateral (injured) and contralateral hemisphere were measured at 24 and 48 hours after TBI. Data are expressed as the mean ± SEM, and were analyzed by one-way analysis of variance followed by Dunnett's *t*-test (6 mice in each group). $**P < 0.01$, *vs.* TBI group; $##P < 0.01$, *vs.* sham group. Contra: Contralateral; Ipsi: ipsilateral; NS: not significant; TBI: traumatic brain injury.

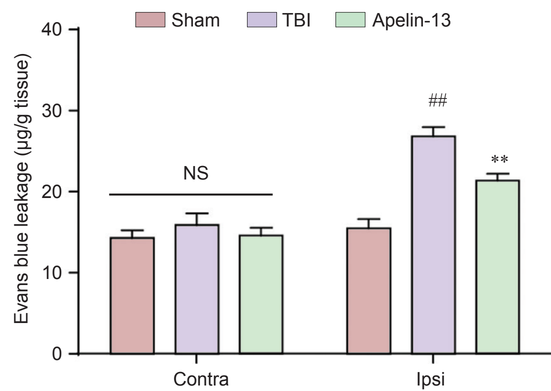

Figure 2 Reduction of blood-brain barrier permeability is affected by apelin-13 at 48 hours after TBI.
Data are expressed as the mean ± SEM, and were analyzed by one-way analysis of variance followed by Dunnett's *t*-test (6 mice in each group). $**P < 0.01$, *vs.* TBI group; $##P < 0.01$, *vs.* sham group. Contra: Contralateral; Ipsi: ipsilateral; NS: not significant; TBI: traumatic brain injury.

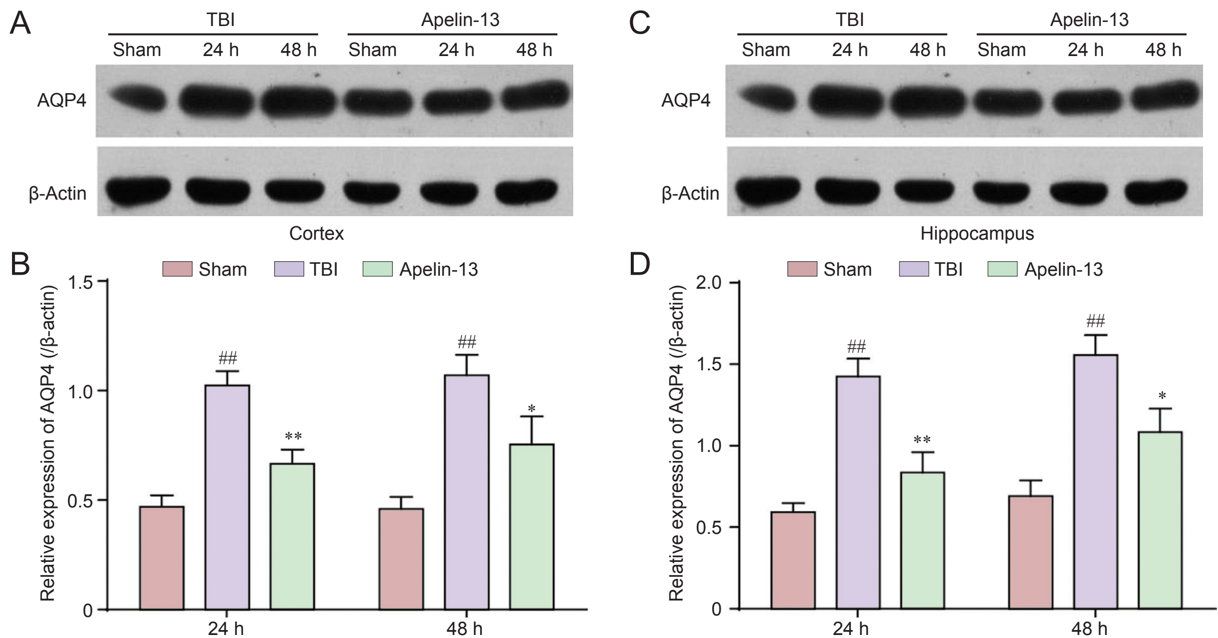

Figure 3 Apelin-13 acutely reduces AQP4 protein expression in the cortex and hippocampus at 24 and 48 hours post-TBI.
(A, C) Representative western blots of AQP4 protein the cortex (A) and hippocampus (C) were detected by western blot assay. (B, D) Quantitative analysis of AQP4 protein expression in the cortex (B) and hippocampus (D). Optical density of the respective protein bands were analyzed by Quantity One (Bio-Rad) and normalized to β-actin. Data are expressed as the mean ± SEM (6 mice in each group). Statistical comparisons were performed by analysis of variance followed by Dunnett's *t*-test. $*P < 0.05$, $**P < 0.01$, *vs.* TBI group; $##P < 0.01$, *vs.* sham group. AQP4: Aquaporin-4; TBI: traumatic brain injury; h: hours.

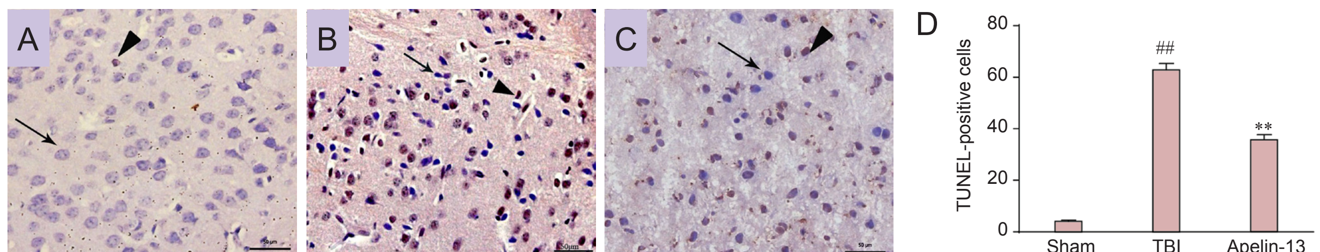

Figure 4 Apelin-13 reduces TBI-induced cell apoptosis in the brain 48 hours after TBI.
Arrows show normal cells and arrowheads point to TUNEL-positive cells in the sham (A), TBI (B), and apelin-13 (C) groups . Scale bars: 50 μm. (D) Quantification of TUNEL-positive cells in the injured cortex (in 400-fold fields). Data are presented as the mean ± SEM (6 mice in each group), and were analyzed by the rank-sum test. $**P < 0.01$, *vs.* TBI group; $##P < 0.01$, *vs.* sham group. TBI: Traumatic brain injury; TUNEL: terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling.

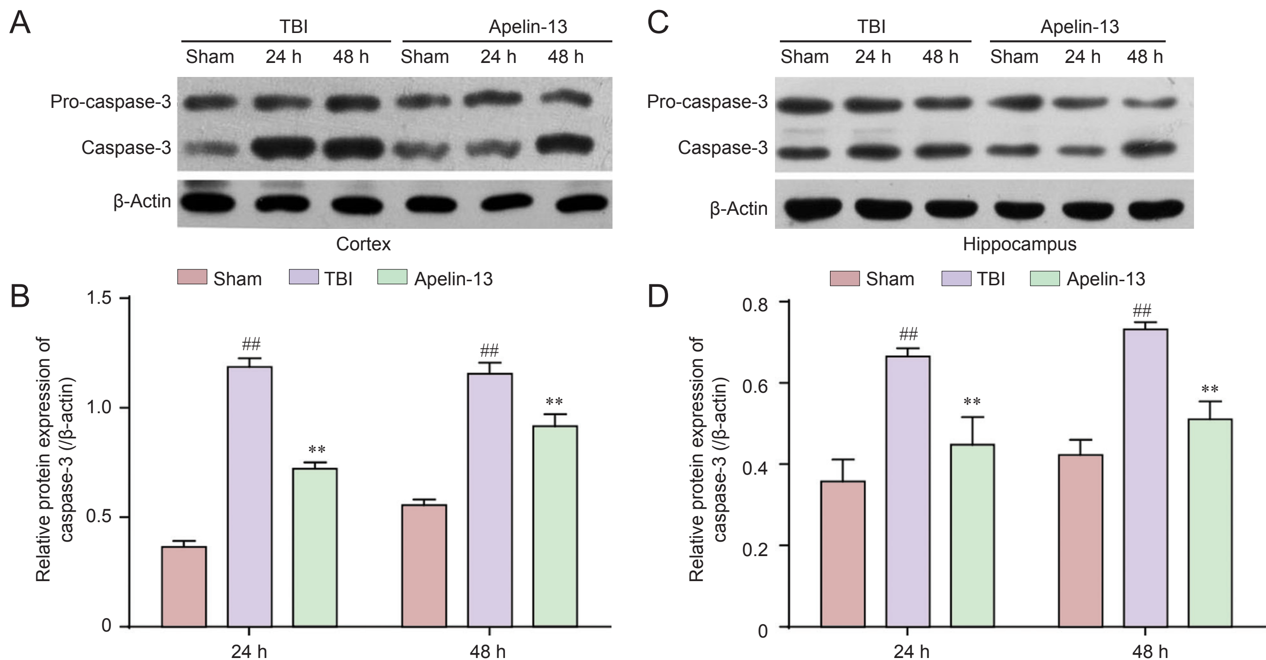

Figure 5 Inhibition of TBI-induced caspase-3 expression in the cortex and hippocampus by apelin-13 at 24 and 48 hours post TBI.
(A, C) Representative western blots of caspase-3 protein in the cortex (A) and hippocampus (C) were detected by western blot assay. (B, D) Quantitative analysis of caspase-3 protein expresssion in the cortex (B) and hippocampus (D). Data are expressed as the mean ± SEM (6 mice in each group). Statistical comparisons were performed by analysis of variance followed by Dunnett's *t*-test. ***P* < 0.01, *vs*. TBI group; ##*P* < 0.01, *vs*. sham group. TBI: Traumatic brain injury; h: hours.

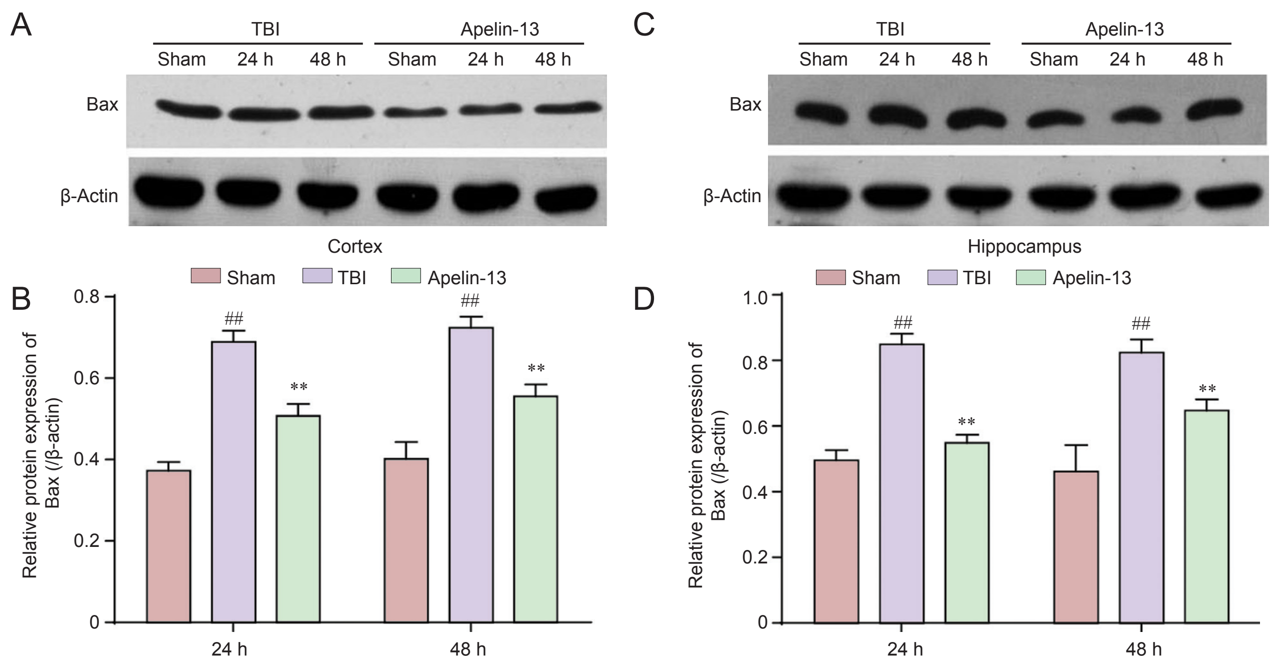

Figure 6 Inhibition of TBI-induced Bax expression in the cortex and hippocampus by apelin-13 at 24 and 48 hours post TBI.
(A, C) Representative western blots of Bax protein in the cortex (A) and hippocampus (C) were detected by western blot assay. (B, D) Quantitative analysis of Bax protein expresssion in the cortex (B) and hippocampus (D). Data are expressed as the mean ± SEM (6 mice in each group). Statistical comparisons were perfomred by analysis of variance followed by Dunnett's *t*-test. ***P* < 0.01, *vs*. TBI group; ##*P* < 0.01, *vs*. sham group. TBI: Traumatic brain injury.

10% electropheresis gels. Proteins were then transferred to polyvinylidene fluoride membranes using a semidry electrotransferring unit (Bio-Rad, Hercules, CA, USA). Membranes were incubated in antibodies against aquaporin 4 (AQP4) (1:600; rabbit polyclonal IgG; Santa Cruz Biotechnology), pro-caspase-3/caspase-3 (1:1,000; rabbit monoclonal IgG; Cell Signaling Technology, Danfoss, MA, USA), BCL2-associated X protein (Bax) (1:1,000; rabbit monoclonal IgG; Cell

Signaling Technology), and β-actin (1:2,000; rabbit monoclonal IgG; Bioworld Technology, Minneapolis, MN, USA), in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk overnight at 4°C. After overnight incubation with primary antibodies, membranes were washed and incubated for 2 hours in horseradish peroxidase conjugated goat anti-rabbit IgG (1:2,000; Bioworld Technology) in Tris-buffered saline containing 0.1% Tween-20. PageRulerTM Prestained Protein Ladder (5 μL; Thermo Fisher Scientific Inc.) was used according to the manufacturer's instructions. Immunoreactivity was tested by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL, USA), according to the manufacturer's instructions. After stripping, membranes were reprobed using β-actin. The signal intensity for binding of each primary antibody was quantitatively analyzed from optical density values using Quantity One (Bio-Rad). Results were normalized to β-actin.

Statistical analysis

Data are presented as the mean ± SEM, and were analyzed using SPSS.13.0 software (SPSS, Chicago, IL, USA). The TUNEL-positive cell count was analyzed using the rank-sum test. Brain water content, Evans Blue stain, and western blot data were analyzed by one-way analysis of variance with Dunnett's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Apelin-13 reduced brain edema in TBI mice

Compared with the sham group, brain water content was higher ($P < 0.01$) and Evans blue leakage reduced ($P < 0.01$) in the TBI group. Additionally, apelin-13 significantly reduced brain water content in the ipsilateral hemisphere ($P < 0.01$) and increased Evans blue leakage in the injured hemisphere at 24 and 48 hours after TBI ($P < 0.01$; **Figures 1**, **2**).

To further investigate the mechanism of apelin-13 attenuating brain edema after TBI, AQP4 protein levels were also measured. Apelin-13 significantly diminished AQP4 protein expression in the injured cortex and hippocampus ($P < 0.05$ or $P < 0.01$; **Figures 3**).

Apelin-13 decreased TBI-induced cell apoptosis

Compared with the TBI group, apelin-13 led to a striking reduction of TUNEL-positive cells 48 hours after TBI ($P < 0.01$; **Figure 4**).

To further investigate the role of apelin-13 TBI-induced apoptosis, caspase-3 and Bax protein levels were examined in the injured cortex and hippocampus. Lower caspase-3 protein levels were found in the apelin-13 group compared with the TBI group ($P < 0.01$; **Figure 5**). Additionally, Bax is a key protein in regulation of apoptosis (Plesnila et al., 2007), and its protein levels were significantly lower in the apelin-13 group compared with the TBI group at 24 and 48 hours post-TBI (**Figure 6**).

Discussion

We have investigated the effect of apelin-13 on secondary events, specifically, brain edema and apoptosis after TBI. To our knowledge, these are the first observations showing that apelin-13 restores the blood-brain barrier, attenuates brain edema, regulates AQP4 expression, and suppresses apoptosis in an *in vivo* TBI model.

Brain edema and subsequent increased intracranial pressure are severe complications that increase mortality and long-term disability in patients (Rangel-Castillo et al., 2008; Eghwrudjakpor and Allison, 2010). The potential therapeutic benefits of apelin-13 for ischemia/reperfusion injury, and its neuroprotective effects, have previously been recognized (Aboutaleb et al., 2013; Xin et al., 2015; Yan et al., 2015). However, whether apelin-13 can alleviate brain edema after TBI remains unclear.

Blood-brain barrier disruption occurs at a high incidence after TBI and contributes to pathological changes such as brain edema, inflammation, and loss of neuronal viability or function after brain injury (Khan et al., 2009). Here, we found that apelin-13 prevents blood-brain barrier disruption following TBI and alleviates brain edema.

Previous studies have shown that brain water content reaches a peak at 24 to 48 hours after TBI. Interestingly, intracranial pressure also peaks at this time (Bao et al., 2012; Zhang et al., 2014). Accordingly, we determined water content in the injured hemisphere at 24 and 48 hours after TBI. Our results show that brain water content is markedly decreased in the ipsilateral hemisphere of the apelin-13 group 24 and 48 hours after TBI, compared with the TBI group, which is consistent with the Evans blue leakage outcome.

Aquaporins are involved in intracranial edema and permit selective bidirectional water movement (Verkman and Mitra, 2000; Marmarou, 2007; He and Lu, 2015). The predominant brain isoform, AQP4, is found within the perivascular end feet of astrocytes and can regulate fast water transport (Nicchia et al., 2004). We found that TBI up-regulates AQP4 protein expression at 24 and 48 hours post-TBI. These results are consistent with our profile of TBI-induced brain water content, which indicates that AQP4 up-regulation may exacerbate brain edema. In contrast, AQP4 protein levels are down-regulated in the apelin-13 group at 24 and 48 hours post-TBI. Despite the controversial role of AQP4 in TBI-induced brain edema (Ke et al., 2001; Saadoun et al., 2003; Sun et al., 2003), our results suggest that an apelin-13 effect on brain edema may be involved in AQP4 regulation.

Further, we counted TUNEL-positive cells to confirm apoptosis after TBI affected by apelin-13. Additionally, we also measured apoptosis-associated protein levels of caspase-3 and Bax. Our previous research has shown that apelin-13 results in a marked increase of Bcl-2 protein levels (Bao et al., 2014). However, Bcl-2 was also the first identified oncogene protein involved in blocking apoptotic cell death. Caspase-3 would be cleaved in both of the two pathways (Zhang et al., 2005). We found that cell apoptosis was inhibited, and caspase-3 and Bax down-regulated by apelin-13 after TBI. These results show that apoptosis may be involved in the protective function of apelin-13.

Author contributions: *HJB and YPW designed experiments. HYQ, JXK, SXW, and HBP carried out experiments. WCH analyzed experimental results. HJB wrote the paper. CJS and CQW assisted with experiments and paper writting. All authors approved the final version of the paper.*
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Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
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