Article

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TWEAK Knockdown Alleviates Post-Cardiac Arrest Brain Injury via the p38 MAPK/NF-κB Pathway

Haifang Zhang[1], Ran Wang[2,*]

[1]Handan Emergency Rescue Command Center, 056002 Handan, Hebei, China
[2]Department of Emergency, The Affiliated Hospital of Hebei University of Engineering, 056002 Handan, Hebei, China
*Correspondence: WangRan__1023@126.com (Ran Wang)
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Background: Cardiac arrest (CA) and subsequent cardiopulmonary resuscitation (CPR) can cause brain injury, which is one of the factors affecting the recovery of brain function in CA patients. There is increasing evidence that tumor necrosis factor-like weak apoptosis-inducing factor (TWEAK) is associated with the brain injury diseases. This study was aimed to investigate the modulation mechanism of TWEAK involved in brain injury after cardiac arrest/subsequent cardiopulmonary resuscitation (CA/CPR).

Materials and Methods: For *in vivo* experiments, healthy male Sprague-Dawley (SD) rats were applied to establish CA/CPR model, and oxygen-glucose deprivation/reoxygenation (OGD/R)-stimulated neurons model was established *in vitro*. TWEAK short hairpin RNAs (shRNAs) were injected into the lateral ventricle of CA/CPR rats or transfected into OGD/R cell culture to analyze the consequent alteration in neurological scores, behavioral tests, cell proliferation, cell apoptosis, and neuroinflammation through immunofluorescence staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and enzyme linked immunosorbent assay (ELISA).

Results: There were high expressions of TWEAK and fibroblast growth factor-inducible 14 (Fn14) in the cerebral cortex of CA/CPR rats and OGD/R-stimulated neuronal cells. TWEAK knockdown attenuated cell apoptosis, inflammation and showed better behavioral tests in CA/CPR rats. Furthermore, TWEAK shRNAs obviously facilitated cell proliferation, suppressed apoptosis and inflammation after OGD/R injury. Western blotting results stated that TWEAK silencing promoted phosphorylated p38 (p-p38) and phosphorylated p65 (p-p65) expressions.

Conclusions: TWEAK might be involved in the pathogenesis of CA/CPR through inhibiting p38 MAPK/NF-κB pathway.

Keywords: TWEAK; Fn14; CA/CPR; brain injury; p38 MAPK/NF-κB

Introduction

Cardiac arrest (CA) has a relatively high fatality rate, with more than 500,000 deaths per year [1]. Cardiopulmonary resuscitation (CPR) after CA can result in various injuries such as energy metabolism disorders, severe ischemia, and hypoxia. Although great progress has been made in CA/CPR treatment, the prognosis of patients after resuscitation is still poor, especially the brain function [2]. According to previous reports [3], the restoration of spontaneous circulation (ROSC) in hospitalized CA patients rarely exceeds 30%, and only 15% of these surviving patients have intact neurological function, which seriously affects the quality life of the patients [4,5].

CA/CPR can be regarded as a cerebral ischemia process after resuscitation, which leads to multiple organ dysfunction and post-cardiac arrest syndrome (PCAS) [6]. Since the brain is extremely sensitive to ischemia, brain injury is particularly common after CA/CPR, accounting for approximately 60% of deaths after ROSC [7]. The molecular mechanism of brain injury in CA/CPR is very complex, including reactive oxygen species production, inflammatory response, neuronal apoptosis, excitatory amino acid damage, abnormal mitochondrial energy metabolism, loss of blood-brain barrier integrity, and intracellular calcium overload. Although many drugs have been used to prevent brain injury after CPR, the effect is not significant. Thus, seeking innovative and specific treatment methods for brain neuroprotection after CA/CPR is needed.

Tumor necrosis factor-like weak apoptosis-inducing factor (TWEAK) is a type II transmembrane protein encoded by tumor necrosis factor superfamily (*TNFSF*) gene, also known as TNFSF12, which is present in neurons and microglia of the central nervous system, perivascular astrocytes, and endothelial cells [8]. It has biological activities such as stimulating cell proliferation and apoptosis, promoting angiogenesis, inducing expression of pro-inflammatory cytokines and chemokines [9]. TWEAK binds to its receptor fibroblast growth factor-inducible 14 (Fn14) and activates MAPK/NF-κB/PI3K/AKT downstream signaling, resulting in modulation of cell proliferation, differentiation, apoptosis, neovascularization and inflammatory response

[10–12]. According to previous reports, TWEAK is closely related to neurodegeneration-related disorders, traumatic brain injury, and myocardial infarction, and its high expression is bound up with the cellular and inflammatory responses of these diseases [13–15], suggesting that TWEAK may have the potential to inhibit the inflammation and cell apoptosis, and promote angiogenesis and proliferation. For instance, Akahori *et al*. [16] revealed that TWEAK interacted with CD163 to regulate tissue regeneration after ischemic injury. Moreover, Eyyupkoca *et al*. [17] clarified that high expression of TWEAK contributed to the development of adverse remodeling in myocardial infarction. More importantly, another study discovered that TWEAK-Fn14 signaling was involved in protecting myocardial or cerebral ischemia injury [18]. However, it remains to be further studied whether TWEAK has a protective effect on the CA/CPR-induced brain injury, especially in terms of proliferation, apoptosis and inflammation response.

It has been reported that p38 MAPK pathway modulates varieties of important cellular physiological/pathological processes such as cell growth, differentiation, apoptosis, and inflammatory response [19,20]. Protein interaction network analysis revealed that the eukaryotic transcription factor NF-κB, which is involved in cellular functions such as inflammation, immune response, cell proliferation and apoptosis, interacted with the p38 protein [21]. Moreover, p38 MAPK acts upstream of the NF-κB signaling pathway in neuropsychiatric diseases [22]. Based on the regulation of the p38 MAPK/NF-κB pathway on inflammation and apoptosis, presumably, this pathway may have something to do with CA/CPR development. A previous study discovered an increased expression of p-p38 in CA/CPR rats at 3 days [23]. Another study showed that the phosphorylation of p38 was decreased in the rat model of ischemic stroke at 7 days and cell model of oxygen-glucose deprivation/reoxygenation (OGD/R) 24 h [24,25]. Besides which, a report discovered an increase in TWEAK expression and activation of MAPK signaling pathway in intracerebral hemorrhage [26]. Hence, the interaction of TWEAK with p38 MAPK/NF-κB signaling pathway may activate a pro-inflammatory cell signaling pathway that is associated with cell death during cerebral ischemia. Thus, we speculated that TWEAK was increased in CA/CPR and it participated in CA/CPR development by regulating the p38 MAPK/NF-κB pathway.

Based on the literature described above, we hypothesized that TWEAK exhibited a critical role in the progression of CA/CPR. This study was aimed to analyze the relationship between TWEAK and CA/CPR *in vitro* and *in vivo*. p38 MAPK/NF-κB signaling pathway activated by TWEAK was investigated as well. Results showed that TWEAK knockdown attenuated cell apoptosis and neuroinflammation in CA/CPR via modulating p38 MAPK/NF-κB pathway, which might provide a potential therapeutic approach for CA/CPR in clinical medicine.

Materials and Methods

Animals

Ninety-five healthy male Sprague-Dawley (SD) rats, about 9–12 weeks old, weighing about 300 g, were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The animals were reared at a constant temperature of 24–26 °C and a constant humidity of 40%–50%, given sterile water and pelleted feed for one week.

Preparation of CA/CPR Model

SD rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (45 mg/kg). The Electrocardiogram (ECG) was applied for monitoring heart rhythm. A PE50 tube was inserted into the artery to detect the right atrial pressure (RAP) and mean arterial pressure (MAP). Moreover, a pressure converter was applied for monitoring blood pressure. Rectal temperature was maintained with a heat lamp (36.5 $\pm$ 0.5 °C).

CA was initiated by percutaneous electrical stimulation of the epicardium as previously described [27]. In brief, ventricular fibrillation (VF) was induced by transthoracic alternating current. When the invasive blood pressure dropped to $<$30 mmHg and the ECG showed a VF waveform, indicating that the CA model was successfully established. Global ischemia is defined as eye discoloration to white, pupil dilation and loss of light reflexes. CPR was started when CA lasted for 7 min. Mechanical chest compressions were performed at a rate of 180 times/min, and the depth of compressions was 25–30% of the front and back diameter of the chest. Meanwhile, epinephrine 2 µg/100 g/3 min was given through the right femoral vein for 2 min. The definition of ROSC is that MAP recovers above 60 mmHg at least for 5 min. The sham group received anesthesia, mechanical ventilation, PE50 catheter placement, skin incision and postoperative analgesia without CA/CPR. After the 120-minute experiment, rats were returned to cages. At 1, 3, 7 days post-ROSC, rat survival status was recorded.

Intracerebroventricular Infusion of TWEAK shRNAs

The lentivirus vectors expressing short hairpin RNAs targeting TWEAK (sh-TWEAK, sc-37522-SH, 5′-GAGGGAGAAUUUAUUAAUATT-3′) and the corresponding scrambled negative control (NC) short hairpin RNAs (shRNAs) (sh-NC, sc-37007, 5′-UUCUCCGAACGUGUCACGU-3′) (Santa Cruz Biotechnology) were carefully diluted following the guidelines [28]. A microsyringe was inserted at 0.5 mm posterior, 1.0 mm lateral to the bregma, and 3.0 mm ventral to the skull under the guidance of a stereotaxic instrument. Single-dose sh-TWEAK or sh-NC were injected into the lateral ventricles 20 min before CA.

Evaluation of Neurological Function

Neurological Deficit Score (NDS)

NDS was applied for evaluating the neurological deficits of CA/CPR rats, including movement, sensation, balance, and reflection. 0-point is defined as normal neurological function. The higher the degree of neurological impairment, the higher the score.

Open Field Test

The rats were placed in the center of the open wooden box (the bottom of the wooden box was divided into 5 $\times$ 5 squares of equal size), and they were moved freely and recorded by the camera. The number of squares crossed (horizontal score) and the number of times that the front paw left the ground and the hind paw stood (vertical score) were recorded within 5 min. After each test, the wooden box was cleaned and the odor was removed to prevent it from affecting the results of subsequent rat experiments.

Rotarod Test

The motor coordination ability of rats was tested by a model XR1514 rotary rod fatigue tester (Shanghai, China). The speed was set to 24 r/min and the time was set to 5 min. The movement time of the rats on the rotating rod was recorded, and the average value of three experiments was taken. The rats were trained three times before CA/CPR surgery.

Culture of Primary Neurons

As previously mentioned, cerebral cortex samples were obtained from neonatal rats (day 1) cortex and then collected into cold D-Hanks solution. The chopped tissues were incubated in 0.25% trypsin (Beyotime, Beijing, China) at 37 °C for 20 min. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was applied to terminate enzymatic digestion. The 200-mesh nylon was used to filter entire cell suspension. The filtrate was centrifuged at 1000 g for 8 min, then the cell precipitates were re-suspended in DMEM containing 10% FBS and 1% penicillin/streptomycin. The cells underwent stringent mycoplasma testing.

1×10^5 cells/cm^2 cells were placed in polylysine-coated dishes at 37 °C for 24 h. The medium was then replaced with serum-free neural basal medium containing 1% L-glutamine and 2% B27. Half of the medium was replaced with neural basal medium containing L-glutamine and B27 every 2 days, and cell morphology was observed under a phase-contrast inverted microscope (Leica, DM IL, Munich, Germany). After 7 days of growth, the neurons were collected to detect the purity by immunofluorescence assay. The positively cells from 5 non-overlapping fields were counted by Image J software 5.2.1 (NIH, Bethesda, MD, USA). The purity of primary neuron (%) was calculated by the percentage of β III Tubulin-positive cells in the total number of cells (4',6-diamidino-2-phenylindole (DAPI)-positive cells).

Cell Transfection

Primary neurons were trypsinized and plated into 6-well plates. Neuronal cells were transfected with sh-TWEAK (sc-37522-V), or scrambled control shRNAs (sc-37007) (Santa Cruz Biotechnology, Beijing, Shanghai), respectively, using Lipofectamine 2000 in OptiMEM, in line with the manufacturer's introductions [29]. After 24 h of transfection, real time quantitative polymerase chain reaction (RT-qPCR) was applied for measuring the transfection efficiency.

Preparation of OGD/R Model

(1) Preparation of anoxic D Hank solution. D Hank solution was prepared by injecting D Hank solution into anoxic gas containing 95% N_2 and 5% CO_2 in self-designed assembly bottle. The oxygen concentration of D Hank solution was determined by blood gas analyzer. (2) Preparation and application of anoxic tank. An anoxic tank was made by passing a vacuum dryer into anoxic gas, and the oxygen content in the tank was measured by anoxic gas analyzer. (3) Establishment of neural oxygen-glucose deprivation/reoxygenation (OGD/R) model. The neurons cultured on 7 days were incubated with anoxic D Hank solution and placed in anoxic tank. The anoxic tank was placed in a 37 °C incubator for 12 h, 24 h, 48 h, and then reoxygenated for 24 h. The neurons of control group were cultured in normal-glucose DMEM under normal oxygen conditions.

Real-Time qPCR

All RNAs were isolated from brain tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT Master Mix kit (TaKaRa, Dalin, China) following the guidance. cDNA was amplified by SYBR Premix Ex Taq Mix Kit (TaKaRa, Dalian, China). qPCR was performed using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Inc. Waltham, MA, USA). The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as an endogenous control. Primer sequences were shown as in Table 1.

CCK-8 Assay

The 96-well plates were equipped with 100 µL neuronal cell suspension, and pre-cultured in an incubator for 24 h at 37 °C with 5% CO_2. Then, the cell counting kit-8 (CCK-8) solution was incubated at 37 °C for 3 h. The absorbance value at 450 nm was measured with a microplate reader (Bio-Tek, Winooski, VT, USA), which indirectly reflected the number and activity of surviving neurons.

Table 1. Primers sequences for RT-qPCR.

Proteins	Primers Sequences (5′-3′)
TWEAK	Forward: AGGAGGAGCTGACAG Reverse: CCTCATAATGGGCTG
Fn14	Forward: GTGTTGGGATTCGGCTTG Reverse: GCAGAAGTCGCTGTGTGGT
Bax	Forward: TCACCCAACCACCCTGGTCTT Reverse: TCACCCAACCACCCTGGTCTT
Bcl-2	Forward: AGAACCTTGTGTGACAAATGAGAAC Reverse: TACCCATTAGACATAT CCAGCTTGA
Cleaved-caspase-3	Forward: CGGACCTGTGGACCTGAAAA Reverse: AGTCAGACTCCGGCAGTAGT
Pro-caspase-3	Forward: ACAGTGGAACTGACGATGATATG Reverse: TCCCTTGAATTTCTCCAGGAATAG
Cleaved-caspase-8	Forward: ACAGATGCCAGAATCCGAAGG Reverse: CGGGAGATGTGGGTACAAGG
Pro-caspase-8	Forward: ACTGGCTGCCCTCAAGTTCCTGTGC Reverse: TCCCTCACCATTTCCTCTGGGCTGC
GAPDH	Forward: AGAAGGCTGGGGCTCATTTG Reverse: AGGGGCCATCCACAGTCTTC

RT-qPCR, real time quantitative polymerase chain reaction; TWEAK, tumor necrosis factor-like weak apoptosis-inducing factor; Fn14, fibroblast growth factor-inducible 14; Bax, B-cell lymphoma-2 associated protein X; Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TUNEL Assay

The neuronal cells were placed in a 24-well plate, fixed with 4% paraformaldehyde, and incubated in an ice bath for 2 min. Afterwards, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) solution (Roche, Mannheim, Germany) was added and incubated in the dark at 37 °C for 60 min. After washing with phosphate buffer saline (PBS), the cells were observed under a fluorescence microscope. Ten fields of view were randomly selected to determine the ratio of apoptotic cells to total cells, that is, the apoptotic rate.

Western Blotting

After being washed 3 times with PBS, radioimmunoprecipitation assay (RIPA) lysate was added to lyse neuronal cells. Bradford method was applied to determine protein concentration. Afterwards, 50 μg proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to poly-vinylidene difluoride membranes. Then, the membranes were blocked with 5% fat-free milk solution for 1 h at 37 °C and incubated with the following rabbit anti-primary antibodies, TWEAK (1:1000; ab37170), Fn14 (1:1000; #4403, CST), B-cell lymphoma-2 (Bcl-2) (1:1000; ab32124), B-cell lymphoma-2 associated protein X (Bax) (1:1000; ab32503), cleaved caspase-3 (1:500; ab32042), pro-caspase-3 (1:1000; ab32150), cleaved caspase-8 (1:1000; #8592, CST), pro-caspase-8 (1:1000; ab108333), p38 (1:1000; #8690, CST), phosphorylated p38 (p-p38) (1:1000; #4511, CST), p65 (1:1000; ab16502), phosphorylated p65 (p-p65) (1:1000; ab76302), and GAPDH (1:1000; ab9485) at 4 °C overnight. Then, they were incubated with secondary antibodies goat anti-rabbit IgG H&L (HRP) (1:2000; ab205718) for 1 h at 37 °C. Finally, the target protein was exposed and imaged using ECL developer (Millipore, Bedford, MA, USA). ImageJ software 5.2.1 (NIH, Bethesda, MD, USA) was used to quantify the band intensity, and the protein expression levels of each gene were normalized by GAPDH.

Enzyme Linked Immunosorbent Assay (ELISA)

The cell supernatant and brain tissues were collected from neuronal cells and CA/CPR rats. Then, the contents of Interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and neurofilament-200 (NF200), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) were measured using the corresponding ELISA kits, in line with the manufacturer's guides [30]. The iMark Microplate Reader (BIO-RAD 680, Irvine, CA, USA) was applied for analyzing absorbance values at 450 nm. Data were expressed as pg/mL.

Immunofluorescence Assay

The NeuN+ cells and iba1+ cells in brain tissues after CA/CPR were detected using immunofluorescence assay. In brief, the coronal frozen slices (20 µm) were fixed with 4% paraformaldehyde and then permeated with 0.3% Triton X-100. Afterwards, the slices were blocked with 5% goat serum for 30 min. Subsequently, the slices were incubated with rabbit anti-NeuN (1:200, ab177487), rabbit anti-iba1 (1:100, ab178847) and rabbit anti-β III Tubulin (1:500, ab18207) overnight at 4 °C, followed by the secondary antibodies goat anti-rabbit IgG H&L (Alexa Fluor® 647) (1:200; ab150079) or (Alexa Fluor® 488) (1:200; ab150077) for 1 h. Finally, the slices were stained with DAPI for 10 min. The Olympus fluorescence microscopy (Olympus, Hilden, Germany) was used for capturing the stained images and Image J software (NIH, Bethesda, MD, USA) was applied for counting the numbers of positively stained cells from more than 8 non-overlapping fields.

Statistical Analysis

Results were obtained from at least three independent experiments. The statistical analysis was performed with SPSS 20.0 statistical software (IBM, Armonk, NY, USA) and the data were analyzed by using Prism 7 software (GraphPad, LaJolla, CA, USA). The results were expressed as mean $\pm$ standard deviation. The comparisons between two groups were performed using the Student's *t* test, and analysis of variance followed by post-hoc analysis was performed for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Morphological Observation and Purity Identification of Primary Neurons

Results from phase-contrast inverted microscope showed that the newly inoculated neurons were round, transparent, and uniformly distributed individually. After 3 days of culture, the cell body was enlarged and most of the cells were fusiform, and the protrusions were connected with each other, forming sparse networks. After 7 days of culture, the volume of neurons were further increased, with more protrusions and many branches, forming obvious networks (Fig. 1A). The primary neurons were immunostained with anti-β III Tubulin. Results showed that β III Tubulin positive cells accounted for 96.3% (Fig. 1B). The purity of primary neuron was more than 95%, which can be used as experimental cell model *in vitro*.

TWEAK and Fn14 were Up-Regulated in CA/CPR

To probe into the association between TWEAK and CA/CPR, TWEAK and Fn14 expressions in the cerebral cortex of CA/CPR rats for 1, 3, or 7 days were measured. Results of RT-qPCR showed a time-dependent up-regulation of TWEAK and Fn14 expression in CA/CPR rats ($p < 0.05$, Fig. 2A,B), and western blotting confirmed the same results ($p < 0.05$, Fig. 2C,D). Furthermore, TWEAK and Fn14 levels in neuronal cells were measured at 12, 24, and 48 h of OGD/R operation. Through RT-qPCR and western blotting detection, it was also found that they were increased in a time dependent manner ($p < 0.05$, Fig. 2E–H). Taken together, TWEAK and Fn14 were up-regulated upon CA/CPR *in vitro* and *in vivo*, suggesting its potential role in CA/CPR. According to the above results, 7 days CA/CPR and 48 h OGD/R were selected for further study.

Down-Regulation of TWEAK Alleviated the CA/CPR-Mediated Brain Injury

To investigate TWEAK effect on the neurological function of CA/CPR rats, TWEAK knockdown lentiviral (sh-TWEAK) was injected into the lateral ventricles of CA/CPR rats. Results showed that TWEAK was significantly decreased after treatment with sh-TWEAK ($p < 0.001$, Fig. 3A). Compared with CA/CPR group, the neurologic scores ($p < 0.05$, Fig. 3B), open field test distance ($p < 0.05$, Fig. 3C), and rotarod time ($p < 0.05$, Fig. 3D) were significantly up-regulated in sh-TWEAK group, indicating significantly better behavioral tests on day 7 in CA/CPR rats after treatment with sh-TWEAK. Besides which, compared with sham group, the NeuN+ cells in the CA/CPR group were significantly decreased, while the TUNEL-positive cells were obviously increased. However, inhibition of TWEAK further increased NeuN+ cells and decreased TUNEL-positive cells ($p < 0.05$, Fig. 3E,F). Moreover, results of immunofluorescence showed that the number of activated microglia/macrophages (labeled by iba1) was decreased in sh-TWEAK group, which was induced by CA/CPR operation ($p < 0.05$, Fig. 3G). Furthermore, the expressions of IL-1β, TNF-α, IL-6 and NF200, BDNF, NGF were detected by ELISA assay. Results showed that the expressions of IL-1β, TNF-α, and IL-6 were significantly increased, and NF200, BDNF and NGF expressions were slightly increased in the CA/CPR group, when compared with sham group. However, knockdown of TWEAK inhibited the expressions of neuroinflammatory factors, while facilitated the expressions of neurotrophic factors ($p < 0.05$, Fig. 3H,I). These data indicate that TWEAK down-regulation improved the neurological function of CA/CPR rats.

Down-Regulation of TWEAK Improved OGD/R-Mediated Neuronal Impairment and Inflammation Injury

Then, the loss of function experiments in neuronal cells upon OGD/R treatment was executed. TWEAK expression was measured by RT-qPCR and western blotting in sh-TWEAK transfected neuronal cells prior to OGD/R. Results showed that the expression of TWEAK was notably inhibited by sh-TWEAK ($p < 0.001$, Fig. 4A). Results of CCK-8 displayed that the neuronal cells viabil-

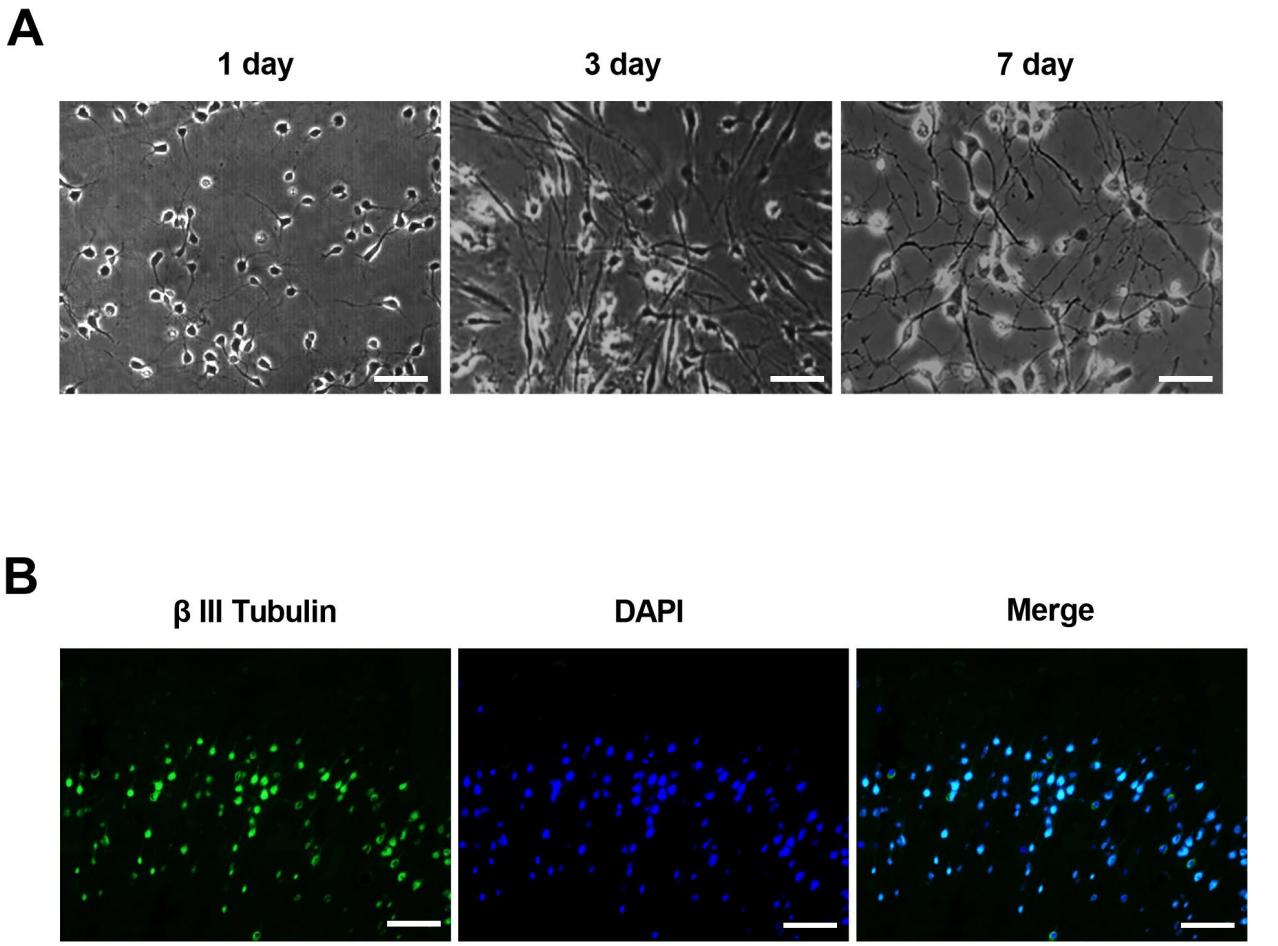

Fig. 1. Morphological and purity identification of primary neurons. (A) Morphological of primary neurons cultured for 1, 3, 7 days (Scale bar = 20 μm). (B) Primary neurons were purified by β III Tubulin (Scale bar = 25 μm). DAPI, 4',6-diamidino-2-phenylindole.

ity was down-regulated after OGD/R injury, but down-regulation of TWEAK enhanced the decreased cell viability of neurons ($p < 0.05$, Fig. 4B). Furthermore, the related apoptosis proteins were measured by RT-qPCR and western blotting assays. Results showed that the Bax, cleaved-caspase-3, and cleaved-caspase-8 levels were remarkably up-regulated, while Bcl-2 level was down-regulated in neuronal cells after OGD/R injury. However, after treatment with sh-TWEAK, these levels were partially revered ($p < 0.05$, Fig. 4C,D). Meanwhile, TUNEL staining discovered that the percentage of apoptosis cells was markedly elevated in OGD/R neuronal cells. However, the increases were significantly declined after treatment with sh-TWEAK ($p < 0.05$, Fig. 4E). In addition, ELISA assay displayed that the expressions of inflammatory cytokines in neuronal cells upon OGD/R were obviously increased, but these levels were significantly decreased by treatment with sh-TWEAK ($p < 0.05$, Fig. 4F). In conclusion, these data indicate that TWEAK knockdown has neuroprotective effects on OGD/R neurons.

Down-Regulation of TWEAK Promoted the Activation of p38 MAPK/NF-κB Pathway in CA/CPR Rats and OGD/R Neuronal Cells

To investigate TWEAK effect on the p38 MAPK/NF-κB pathway in CA/CPR, western blotting assay was applied for examining the related proteins of the p38 MAPK/NF-κB pathway in the cerebral cortex of CA/CPR rats and OGD/R neuronal cells. Results represented that the protein levels of phosphorylated p38 and NF-κB p65 were signally declined in the cerebral cortex of CA/CPR rats versus to sham group. However, the abovementioned protein levels were raised significantly after sh-TWEAK treatment ($p < 0.05$, Fig. 5A). Meanwhile, the protein levels of phosphorylated p38 and NF-κB p65 were obviously decreased in neuronal cells after OGD/R injury in comparison with control group. However, the abovementioned protein levels were elevated in sh-TWEAK group versus to that of the OGD/R group ($p < 0.05$, Fig. 5B). This phenomenon suggest that TWEAK down-regulation facilitated the activation of p38 MAPK/NF-κB pathway in CA/CPR rats and OGD/R-treated neuronal cells.

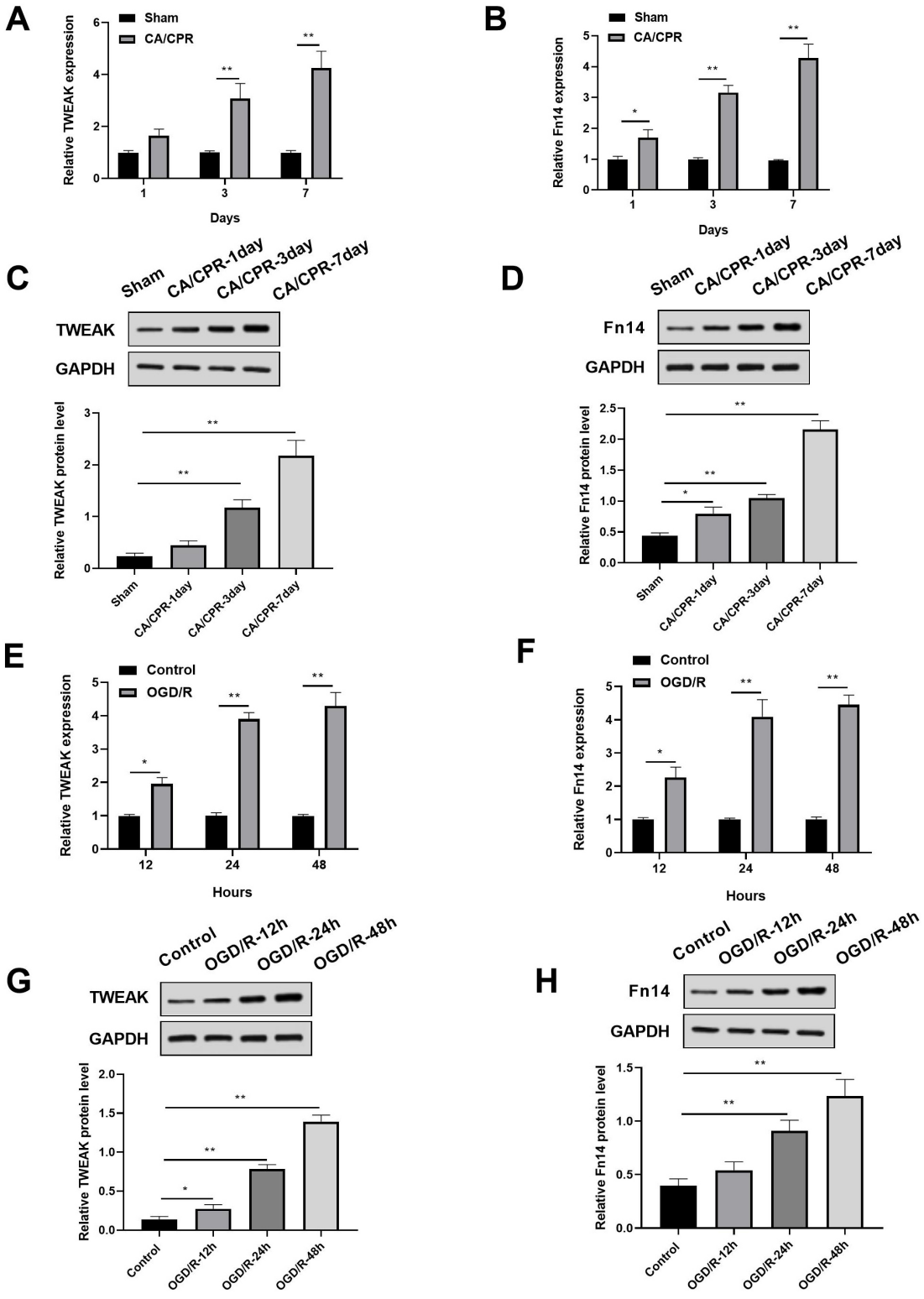

Fig. 2. TWEAK (tumor necrosis factor-like weak apoptosis-inducing factor) and Fn14 (fibroblast growth factor-inducible 14) were up-regulated in CA/CPR (cardiac arrest/subsequent cardiopulmonary resuscitation). (A,B) TWEAK and Fn14 expressions were measured in CA/CPR rats at 1, 3, and 7 days by RT-qPCR (real time quantitative polymerase chain reaction) (n = 5). (C,D) Western blotting measured the levels of TWEAK and Fn14 at 1, 3, and 7 days after CA/CPR in rats (n = 5). (E,F) TWEAK and Fn14 expressions were detected in OGD/R (oxygen-glucose deprivation/reoxygenation) neuronal cells at 12, 24, and 48 h (n = 3). (G,H) Western blotting measured the levels of TWEAK and Fn14 in neuronal cells at 12, 24, and 48 h after OGD/R (n = 3). *$p < 0.05$, **$p < 0.01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

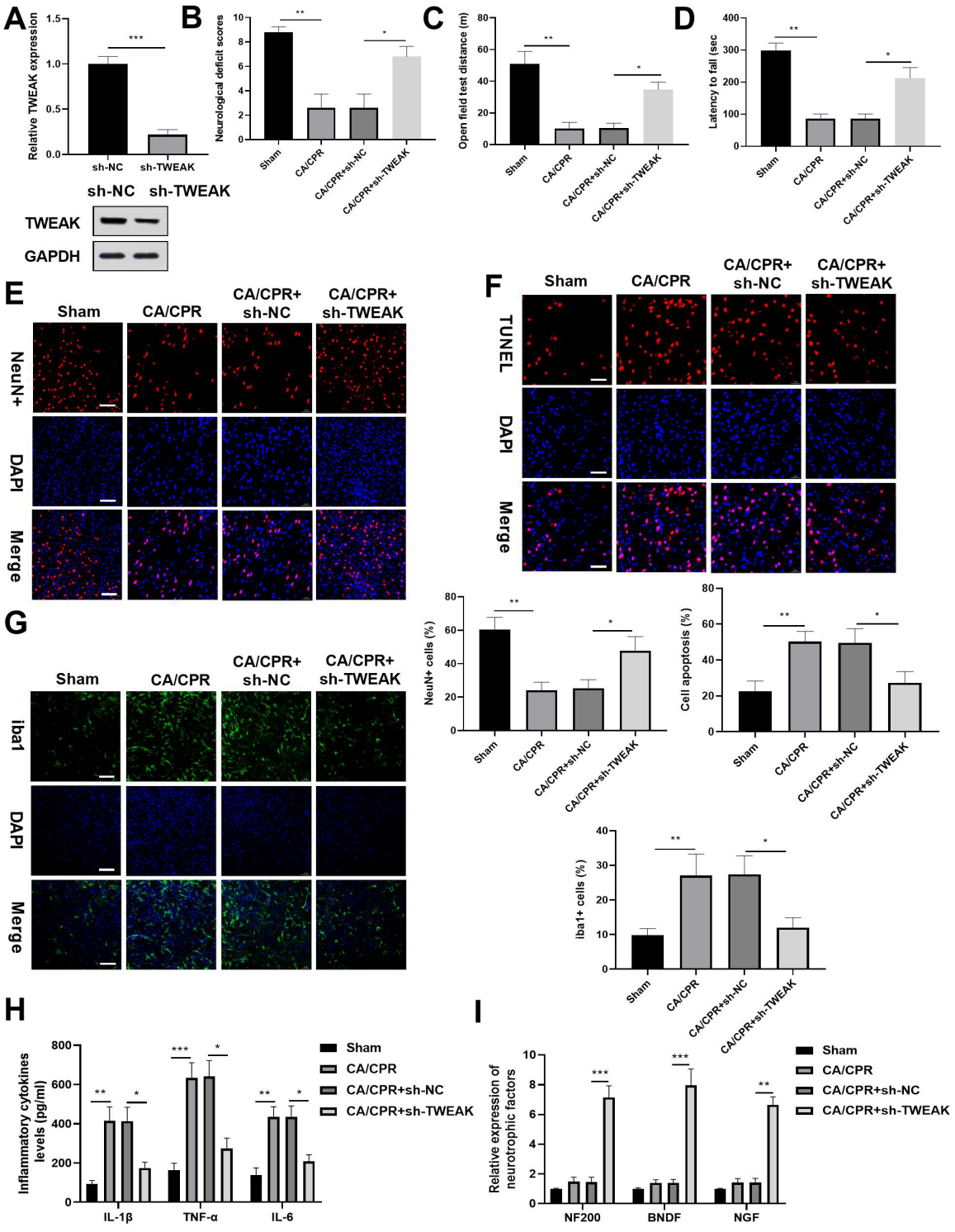

Fig. 3. TWEAK down-regulation alleviated the CA/CPR-mediated brain injury. The neurological function was detected in CA/CPR rats after treatment with sh-TWEAK at 7 days. (A) RT-qPCR and western blotting analysis of TWEAK expression. (B) The neurologic scores, (C) open field test distance, and (D) rotarod time were measured at 7 days after CA/CPR. (E) Immunofluorescence staining showed the number of NeuN+ cells (Scale bar = 25 μm). (F) TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining displayed the apoptosis cells in the CA/CPR rats (Scale bar = 25 μm). (G) Immunofluorescence staining showed the number of activated microglia/macrophages (Scale bar = 75 μm). (H) The expressions of IL (Interleukin)-1β, TNF (tumor necrosis factor)-α, IL-6 and (I) NF200 (neurofilament-200), BDNF (brain derived neurotrophic factor), NGF (nerve growth factor) were detected by ELISA (enzyme linked immunosorbent assay) (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

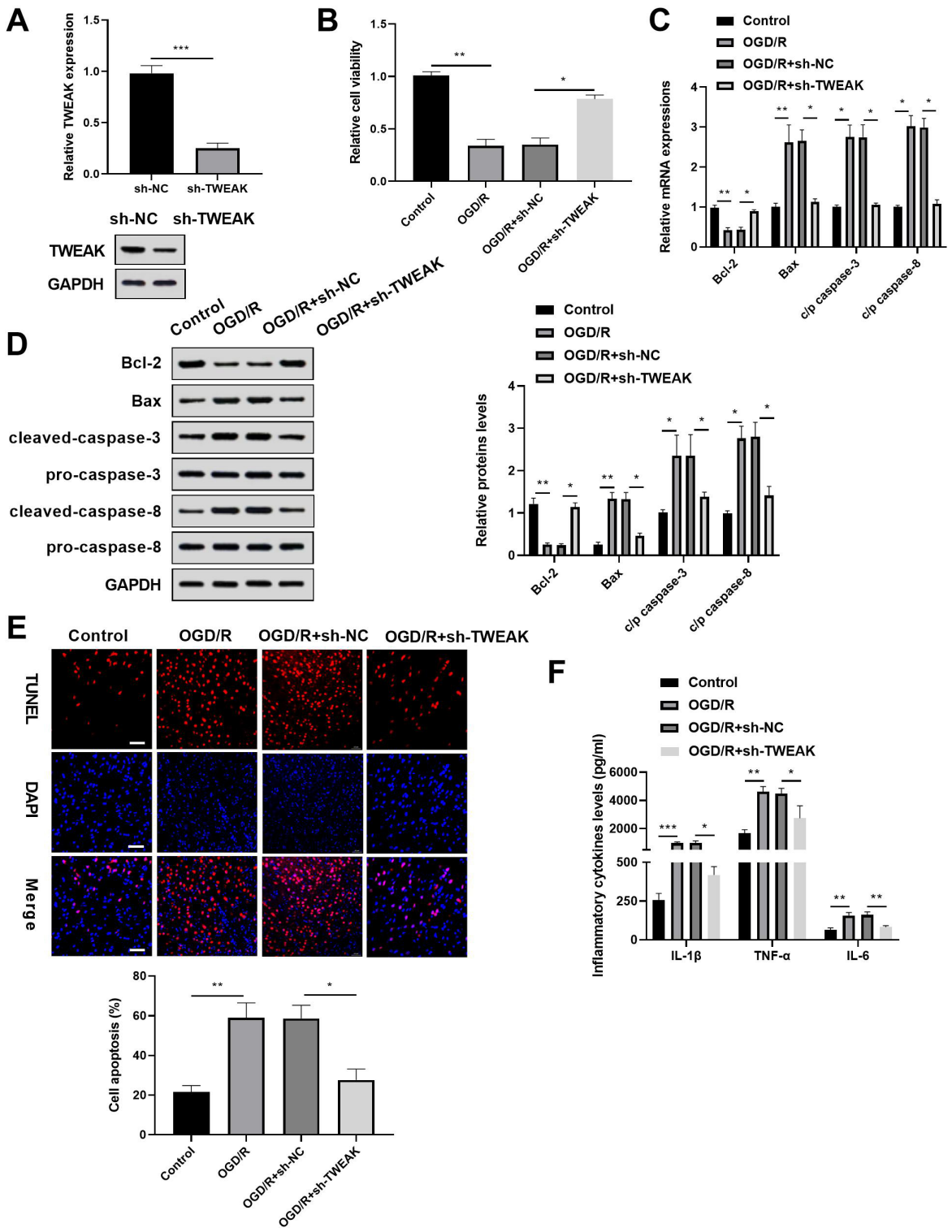

Fig. 4. TWEAK silencing improved OGD/R-mediated neuronal impairment and inflammation injury. Neuronal cells were treated with sh-TWEAK prior to OGD/R. (A) Detection of TWEAK expression by RT-qPCR and western blotting. (B) Detection of neuronal cell viability by CCK (Cell Counting Kit)-8 assay. (C) Measurement of the levels of apoptosis proteins by RT-qPCR assay and (D) western blotting assay. (E) Measurement of the percentage of apoptosis cells by TUNEL staining (Scale bar = 25 µm). (F) ELISA assay analysis of the expressions of IL-1β, TNF-α, and IL-6 (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Bcl-2, B-cell lymphoma-2; Bax, B-cell lymphoma-2 associated protein X; NC, negative control.

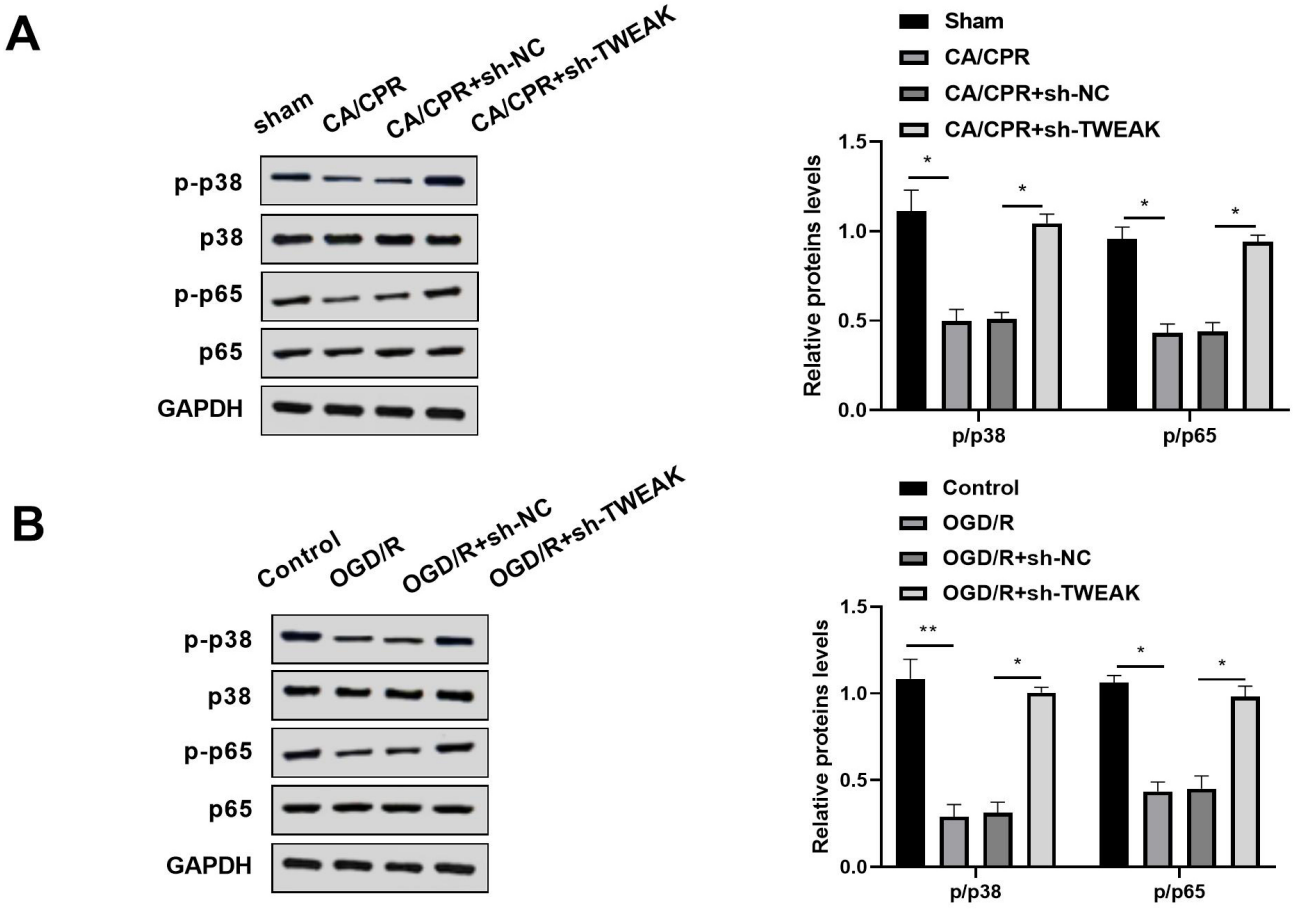

Fig. 5. TWEAK silencing promoted the activation of p38 MAPK/NF-κB pathway in CA/CPR rats and OGD/R neuronal cells. (A) Measurement of the protein levels of p-p38 (phosphorylated p38) and p-p65 (phosphorylated p65) in the cerebral cortex of CA/CPR rats after sh-TWEAK treatment (n = 5). (B) Measurement of p-p38 and p-p65 levels in OGD/R neuronal cell after sh-TWEAK treatment (n = 3). *$p < 0.05$, **$p < 0.01$.

The p38 MAPK Signaling Pathway Inhibitor Reversed the Neuroprotective Effect of sh-TWEAK on OGD/R Injury

To determine whether p38 MAPK signaling pathway inhibition mediated the neuroprotective effect of sh-TWEAK on OGD/R injury, neuronal cells were pretreated with the p38 MAPK signaling pathway inhibitor SB203580 or along with sh-TWEAK prior to OGD/R operation. Results displayed that SB203580 attenuated the protein levels of p-p38 and p-p65 induced by sh-TWEAK treatment ($p < 0.05$, Fig. 6A). Moreover, CCK-8 assay showed that SB203580 overturned the promotion effect of sh-TWEAK on cell viability ($p < 0.05$, Fig. 6B). Bides which, TUNEL assay demonstrated that SB203580 partly restored the inhibitory effect of sh-TWEAK on neuronal cell apoptosis ($p < 0.05$, Fig. 6C). In addition, results of ELISA assay revealed that SB203580 reversed the suppression effect of sh-TWEAK on inflammatory cytokines ($p < 0.05$, Fig. 6D). Collectively, these results suggest that the p38 MAPK signaling pathway inhibitor reversed the neuroprotective effects of sh-TWEAK on OGD/R injury.

Discussion

Brain injury after CA is a major cause of poor prognosis in patients. Therefore, the ultimate goal of CPR is the recovery of brain function [31,32]. Previous studies have shown that the dysregulation of TWEAK/Fn14 is associated with the pathophysiology of the neurodegeneration-related disorders [33]. Although it has been demonstrated that brain injury causes a significant increase in TWEAK/Fn14 expression and TWEAK/Fn14 axis acts as a common target for protection of brain injury [18], the functional role of TWEAK in the brain injury induced by CA/CPR remains unknown. Herein, a CA/CPR rat model and an OGD/R model were constructed to simulate the pathology of CA/CPR patients. Results displayed that TWEAK down-regulation attenuated CA/CPR-mediated brain injury and attenuated neuronal cell apoptosis and inflammation. Its brain protective effect was mainly realized through the p38 MAPK signaling pathway, which provided an important therapeutic target for the treatment and prognosis of CA/CPR.

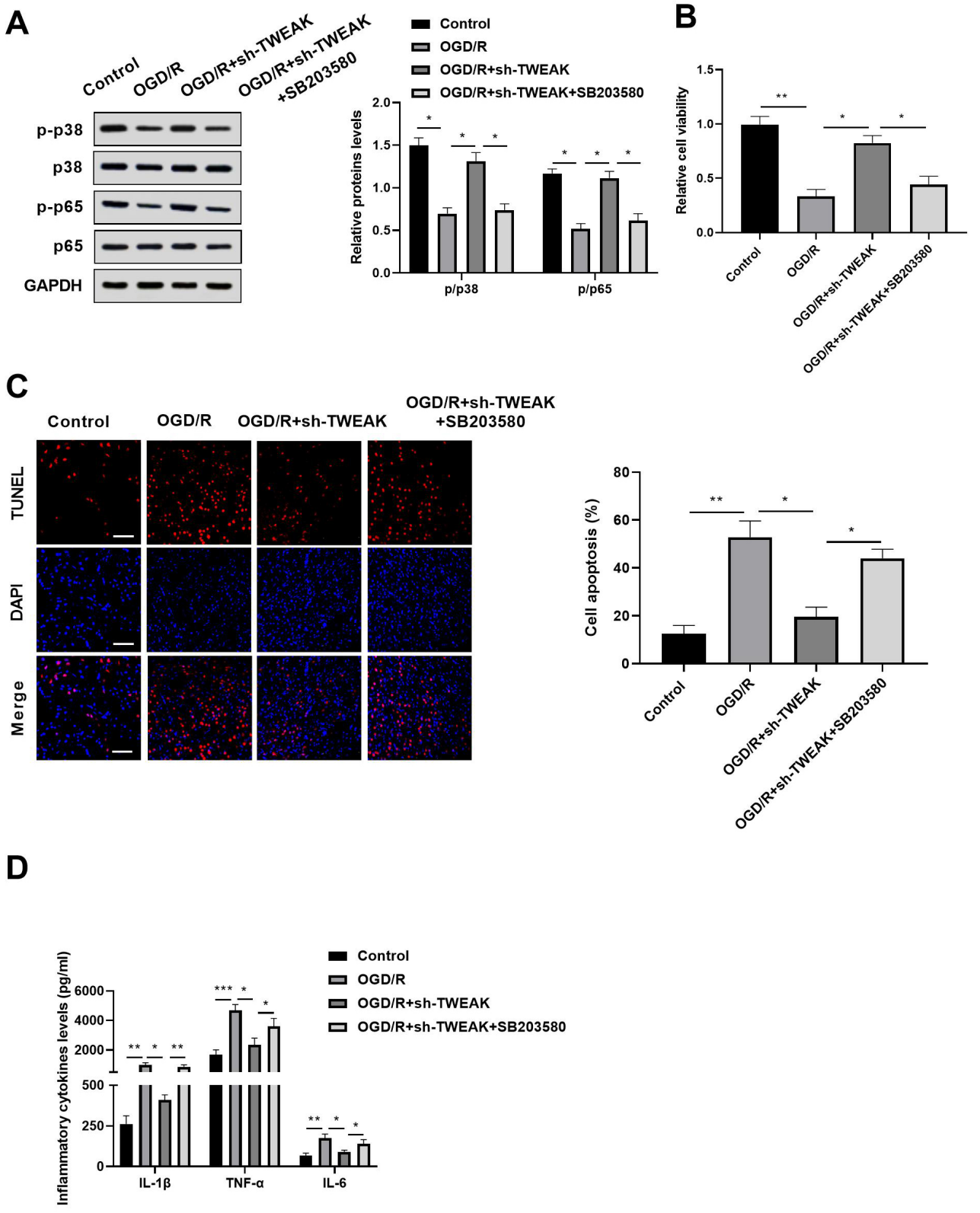

Fig. 6. The p38/MAPK signaling pathway inhibitor reversed the neuroprotective effects of sh-TWEAK on OGD/R injury. Neuronal cells were pretreated with the SB203580 or along with sh-TWEAK prior to OGD/R operation (A) Western blotting analysis of the levels of p-p38 and p-p65. (B) CCK-8 analysis of neuronal cells viability. (C) TUNEL analysis of neurons apoptosis (Scale bar = 25 μm). (D) ELISA analysis of the expressions of inflammatory cytokines (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

A prior study discovered that TWEAK and Fn14 were up-regulated in ischemic stroke patients and mice [34]. Another study showed that the TWEAK/Fn14 axis was involved in the pathogenesis of ischemic stroke by mediating neuronal cells apoptosis and blood-brain barrier disruption [35]. Moreover, a report showed that intracerebroventricular administration of TWEAK in adult mice triggered neuronal apoptosis, resulting in cognitive dysfunction [36]. Based on the above results, we guessed that TWEAK might be taken part in the development of CA/CPR-induced brain injury. Here, our findings showed that TWEAK and Fn14 were up-regulated in the cerebral cortex of CA/CPR rats and OGD/R neuronal cells. Moreover, down-regulation of TWEAK promoted neuronal cell viability, alleviated apoptosis and inflammation upon CA/CPR *in vitro* and *in vivo*, exhibiting significantly better neuroprotective function after CA/CPR.

Substantial evidence suggest that TWEAK activates the p38 MAPK signaling pathway, which can promote inflammation and apoptosis [37]. A previous study has reported that after brain injury, inflammation can rapidly activate microglia and induce the production of inflammatory cytokines, leading to secondary inflammation-related brain injury, and inhibition of p38 MAPK pathway can reduce microglia activation and inflammatory factor overexpression caused by oxidative stress injury [38]. However, p38 MAPK has also been reported to play a neuroprotective role in cortical cell apoptosis during acute and subacute brain injury [39,40]. Moreover, the upregulation of p38 MAPK signaling plays a neuroprotective role in the apoptotic injury of subacute cerebral ischemia (7 days) [41]. In view of the role of TWEAK knockdown in protecting the brain from ischemic injury reported by Echeverry *et al.* [42], we hypothesized that TWEAK played an important role in brain injury after CA/CPR via p38 MAPK/NF-κB pathway. Our results revealed that TWEAK knockdown facilitated the activation of the p38 MAPK/NF-κB pathway in the cerebral cortex of CA/CPR rats and OGD/R-treated neuronal cells. Intervention with p38 MAPK inhibitor significantly weakened the effects of sh-TWEAK on OGD/R injury. The findings suggest that down-regulation of TWEAK indeed improved brain injury after CA/CPR via activating the p38 MAPK/NF-κB pathway.

There were limitations in this present study, this study has not yet interfered with the p38 MAPK/NF-κB signaling pathway *in vivo* to verify the mechanism of TWEAK. At the same time, the role of TWEAK in regulating neurogenesis and angiogenesis still needs to be further explored. We will further explore these shortcomings in follow-up experiments.

Conclusions

Our study confirmed that TWEAK had a significant effect on CA/CPR-mediated brain injury. Down-regulation of TWEAK markedly elevated neuronal cell viability and attenuated apoptosis after OGD/R injury. Furthermore, TWEAK knockdown attenuated cell apoptosis and inflammation in CA/CPR rats, exhibiting significantly better behavioral tests after CA/CPR through the p38 MAPK/NF-κB signaling pathway. This study might provide an important reference for the treatment and prognosis of brain injury in CA survivors.

Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author Contributions

RW designed and supervised the research. HZ performed the research and drafted the manuscript. RW and HZ performed data analysis and statistical analysis. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Hebei University of Engineering (approval no. HEU2020021008).

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Conflict of Interest

The authors declare no conflict of interest.

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