Targeted delivery of Neurogenin-2 protein in the treatment for cerebral ischemia-reperfusion injury

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A B S T R A C T

Neurogenin-2 (Ngn2), as a proneural gene that promotes the survival and differentiation of neural precursor cells, is an attractive candidate for therapy against cerebral ischemia-reperfusion injury. However, the delivery approach limits its clinical application. To deliver Ngn2 protein into the cerebral ischemic region and exert a therapeutic effect on injured neurons after ischemia, we here reported that the fusion protein TAT-LBD-Ngn2 was constructed by fusing a transactivator of transcription (TAT) domain and a laminin-binding domain (LBD) to Ngn2. TAT-LBD-Ngn2 promoted the outgrowth of neuronal neurite, increased the survival rate and alleviated apoptosis of hippocampal neurons exposed to oxygen glucose deprivation in vitro. Furthermore, a focal cerebral ischemia model in C57BL/6 mice showed that TAT-LBD-Ngn2 efficiently crossed the blood brain barrier, aggregated in the ischemic zone and was consistently incorporated into neurons. Moreover, TAT-LBD-Ngn2 transduced into brains attenuated neuronal degeneration and apoptosis in the ischemic zone. TAT-LBD-Ngn2 treatment resulted in a reduction of infarct volume that was associated with a parallel improvement in neurological functional outcomes after reperfusion. In conclusion, the targeted delivery of TAT-LBD-Ngn2 into the ischemic zone attenuated cerebral ischemia-reperfusion injury through the inhibition of neuronal degeneration and apoptosis, suggesting that TAT-LBD-Ngn2 is a promising target candidate for the treatment of ischemic stroke.

1. Introduction

Stroke remains a vexing public health problem. Stroke is currently the second leading cause of death in the Western world and causes 10% of deaths worldwide. Stroke, which disables 75% of its survivors, is also the leading cause of adult disability in the United States and Europe [1]. However, limited advances have been made in developing therapies to counter the deleterious effects of ischemic stroke, with the only available treatment being thrombolyis with tissue plasminogen activator (tPA) [2]. Unfortunately, due to the narrow therapeutic window (<4.5 h) and safety concerns, less than 5% of all patients are treated with tPA. Thus, the development of treatment strategies for cerebral ischemic injury is a high priority.

Neurogenin-2 (Ngn2), a gene encoding a basic-helix-loop-helix (bHLH) transcription factor located on chromosome 3, regulates signaling pathways that identity and specify neuronal fate in the central nervous system [3,4]. Many studies have demonstrated that Ngn2 can promote neuronal differentiation, subtype specification, maturation and integration in different regions of the brain [5–8]. Moreover, transplanting Ngn2-transduced neural precursor cells (NPCs) enhanced neuronal yield and donor cell survival [9], and the transitory expression of Ngn2 in embryonic progenitors is sufficient to stimulate axonal sprouting and improve motor recovery after severe spinal cord injury [10]. These results suggest that Ngn2 may be an attractive candidate for the treatment of neurological disease. However, the functions of Ngn2 were investigated only in neurons that were transiently transfected with Ngn2 gene in vitro [9,11].

A popular treatment strategy involves gene therapy, but its use in the clinic is hampered by barriers to gene transfection and...
efficient expression. To avoid the shortcomings of gene therapy, many reconstructive protein drugs have been introduced for clinical applications, including insulin [12], erythropoietin (EPO) [13] and interferon [14]. Thus, in the present study, we investigated the potential use of Ngn2 protein to treat cerebral ischemic injury.

However, in practice, the delivery of large therapeutic proteins into the brain parenchyma has been hampered by the presence of the blood–brain barrier (BBB) [15]. Another hurdle is whether these therapeutic proteins can aggregate in the cerebral ischemic zone. It has been demonstrated that fusion proteins containing the protein transduction domain (PTD) derived from a human immunodeficiency virus (HIV) transactivator of transcription (TAT) are able to cross cell membranes and the BBB after systemic administration, which represents a promising strategy for treating experimental brain injury, as reported by us and others [16–18]. Laminin in the ischemic region could be viewed as a potential therapeutic binding target for the delivery of exogenous protein to ischemic brain parenchyma [19]. The laminin-binding domain (LBD) from the first 135 amino acids of agrin, which has a high laminin affinity [20], is able to target therapeutic proteins to laminin.

Therefore, this study focused on the generation of a TAT-LBD-Ngn2 fusion protein and the assessment of its biological function. We generated the fusion protein TAT-LBD-Ngn2 in Escherichia coli BL21 and examined the bioactivity of TAT-LBD-Ngn2, especially its cytotoxicity and ability to cross the cell membrane of hippocampal neurons. Then, we evaluated the protective effect of TAT-LBD-Ngn2 in hippocampal neurons that had been exposed to oxygen glucose deprivation (OGD) in vitro. Next, we examined whether TAT-LBD-Ngn2 could aggregate in the ischemic zone through the BBB following middle cerebral artery occlusion (MCAO) injury. Finally, we tested whether TAT-LBD-Ngn2 could alleviate cerebral ischemia injuries after MCAO.

2. Materials and methods

2.1. Preparation of fusion proteins

The mouse Ngn2 peptide sequence consists of 263 amino acid residues. The LBD peptide sequence consists of 134 amino acid residues. LBD and Ngn2 were linked by a linker GGGGS sequence. The LBD-Ngn2 cDNA sequence was synthesized by Genscript Co. (Nanjing, China) and included NcoI and XhoI site sequences at 5′ and 3′. The synthesized product was digested with NcoI and XhoI restriction enzymes (TaKaRa, Japan) and cloned into the NcoI/XhoI sites of the pTAT-HA vector. The final expression plasmid was named pTAT-LBD-Ngn2 and contained a (His)6 tag, a TAT sequence (YGRKKRRQRRR) and an LBD-Ngn2 sequence, as shown in Fig. 1A, B. The pTAT-LBD-Ngn2 was transformed into the E. coli strain BL21 (DE3) pLysS (Stratagene, La Jolla, CA, USA). And then the BL21 strain was induced with 500 μM IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma, USA) at 37°C for 4 h. The TAT-LBD-Ngn2 protein was purified with a Ni2+-nitrilotriacetic acid superflow agarose column according to the protocol described in the literature [21].

TAT-LBD-Ngn2 fusion protein was identified by Western blot. The primary antibodies used were anti-(His)6 mouse monoclonal antibody (1:1000, Abcam, Cambridge, MA, USA) and anti-Ngn2 rabbit monoclonal antibody (1:2000, Abcam, USA), followed by horseradish peroxidase (HRP)-conjugated goat polyclonal antibody to mouse IgG (1:10,000; Abcam, USA) or HRP-conjugated goat polyclonal antibody to rabbit IgG (1:10,000; Abcam, USA). LBD-Ngn2, TAT-Ngn2 and TAT-LBD fusion proteins (as controls) were expressed and purified as TAT-LBD-Ngn2.

![Fig. 1. Construction, expression and purification of the fusion proteins. (A) The schematic diagram of the four types of recombinant plasmid. (B) The schematic diagram of the four types of fusion proteins. (C) The noninduced, induced and purified TAT-LBD-Ngn2 protein lysates were visualized by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and identified by Western blot using anti-(His)6 and anti-Ngn2 antibodies. (D) Effect of the purified fusion proteins on neuronal viability by MTT assay. The data were presented as the mean ± SEM from three times independent experiments.](http://dx.doi.org/10.1016/j.biomaterials.2013.07.076)
2.2. Animals

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and was conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University (X’An, China). C57BL/6 mouse embryos (E18.5) and male C57BL/6 mice (aged 14 weeks, weighing 22–25 g) were provided by the Experimental Animal Center of the Fourth Military Medical University. The protocol of this study was conducted in accordance with the guidelines for the care and use of laboratory animals of The Fourth Military Medical University, and approval was obtained. During the experiments, all efforts were made to minimize animal suffering and the number of animals used.

2.3. In vitro experiment

2.3.1. Primary hippocampal neurons culture

Primary hippocampal neurons were cultured as described previously [22]. In brief, the neurons were cultured from C57BL/6 mouse embryos (E18.5), washed with D-Hank’s solution three times in sterile conditions and then seeded at a density of 1 × 10^5 cells/cm² onto plates coated with poly-(L)-lysine (50 μg/ml; Sigma, USA). These cells were grown in Neurobasal medium (Gibco, Invitrogen Corp, USA) supplemented with 2% B27, 1% glutamine, and 1% penicillin/streptomycin (Sigma, USA) at 37 °C under a humidified incubator in air containing 5% CO₂. The purity of neurons was determined by immunocytochemistry for βIII-tubulin at 5-day after plated, which indicated that 95% of the cells in cultures were positive for βIII-tubulin (1:250; Millipore, Temecula, CA, USA) (data not shown).

2.3.2. Tat-LBD-Ngn2 biological activity assay in vitro

To study the function of Tat-LBD-Ngn2, we evaluated the safety of these purified fusion proteins for neurons in vitro. The neurons were cultured in the presence of Tat-LBD-Ngn2, Tat-LBD or Tat-Ngn2, at a concentration of 125 μg/l for 24 h. Cell viability was assessed using the methyl thiazolyl tetrazolium (MTT) assay, as previously described [23].

Next, the cell transduction ability of Tat-LBD-Ngn2 was assayed by immunofluorescent (IF) analysis [18]. In brief, the neurons were cultured with Tat-LBD-Ngn2 (125 μg/l) or LBD-Ngn2 (125 μg/l) for 6 h, washed 3 times with PBS after fixation in 4% PFA and then incubated simultaneously with 2 primary antibodies, i.e., anti-NeuN mouse monoclonal antibody (1:1000; Abcam, UK) and anti-Ngn2 rabbit monoclonal antibody (1:200; Abcam, USA), overnight at 4 °C. The neurons were washed 3 times with PBS and then incubated with two secondary antibodies, i.e., FITC-labeled goat anti-Rabbit IgG (1:200; Vector, Burlingame, CA, USA) and Alexa-594-labeled donkey anti-mouse IgG (1:500; Molecular Probes, Rockford, IL, USA), for 1 h at 37 °C. Nuclei were visualized by DAPI staining. Images were viewed with a confocal laser scanning microscope (BEGO; Olympus, Japan).

Neurite outgrowth was observed when the neurons were cultured with Tat-LBD-Ngn2 (125 μg/l), LBD-Ngn2 (125 μg/l) or Tat-Ngn2 (125 μg/l) for 72 h by immunostaining, using anti-βIII-tubulin mouse monoclonal antibody (1:500; Millipore, USA) and Alexa-594-labeled donkey anti-mouse IgG secondary antibody (1:500; Molecular Probes, USA). The neurite growth was measured as the linear distance from the point of exit to the end of the longest branch of that neurite [24]. These measurements were made on cells present in 10 randomly selected fields in each experiment and were repeated in at least three independent cultures.

2.3.3. Oxygen glucose deprivation (OGD) model

To test the neuroprotective function of Tat-LBD-Ngn2 against ischemic injury in vitro, the OGD model was used in our study as previously described [23]. In brief, we designed several groups: Control (no OGD), OGD+Tat-LBD-Ngn2 (125 μg/l) and OGD+Tat-LBD-Ngn2 (125 μg/l) and OGD+TAT-LBD-Ngn2 (250 μg/l) after reperfusion. At 6 h, 24 h, 48 h, or 72 h after reperfusion, the mice were anesthetized, and the brains were acquired. Brain sections were washed with PBS, permeabilized in 0.3% (v/v) Triton X-100 and 1% (v/v) bovine serum albumin (BSA) in PBS for 30 min, blocked for 30 min in blocking buffer [4% (v/v) normal mouse serum and 1% (v/v) BSA], and incubated for 1 h in blocking buffer containing rabbit anti-laminin antibody (1:500, Sigma, USA). Sections were further washed with PBS and incubated for 1 h in blocking buffer containing secondary antibody (FITC-labeled goat anti-rabbit IgG, 1:500; Molecular Probes, USA). Images were acquired and analyzed as described above.

2.4. In vivo experiment

2.4.1. Transient focal cerebral ischemia

In this study, focal cerebral ischemia was induced with a middle cerebral artery occlusion (MCAO) model in mice using an intraluminal filament technique, as described previously [26]. Reperfusion was accomplished by withdrawing the suture after 60 min of ischemia, and the wounds were sutured.

2.4.2. The expression of laminin in the cerebral ischemic region in the MCAO model

We observed a change in laminin expression in the cerebral ischemic region after reperfusion, using IF and Western blot as previously described [27]. In brief, C57BL/6 male mice (n = 5) were subjected to transient focal cerebral ischemia. At 1 h, 6 h, 24 h, 48 h, or 72 h after reperfusion, the mice were anesthetized, and the brains were acquired. Brain sections were washed with PBS, permeabilized in 0.3% (v/v) Triton X-100 and 1% (v/v) bovine serum albumin (BSA) in PBS for 30 min, blocked for 30 min in blocking buffer [4% (v/v) normal mouse serum and 1% (v/v) BSA], and incubated for 1 h in blocking buffer containing rabbit anti-laminin antibody (1:500, Sigma, USA). Sections were further washed with PBS and incubated for 1 h in blocking buffer containing secondary antibody (FITC-labeled goat anti-rabbit IgG, 1:500; Molecular Probes, USA). Images were acquired and analyzed as described above.

The total protein concentration of brain tissues was analyzed using a BCA kit (Nanjing Jiancheng, China). Blots were probed with mouse monoclonal antibodies against Bcl-2 and Bax (1:1000, Santa Cruz, CA, USA) and β-actin (1:2000; Anbo, San Francisco, CA, USA). The appropriate secondary antibodies were used. Three times independent experiments were performed.

2.4.3. Distribution and total concentration of Tat-LBD-Ngn2 aggregation in the ischemic region

To assess the degree of Tat-LBD-Ngn2 and Tat-Ngn2 aggregation in the ischemic region in vivo, C57BL/6 male mice (n = 5 for each group) were subjected to MCAO and received an intraperitoneal injection of Tat-LBD-Ngn2 (250 μg/kg), Tat-Ngn2 (250 μg/kg) or LBD-Ngn2 (250 μg/kg) after reperfusion. At 6 h, 24 h, 48 h, or 72 h after reperfusion, the mice were anesthetized, and the brains were removed for IF and Western blotting as described above [27]. The primary antibody was a mouse monoclonal antibody against His (His₁₉, 1:1000; Abcam, USA), and the secondary antibody was Alexa Fluor 594-conjugated anti-mouse IgG (1:1000; Molecular Probes, USA). The images were acquired and analyzed as described above.

The total protein concentration of brain tissues was analyzed using a BCA kit (Nanjing Jiancheng, China). Blots were probed with mouse monoclonal antibodies against His (His₁₉, 1:1000; Abcam, USA), and β-actin (1:2000; Anbo, USA), followed by HRP-conjugated goat secondary antibody against rabbit IgG (1:10,000; Abcam, USA).

2.4.4. Assay the incorporation of Tat-LBD-Ngn2 in neurons in the ischemic brain

To test whether the protein was located in neurons, double-labeling experiments were performed in ischemic cerebral cortex sections by combining anti-(His)6 with immunohistochemistry for Neuonal Nuclei (NeuN) to confirm that Tat-LBD-Ngn2 was incorporated into neurons. In brief, C57BL/6 male mice (n = 5) were...
subjected to transient focal cerebral ischemia and received an intraperitoneal injection of TAT-LBD-Ngn2 (250 μg/kg) after reperfusion. At 6 h, 24 h, 48 h, or 72 h after reperfusion, the mice were anesthetized, and ischemic brains were acquired. Brain sections were washed with PBS, permeabilized in 0.2% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min, blocked for 30 min in blocking buffer [4% (v/v) normal mouse serum and 1% (w/v) BSA], and incubated for 16 h in blocking buffer containing mouse monoclonal antibody against (His)6 (1:1000; Abcam, USA) and rabbit monoclonal antibody against NeuN (1:20,000; Abcam, USA). Sections were further washed with PBS and incubated for 1 h in blocking buffer containing secondary antibody (FITC-labeled goat anti-rabbit IgG, 1:2000; Molecular Probes, USA, and Alexa Fluor 594-conjugated anti-mouse IgG, 1:1000; Molecular Probes, USA). We also performed the double-labeling experiments in the intact ischemic cerebral cortices as above. The brain sections containing intact ischemic cerebral cortices were mainly selected for the image capture and double-labeling cell counts.

2.4.5. Assessment of the neuroprotective effect of TAT-LBD-Ngn2 in vivo

To assess the neuroprotective effect of TAT-LBD-Ngn2 in vivo, we used Fluoro-Jade C (FJC) staining, which can label dying or degenerative neurons in the brain. FJC was purchased from Chemicon (AG325, Chemicon, USA). After the induction of MCAO, C57BL/6 mice (n = 8 for each group) were immediately injected intraperitoneally with TAT-LBD-Ngn2 (250 μg/kg), TAT-Ngn2 (250 μg/kg) or PBS, and the injection was repeated daily for 3 days. At 72 h after reperfusion, the ischemic brains were acquired and the test steps of FJC were described as previous studies [28,29]. The FJC-positive stain exhibited strong green color under the same filter system used for activating fluorescein. The FJC-stained slides were observed and counted under a fluorescence microscope or a laser scanning confocal microscope (LSCM, Olympus, FV300).

Apoptosis in the ischemic region was also assessed in situ by TUNEL staining. The slides were observed and counted under a microscope. The TUNEL staining was quantitatively evaluated using the method as described in our previous study [27]. Using the previously described treatment, at 72 h after reperfusion, mice from the three groups (n = 8 for each group) were decapitated, and 1-mm-thick coronal sections from throughout the brain were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) to evaluate the infarct volume [30].

2.5. Statistical analysis

SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The neurological deficit scores (such as Longa scores) were expressed as the median (range) and were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney U statistic with Bonferroni's correction. Other values were reported as the mean ± SEM and were analyzed among groups by multiple one-way analysis of variance. When indicated by a significant F ratio, post-hoc testing was performed with Scheffé’s test. Statistical significance was defined as P < 0.05.

3. Results

3.1. Preparation of the TAT-LBD-Ngn2 fusion protein

As shown in Fig. 1C, TAT-LBD-Ngn2 protein was effectively induced and purified, as confirmed by SDS-PAGE. Meanwhile, the specificity of the expressed TAT-LBD-Ngn2 was further
Fig. 3. Effect of TAT-LBD-Ngn2 on cell viability, LDH release and cell apoptosis in hippocampal neurons exposed to OGD. (A) The cell viability. (B) LDH release. The Control group (normal cell group) was defined as 100%. (C) Representative fluorescent micrographs of TUNEL staining. The TUNEL-positive neurons were shown in green, and the nuclei were stained blue with DAPI. Scale bars = 100 μm. (D) Statistical analysis of the numbers of TUNEL-positive neurons in each group. *P < 0.05; **P < 0.01; ***P < 0.001.

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verified by Western blot using specific anti-(His)_6 and anti-Ngn2 antibodies. We also produced and purified other control proteins: LBD-Ngn2, TAT-Ngn2, and TAT-LBD (data not shown). Furthermore, we assessed the safety of the four purified fusion proteins in normal hippocampal neurons. These proteins did not show toxicity in normal hippocampal neurons upon incubation for 24 h (Fig. 1D).

3.2. The effect of TAT-LBD-Ngn2 on neurite outgrowth

To assess the transducible ability of TAT-LBD-Ngn2 in vitro, the hippocampal neurons were incubated with TAT-LBD-Ngn2 or LBD-Ngn2 at a concentration of 125 μg/l for 6 h. TAT-LBD-Ngn2 was transduced into the cells and accumulated in the cell body, whereas fluorescence signals were absent in cells treated with LBD-Ngn2 (Fig. 2A). These results indicated that the TAT-mediated LBD-Ngn2 fusion protein had the ability to be transduced into hippocampal neurons.

To assess the effect of TAT-LBD-Ngn2 on outgrowth of neuronal neurites in vitro, the hippocampal neurons were incubated with TAT-LBD-Ngn2, TAT-Ngn2 and LBD-Ngn2 (125 μg/l) for 72 h. As shown in Fig. 2B, C, the neurites were longer in both TAT-LBD-Ngn2 and TAT-Ngn2 groups than those in the Control or LBD-Ngn2 groups (P < 0.05), suggested that the Ngn2 protein can promote the outgrowth of neuronal neurites.

3.3. Assay of hippocampal neurons survival exposed to OGD

The cell viability in the TAT-LBD-Ngn2 (62.5, 125 or 250 μg/l) group was significantly higher than in either the OGD group

Fig. 4. Effect of TAT-LBD-Ngn2 on caspase-3, Bax and Bcl-2 in hippocampal neurons exposed to OGD. (A) Caspase-3 activity at 24 h after OGD exposure. (B) Expression of Bcl-2 and Bax protein at 24 h after OGD exposure. (C, D) Statistical analysis of Bcl-2 and Bax protein expression. *P < 0.05, **P < 0.01.

Fig. 5. The temporal expression of laminin in the cerebral ischemic region in mice (n = 5). (A) The temporal expression of laminin after reperfusion. (B) Statistical analysis of laminin expression. *, P < 0.05; **, P < 0.01. (C) Immunofluorescence (IF) for laminin in the cerebral ischemic region at 6 h after reperfusion. The areas of the ischemic region are represented in the cartoons as gray shading on the lower left of the picture. Scale bars = 100 μm.

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(P < 0.01) or the TAT-LBD (P < 0.01) group (Fig. 3A). To further confirm the protective effect of TAT-LBD-Ngn2, an LDH assay was performed. TAT-LBD-Ngn2 treatment at different concentrations decreased OGD-induced LDH release (Fig. 3B).

### 3.4. Assay of hippocampal neurons apoptosis exposed to OGD

At 24 h after exposure to OGD, the number of TUNEL-positive cells in the OGD group was significantly higher than in the Control group (P < 0.001). However, the number of TUNEL-positive cells in TAT-LBD-Ngn2 groups was lower than in the OGD or the TAT-LBD groups (P < 0.05) (Fig. 3C, D). Moreover, OGD-induced caspase-3 activation was alleviated by different concentrations of TAT-LBD-Ngn2 treatment (P < 0.05), not by the TAT-LBD (Fig. 4A). These results indicated that TAT-LBD-Ngn2 can alleviate neurons apoptosis following OGD injury.

We also assayed the expression of Bax and Bcl-2 proteins 24 h after exposure to OGD injury (Fig. 4B). Treatment with TAT-LBD-Ngn2 increased the level of Bcl-2 protein (Fig. 4B, C) whereas decreased Bax expression (Fig. 4B, D) in a dose-dependent manner. However, the TAT-LBD (125 µg/l) did not affect the levels of Bax and Bcl-2 expression.

### 3.5. Aggregation effect of TAT-LBD-Ngn2 in the ischemic region

In order to explore the targeted delivery of TAT-LBD-Ngn2, the expression of laminin in the cerebral ischemic region was assessed at first. Our results showed that the expression of laminin was upregulated remarkably at 6 h, and lasted to 72 h after reperfusion (Fig. 5).

Next, the distribution and accumulation of the fusion proteins in the ischemic region were determined after a single dose (250 µg/kg) of injection. The results showed that both TAT-LBD-Ngn2 (Fig. 6A) and TAT-Ngn2 (Fig. 6B), but not LBD-Ngn2 (Fig. 6C) distributed in the cerebral ischemic region. Meanwhile, the accumulation of TAT-LBD-Ngn2 in the cerebral ischemic region was more abundant than the accumulation of TAT-Ngn2. Furthermore, the amount of TAT-LBD-Ngn2 peaked at 24 h after reperfusion and decreased gradually in subsequent time (Fig. 6D, E).

Then, we examined whether neurons in the cerebral ischemic region could incorporate TAT-LBD-Ngn2. The result showed that NeuN/His double-labeled neurons were not only in intact cortex but also in ischemic region (Fig. 7A, B). Meanwhile, the number of NeuN/His double-labeled neurons in the cerebral ischemic region was increasing from 6 h to 24 h after reperfusion but decreased in subsequent time (Fig. 7D).

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![Fig. 6. The levels of TAT-LBD-Ngn2, TAT-Ngn2 and LBD-Ngn2 in the cerebral ischemic region in mice (n = 5 for each group). Representative IF staining of TAT-LBD-Ngn2 (A), TAT-Ngn2 (B) and LBD-Ngn2 (C) using anti-(His)6 monoclonal antibody at 24 h after reperfusion. The arrow denotes the positive signal. Scale bars = 100 µm (D) TAT-LBD-Ngn2 protein levels in the cerebral ischemic region after reperfusion. (E) Statistical analysis of TAT-LBD-Ngn2 protein levels.](http://dx.doi.org/10.1016/j.biomaterials.2013.07.076)
3.6. Neuronal degeneration and apoptosis analysis in vivo

To assess neuroprotection of TAT-LBD-Ngn2, the FJC and TUNEL staining on ischemic brain sections at 72 h after reperfusion were performed. The number of FJC-positive neurons was fewer in TAT-LBD-Ngn2 and TAT-Ngn2 groups. Importantly, fewer FJC-positive neurons were observed in the TAT-LBD-Ngn2 group than in the TAT-Ngn2 group ($P < 0.05$) (Fig. 8A, C). Similarly, the number of TUNEL-positive cells was fewer in TAT-LBD-Ngn2 and TAT-Ngn2 groups. Meanwhile, fewer TUNEL-positive cells were observed in the TAT-LBD-Ngn2 group than in the TAT-Ngn2 group ($P < 0.05$) (Fig. 8B, D).

3.7. Infarct volume and neurobehavior evaluation

As shown in Fig. 9A and B, brain infarct volume in the TAT-Ngn2 and TAT-LBD-Ngn2 groups was smaller compared with the MCAO group ($P < 0.05$). Interestingly, the TAT-LBD-Ngn2 treatment reduced brain infarct volume more effectively than TAT-Ngn2 ($P < 0.01$).

The neurobehavioral deficit was assessed by the Longa scores. As shown in Fig. 9C, the Longa scores of the TAT-LBD-Ngn2 group were lower than those of the TAT-Ngn2 group ($P < 0.05$) at 72 h after reperfusion.

Moreover, the motor function recovery was also evaluated by the open-field test. Mice in the TAT-LBD-Ngn2 group traversed a greater distance in the central area than TAT-Ngn2 group ($P < 0.05$) at 72 h after reperfusion (Fig. 9D). Further, the duration of movement in the outer ring was longer in the TAT-LBD-Ngn2 group than in the TAT-Ngn2 group ($P < 0.05$) at 72 h after reperfusion (Fig. 9E).

4. Discussion

Few neurological conditions are as complex and devastating as stroke, which is one of the leading causes of morbidity and mortality worldwide [33,34]. Because of the complex pathophysiological process and limitations in therapies, cerebral ischemic injury claims hundreds of thousands of lives every year worldwide [35].

Ngn2 is an attractive candidate for stroke treatment because it is a proneural gene that has been well studied [36]. A recent study has demonstrated that Ngn2 could activate the expression of NeuroD, which is a bHLH gene that participates in a genetic pathway and regulates the balance between progenitor maintenance and differentiation [37]. Furthermore, Ngn2 could enhance the survival and differentiation of neural precursor cells after transplantation [9]. Another report showed that grafted human embryonic progenitors transfected with a plasmid expressing Ngn2 could stimulate axonal sprouting and improve motor recovery after severe spinal cord injury [10]. Due to the function of Ngn2 in neural development, maturation and stimulating axonal sprouting, Ngn2 may be a valuable molecule in therapy for cerebral ischemic injury.

The delivery of Ngn2 to targeted sites by gene transfection is far from clinical application. In the present study, we investigated the therapeutic benefits of Ngn2 on cerebral ischemic injury through the delivery of Ngn2 recombinant fusion protein instead of gene transfection. However, the therapeutic proteins across the BBB is restricted to short (<6 amino acids) and highly lipophilic peptides [15,38]. Therefore, it is difficult to generate pharmacologically active concentrations of Ngn2 protein that are able to cross the BBB in brain by systemic injection in vivo. It is well known that the PTD
of TAT protein is a promising tool for disease therapy because of its ability to deliver a variety of full-length peptides through the plasma membrane into cells and, most importantly, across the BBB into brain tissue [39,40]. Our study and other studies have shown that various TAT fusion proteins are safe and efficiently transported in vitro and in vivo [16,18,27]. A good example is Tat-NR2B9c, an inhibitor of postsynaptic density-95 protein, which has been demonstrated to safely and efficiently reduce ischemic brain damage in cynomolgus macaques and human beings [41,42]. Thus, using the PTD of TAT for protein transduction represents a promising strategy for treating a majority of neurological diseases including ischemic stroke.

However, in the delivery of protein drugs, ineffective diffusion should be avoided. The concentration of protein drugs in the cerebral ischemic region is the key for therapeutic efficacy [19]. Therefore, targeting delivery is a promising approach for Ngn2 because it would allow effective treatment of the target tissue while reducing adverse effects on normal tissues. Laminin, an important extracellular matrix protein in brain, is upregulated within the ischemic region [19,43] after cerebral ischemic injury.

![Fig. 8. Effects of TAT-LBD-Ngn2 on neuronal degeneration and apoptosis following focal ischemic injury in mice (n = 8 for each group). (A) Representative FJC staining to detect neuronal degeneration in the cerebral ischemic region at 72 h after reperfusion. Scale bars = 100 μm. (B) Representative TUNEL staining at 72 h after reperfusion. Scale bars = 100 μm (C) and (D) Statistical analysis of the numbers of FJC-positive and TUNEL-positive cells respectively. *P < 0.05.]

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Previous studies have shown that the LBD exhibits a high affinity interaction with the coiled-coil domain of laminin [44]. Additionally, the LBD is helpful for the sustained release of the LBD fusion protein from laminin, as reported by Cao et al. [45]. In addition, treatment with LBD-BDNF (brain-derived neurotrophic factor) attenuated neural degeneration after focal cerebral ischemia and showed improved neurological functional outcome and neurogenesis in the hippocampal dentate gyrus [19]. In the current study, laminin was gradually upregulated from 6 h to 72 h after reperfusion in cerebral ischemic region, provided a reasonable window of therapy and supported a model in which laminin could be used for targeting Ngn2 protein delivery after reperfusion.

Therefore, to target Ngn2 delivery to the ischemic zone, we fused TAT and LBD [20] to native mouse Ngn2 and generated a fusion protein, TAT-LBD-Ngn2. Our results showed that TAT-LBD-Ngn2 could not only be efficiently delivered into hippocampal neurons when added exogenously in culture medium but that it could also cross the BBB to aggregate in the ischemic zone after reperfusion when injected intraperitoneally. Moreover, the accumulation of TAT-LBD-Ngn2 in the cerebral ischemic region was higher than that of TAT-Ngn2, and TAT-LBD-Ngn2 was consistently incorporated into neurons in the ischemic region. Our finding indicated that TAT-LBD-Ngn2 was efficiently targeted for delivery into neurons in the ischemic zone, and these findings were consistent with those obtained in previous studies [18,42].

Fig. 9. Effects of TAT-LBD-Ngn2 on infarct size and neurobehavioral function following focal ischemic injury in mice (n = 8 for each group). (A) Representative brain infarct size by TTC staining at 72 h after reperfusion. (B) Statistical analysis of the infarct size in every group (% of contralateral hemisphere). *P < 0.05, **P < 0.01. (C) The Longa scores at 24 h, 48 h and 72 h after reperfusion. *P < 0.05. (D, E) The open-field test was used to assess motor function recovery at 72 h after reperfusion. *P < 0.05.
Cerebral ischemia causes significant damage resulting from the insufficient supply of glucose and oxygen to the brain tissue, thus promoting cellular damage and death [23,46]. At first, the therapeutic benefits of TAT-LBD-Ngn2 against cerebral ischemia injury were evaluated in an OGD model in vitro. Our results showed that TAT-LBD-Ngn2 did not impair the viability and growth of hippocampal neurons in the dosage range used, implying that it was safe and had no cytotoxic effects on hippocampal neurons. Moreover, TAT-LBD-Ngn2 increased neuronal cell viability and reduced LDH release in hippocampal neurons after OGD injury in a dose-dependent manner, suggesting that TAT-LBD-Ngn2 exerted a cytoprotective effect on hippocampal neurons following OGD injury.

Many studies have shown that OGD injury can induce neuronal cell apoptosis [47]. The process of apoptosis includes various changes, such as nuclear and DNA fragmentation, caspases activation and increased expression of apoptosis-associated proteins [48,49]. In the present study, we found that TAT-LBD-Ngn2 inhibited neuronal apoptosis after hippocampal neurons were exposed to OGD. It is well known that apoptosis occurs through two main pathways: extrinsic and intrinsic apoptosis signaling. In the extrinsic or cytoplasmic pathway, caspases, the cytoplasmic aspartic-specific cysteine proteases, play an important role in apoptosis [49]. In the intrinsic or mitochondrial pathway, apoptosis is regulated by the anti-apoptotic Bcl-2 and pro-apoptotic Bax protein families [48]. The activation of caspase-3 and the expression of Bcl-2 and Bax were assessed in our study. The results from these assays revealed that TAT-LBD-Ngn2 inhibited caspase-3 activation, increased Bcl-2 expression and decreased Bax expression in a dose-dependent manner, which ultimately resulted in lower levels of nuclear DNA degradation and cellular apoptosis.

We further examined whether TAT-LBD-Ngn2 could provide neuroprotective effects in a mouse focal cerebral ischemia model induced by transient middle cerebral artery occlusion (MCAO). It has been reported that thousands of neurons degenerate during the early stage of transient focal cerebral ischemia injury by MCAO [29] and that many apoptotic neurons are localized primarily in the cerebral ischemic region in the inner boundary zone of the infarct in mice [32]. In the present study, we used Fluoro-Jade C staining, a reliable and convenient method for identifying neuronal degeneration [28,29], and TUNEL staining to observe neuronal apoptosis at 72 h after reperfusion. Our results showed that TAT-LBD-Ngn2 significantly decreased the numbers of FJC-positive cells and TUNEL-positive cells in the cerebral ischemic region in comparison to TAT-Ngn2, suggesting that TAT-LBD-Ngn2 could rescue neurons undergoing ischemic injury from degeneration and apoptosis. In addition, TAT-LBD-Ngn2 rendered a greater reduction in infarct size and better improvement of motor function than did TAT-Ngn2 treatment. These more beneficial effects compared to TAT-Ngn2 were in accordance with the greater accumulation of TAT-LBD-Ngn2 in the cerebral ischemic region because of LBD presence. Thus, TAT-LBD-Ngn2 provided better neuroprotective effects against ischemic cerebral injuries than TAT-Ngn2.

This study verified that the injection of TAT-LBD-Ngn2 resulted in a lower degree of neuronal apoptosis and degeneration, reduced infarct volume and improved neurological behavior function in a mouse model of MCAO. These data supported this Ngn2-targeting therapy as a useful approach for the treatment of stroke. The new function of Ngn2 was supported by previous studies, in which the transduction of Ngn2 into donor NPCs before transplantation dramatically enhanced the neuronal yield and donor cell survival [9] and in which the transitory expression of Ngn2 in embryonic progenitors improved motor recovery after severe spinal cord injury [10]. In this study, we mainly focused on the therapeutic effects of TAT-LBD-Ngn2 in early stages of focal cerebral ischemia-reperfusion injury. Our data suggest that anti-apoptosis is one of the beneficial effects of TAT-LBD-Ngn2 in focal cerebral ischemia. However, how to attenuate cerebral ischemic injury and improve neurological behavior function with TAT-LBD-Ngn2 needs to be explored. It is well documented that Ngn2 plays a key role in dentate neurogenesis during development and that the expression of Ngn2 increases the population of young neurons among donor NPCs [11]. While a majority of cortical neurons derive from Ngn2+ progenitors, local-circuit cortical interneurons originate in the ganglionic eminences from Ngn2−, Mash1+ progenitors that subsequently migrate dorsally into the neocortex and hippocampus [50]. Thus, the role of neurogenesis in the therapeutic effects of TAT-LBD-Ngn2 on ischemic stroke must be explored in future work.

5. Conclusions

To our knowledge, the present study demonstrates that the fusion protein TAT-LBD-Ngn2, exogenous Ngn2 fused with LBD and TAT domains, can be directly transduced into cultured hippocampal neurons and exhibits a cellular protective function against oxygen and glucose deprivation as well as promotes neurite outgrowth in vitro. Furthermore, using a focal cerebral ischemia model in mice, intraperitoneal injection of TAT-LBD-Ngn2 is efficiently transduced into the brain parenchyma and aggregates in the ischemic zone. TAT-LBD-Ngn2 treatment attenuates neuronal degeneration and apoptosis in the ischemic zone and results in a reduction of the infarct volume that is associated with a parallel improvement in neurological functional outcomes after reperfusion. This finding suggests that the targeted delivery of TAT-LBD-Ngn2 fusion proteins may be an efficient therapeutic agent for neuroprotection and functional recovery in stroke treatment. However, the long-term effect of TAT-LBD-Ngn2 on brain plasticity after focal ischemia deserves further investigation, and the associated molecular mechanisms underlying the TAT-LBD-Ngn2 neuroprotection must be further explored.

Accession numbers

Xingchun Gou, Zhiqing Zhao, Qiang Wang, Lixian Xu conceived and designed the experiments; Bin Deng, Xingchun Gou, Hai Chen, Liya Li, Hao Xu, Qiang Wang performed the experiments and analyzed the data; Bin Deng, Xingchun Gou, Qiang Wang, Lixian Xu wrote the paper.

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