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Original Article

Therapeutic effect of transmembrane TAT-tCNTF via Erk and Akt activation using *in vitro* and *in vivo* models of Alzheimer's disease

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Abstract: Suppressing Alzheimer's disease (AD) progression via its pathological characteristics, namely senile plaques and neurofibrillary tangles, is an efficient treatment approach. Numerous studies have indicated that ciliary neurotrophic factor (CNTF) not only promotes neuronal growth and maintains cell survival but also significantly reduces amyloid beta (Aβ) aggregation and deposition. In this study, transactivator of transcription (TAT) was linked to truncated ciliary neurotrophic factor (tCNTF) and expressed as a fusion protein, TAT-tCNTF, to overcome the transmembrane inability of CNTF. Accordingly, TAT-tCNTF was shown to automatically transport across biomembranes and enter cells mainly by macropinocytosis. Furthermore, TAT-tCNTF increased cell viability in hippocampal neurons treated with Aβ. After intracerebroventricular Aβ injection, mice exhibited amyloid deposits, which were significantly reduced after intraperitoneal TAT-tCNTF injection. Indeed, TAT-tCNTF significantly reduced Aβ-induced tau hyperphosphorylation, and yet barely affected amyloid precursor protein. Accordingly, it was possible to elucidate its potential pharmacological mechanism, with the working effect of TAT-tCNTF shown to be performed by specifically binding to its receptor, CNTFRα, and then activating the Extracellular regulated protein kinases (Erk) and Protein kinase B/Akt pathways exclusive of the Signal transducers and activators of transcription 3 (Stat3) pathway.

Keywords: Alzheimer's disease, TAT-tCNTF, amyloid beta-protein, blood-brain barrier, hippocampal neurons

Introduction

Alzheimer's disease (AD) is among the most complicated and refractory degenerative diseases, causing progressive memory disorders, cognitive impairments, and/or aphasia [1, 2]. In recent years, the incidence rate of AD has been on the rise as the global population ages. Despite urgent medical needs, cholinesterase inhibitors are currently the main therapy for AD, but they have no efficacy on modifying disease progression. It is estimated that more than 100 million people will suffer from AD by 2050 [3]. Consequently, it is vital that a new pharmacological approach is found. Senile plaques (SPs) and neurofibrillary tangles (NFTs) are two important pathological features of AD, with the former being an initiating factor that may cause neuronal degeneration [4-6]. The pathological characteristics of AD are principally detected in hippocampal regions, which are crucial to cognitive function [7]. Owing to the visible appearance of SPs and NFTs many years prior to the clinical symptoms of AD, these features are viewed as typical biomarkers to diagnose and prevent AD in its early stages [8].

SPs are mainly caused by Amyloid beta-protein (Aβ) deposition. In turn, Aβ is derived from the Amyloid precursor protein (APP) through two major enzymatic cleavage steps. Specifically, AD pathogenesis is due to sequential cleavage of APP by β-secretase (BACE1) first, followed by γ-secretase, which generates 39- to 43-amino acid Aβ peptide fragments [9-11]. With regard to Aβ peptides, $A\beta_{1-42}$ is particularly prone to aggregation, and self-assembles to form a heterogeneous mixture of oligomers and protofibrils, with deposition in SPs being fatal to neurons [12]. Increased astrogliosis and interleukin-1β (IL-1β) immunoreactivity is reported in microglia and neurons in animals

receiving $A\beta_{25-35}$ injections [13]. Another study recently revealed that $A\beta_{25-35}$ treatment inhibits the viability of murine hippocampal neurons (namely, HT22 cells) in a dose- and time-dependent manner, as well as dose-dependently impairing learning abilities in C57 mice [14]. This study also suggested that the PI3K/AKT/mTOR/p70S6K pathway was involved in $A\beta_{25-35}$-induced autophagy in both HT22 cells and C57 mice. Generally, Aβ is a complex biological molecule that interacts with many receptor types, which alternates under normal neuronal conditions. Aβ deposition affects synaptic function and leads to plaque formation, which then gradually aggravates neuronal and synaptic damage, followed by a large increase in hyperphosphorylated tau protein, before eventually leading to neurological dysfunction. Appropriately, the neuronal degeneration caused by Aβ is similar to the pathological changes of AD. Thus, in this study Aβ-injured models were adopted as available and efficient disease models to study the therapeutic effect of Transactivator of transcription (TAT) truncated ciliary neurotrophic factor (tCNTF) on AD.

Soluble Aβ oligomers (AβOs) are also associated with AD hallmarks (including phospho-tau, oxidative stress, and synapse loss) in cell-based systems and animal models [15]. Indeed, it has been shown that intra-cerebroventricular (i.c.v.) injection of AβOs into macaque brain induces astrocyte and microglial activation, synapse loss, phospho-tau, and NFT formation in regions associated with cognitive functions and operant behavior [16]. Moreover, using tau knockout mice, another study found that tau ablation protects against Aβ-induced cognitive impairment, hippocampal neuronal loss, and iron accumulation [17]. This study also reported that Aβ toxicity is mediated by tau, with this downstream effector of Aβ promising to be a tractable therapeutic target. Tau is a microtubule-associated protein involved in AD pathogenesis. There are about six different hyperphosphorylated tau isoforms in AD brain that lead to neurodegeneration [3]. The highly phosphorylated forms of tau (and in particular NFTs) are responsible for neuronal loss and neurotoxicity. Given this, some researchers have suggested that a therapeutic approach for AD may be achieved by reducing tau phosphorylation, which would be predicted to prevent tau aggregation and neuronal death [18]. Nevertheless, application of NFTs as therapeutic targets is still confronted with a series of problems, particularly limited blood-brain barrier (BBB) permeability [19].

In the past few years, research on ciliary neurotrophic factor (CNTF) has been an important area of neurobiology, as CNTF shows excellent activity in promoting neuronal growth and exerts a neuroprotective effect in neurons undergoing degeneration [20]. CNTF is a cytokine with neurotrophic and differentiation-inducing effects across a broad spectrum of peripheral and central nervous system cells. It enhances survival of sensory neurons, motor neurons, cerebral neurons, and hippocampal neurons. Additionally, it is widely distributed in the nervous system with various physiological functions, including an important role in neuronal nutrition [21]. Consequently, CNTF is a promising therapeutic medicine for neurodegenerative diseases such as AD. However, due to its short half-life, poor stability, lack of transmembrane domains and high immunogenicity, its clinical application is greatly restricted [22]. In light of these practical problems, structure modification and transformation at the gene level has been performed by expressing a recombinant stabilized protein with no immunogenicity [23].

Recombinant CNTF is only a partial solution for the above problems. Owing to its inability to be automatically transported across biomembranes to reach the target area, its clinical application is greatly limited. Even so, in view of its prospects for AD therapy, many studies are still in progress and some novel methods have been used to solve the delivery issue, e.g., recombinant cells secreting CNTF encapsulated in alginate polymers [24]. Nonetheless, delivery of CNTF to the brain remains problematic because of bioavailability restrictions imposed by the BBB. A cationic undecapeptide fragment derived from human immunodeficiency virus type 1 (HIV-1) TAT protein, termed the TAT protein transduction domain, has been shown to be an invaluable tool for delivering a wide variety of macromolecules across cell membranes and into intact tissues [25, 26]. TAT is an efficient transduction domain without the neurotoxicity [27]. After an initial ionic interaction, TAT-fusion proteins are rapidly internalized by lipid raft-mediated macropinocytosis, enabling previously unavailable large molecules to modulate *in vivo* biology and alleviate dis-

ease [28, 29]. Accordingly, in this study, we used gene recombination to link TAT with tCNTF. The fusion protein (TAT-tCNTF) was expressed in *Escherichia coli* and obtained by separation and purification. Our previous work revealed that TAT-tCNTF reduced amyloid deposition and improved memory and learning ability in Aβ-injured mice [30]. Further, TAT-tCNTF was demonstrated to promote human erythroleukemia cell line (TF-1) cell survival, induced cell differentiation in vitro, and protected SH-SY5Y cells from death following $A\beta_{25-35}$ exposure [31]. However, the transmembrane mechanism of TAT-tCNTF was not clear, nor its therapeutic action in *in vitro* and *in vivo* Aβ-injured models.

Thus, the aim of our present study using *in vitro* and *in vivo* models was to identify the transmembrane mechanism of TAT-tCNTF that enables it to cross biomembranes and exert its pharmacological action against AD. Furthermore, we propose TAT-tCNTF as a new pharmacological approach for AD, in which the advantages of CNTF are maintained and transmembrane capacity is improved.

Materials and methods

Cell culture and reagents

The human neuroblastoma cell (SH-SY5Y) was obtained from Institute of basic medical science, Chinese Academy of Medical Sciences (Beijing, China). SH-SY5Y were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA), supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, USA), and maintained at 37°C in an atmosphere of 5% CO_2. Primary hippocampal neurons were isolated from Wistar rats and cultured *in vitro* as described [32, 33].

CF488A-labeled TAT-tCNTF

TAT-tCNTF was labeled as a fluorescent protein using a Mix-n-Stain CF488A antibody labeling kit (Biotium, USA). The reaction buffer was warmed to room temperature and TAT-tCNTF solution cleaned with PBS before mixing both reagents at a ratio of 1:10. Next, the entire solution was transferred to a vial containing CF488A dye and incubated in the dark for 30 min at room temperature. Subsequently, the fluorescent protein, [CF488A]-TAT-tCNTF, was successfully generated.

Fluorescence microscopy and endocytosis inhibition assay

To examine internalization of TAT-tCNTF within cells, SH-SY5Y cells were incubated with 5 $\mu g \cdot ml^{-1}$ fluorescent CF488A-labeled TAT, recombinant human (rhCNTF), or TAT-tCNTF. After incubation for 1 h, the cells were washed and images acquired using a microscope (Observer A1; Zeiss Inc., Germany). To examine the effect of a macropinocytosis inhibitor on TAT-tCNTF entry to cells, SH-SY5Y cells were treated with 100 μM amiloride hydrochloride (AMI; Calbiochem, Germany) for 30 min, and then incubated with 5 $\mu g \cdot ml^{-1}$ CF488A-labeled TAT-tCNTF for 1 h. Fluorescent images were acquired using a CCD camera (Observer A1; Zeiss Inc., Germany).

Cell viability

Hippocampal neurons were plated onto 96-well plates at a density of 1.0×10^4 cells per well in serum-free medium. $A\beta_{25-35}$ (Sigma-Aldrich, USA) was dissolved in distilled water and incubated at 37°C for 1 week to transform to the aggregated phase before use. The Aβ injury group was treated with 10 μM $A\beta_{25-35}$ for 24 h, while the control group was not treated. TAT-tCNTF (100 ng/ml) was added to half the Aβ injury or control groups, and then incubated for another 24 h. At the end of incubation, 10 μl CCK-8 (Dojindo, Japan) was added to each well and incubated for an additional 2 h at 37°C. Samples were read using a Multiscan Microeliza Reader (iMark; Bio-Rad, USA) at 450 nm, and cell viability assessed by colorimetric assay.

Animal experiments

Six-month-old male C57BL/6 mice (22-26 g) were supplied by Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China) and group-housed in plastic rodent cages and maintained on 12-h light/dark cycles with *ad libitum* access to standard food and water. Chloral hydrate (300 mg/kg) was injected intraperitoneally (i.p.) to anesthetize the mice. In the Aβ injury group, 3 μl $A\beta_{25-35}$ solution was injected (i.c.v.) using a 26-gauge needle (Hamilton, Switzerland) inserted 3 mm beneath the skull surface. In the control group, mice were injected with normal saline at the same dose. After Aβ injection, all mice were treated by BrdU (100 mg/kg/day) injection (i.p.)

at the same time for five consecutive days. Afterwards, the Aβ injury group was treated with TAT-tCNTF (100 μg/kg/day; i.p.) for 10 consecutive days from the 16th day. Animals were sacrificed on the 26th and 45th days of the experiment. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experimental procedures were performed in accordance with the *Guide for* the *Care and Use of Laboratory Animals* published by the Institute of Laboratory Animal Resources of the National Research Council (United States), and approved by the Institutional Animal Care and Use Committee of Academy of Military Medical Sciences, Beijing, China.

Immunohistochemistry (paraffin sections)

The number of Ki67 and NeuN immunoreactive neurons in specific hippocampal regions was quantitatively assessed. The specific primary antibodies and concentrations used for detection of immunoreactivity were: anti-Ki67 (1:1000, ab15580; Abcam, UK) and anti-NeuN (1:1000, MAB377; Millipore, USA). Immunohistochemistry was performed using diaminobenzidine (DAB) as the chromogen to yield a brown nuclear reaction product. For quantification of cells, every section throughout the Dentate Gyrus (DG), and CA1, and CA3 regions was microscopically imaged to visualize hippocampal cell distribution.

Immunofluorescence

Hippocampal sections were blocked with 10% goat serum working solution (ZLI-9056; ZSGB-BIO, China) for 1 h, and incubated with specific antibodies for anti-BrdU (1:300, ab6326; Abcam, UK) and glial fibrillary acidic protein (GFAP) (1:1000, z033429; Dako, Denmark). Immunostaining was visualized using FITC- or PE-conjugated secondary antibodies (Santa Cruz, USA), which were incubated for 2 h at room temperature. Sections were washed with PBS and coverslipped in Vectashield (MPbio, USA), with DAPI added as a nuclear dye. Immunostained sections were examined under a confocal microscope (LSM 510 META; Zeiss Inc., Germany), as described previously [34]. Mean cell count values per section for individual animals were used for statistical analysis. The results were expressed as the ratio of double positive cells to total number of respective neuropeptide-immunoreactive cells per 0.2 mm^2 area of DG, CA1, or CA3.

Western blotting

Expression of APP, tau, p-tau, and CNTF signaling molecules, including Erk (and p-Erk), Akt (and p-Akt), and Stat3 (and p-Stat3), was analyzed by immunoblotting. Samples from control mice, Aβ (i.c.v.) mice, and TAT-tCNTF (i.p.) mice were lysed in RIPA buffer (100 mM Tris-HCl, 300 mM NaCl, 2% Tween-20, and 0.4% NP-40) and 0.025% protease inhibitor (Roche, USA). Total protein (20 μg) was separated by electrophoresis using 10% or 15% SDS-PAGE gels and transferred to nitrocellulose polyvinylidene difluoride (PVDF) membranes. Blots were then blocked with 5% skim milk in PBS with 0.2% Tween-20 (PBS-T), and probed overnight at 4°C with either mouse monoclonal APP (1:1000, 5524-1; Epitomics, USA), tau (1:1000, 4019; Cell Signaling Technology, USA), p-tau (1:1000, 12885; Cell Signaling Technology, USA), Erk (1:1000, 9102; Cell Signaling Technology, USA), p-Erk (1:1000, 4370; Cell Signaling Technology, USA), Akt (1:1000, 4685; Cell Signaling Technology, USA), p-Akt (1:1000, 4060; Cell Signaling Technology, USA), Stat3 (1:200, 7179; Santa Cruz, USA), p-Stat3 (1:2000, 9145; Cell Signaling Technology, USA), or GAPDH (1:1000, sc-130656; Santa Cruz, USA) in bovine serum albumin (0332; Amresco, USA). The blots were next washed with PBS-T, and incubated for 2 hours at RT in horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies (1:4000; ZSGB-BIO, China). Labeled proteins were visualized using Electro-Chemi-Luminescence (ECL) Western Blotting Substrate (29100; Engreen Biosystem, Ltd., New Zealand), with photodevelopment performed using a luminescent image analyzer (GE IMAGE Quant LAS4000 mini, Japan)

Statistical analysis

Data are represented as mean ± SD. Biostatistical analyses were conducted with GraphPad Prism 5.0 and SPSS 16.0 software. The difference of Ki67-positive cell ratio between control group and Aβ group was compared by paired-T test. Comparisons between control group, Aβ group and Aβ+TAT-tCNTF group were performed by one-way ANOVA, with statistical significance determined by post hoc

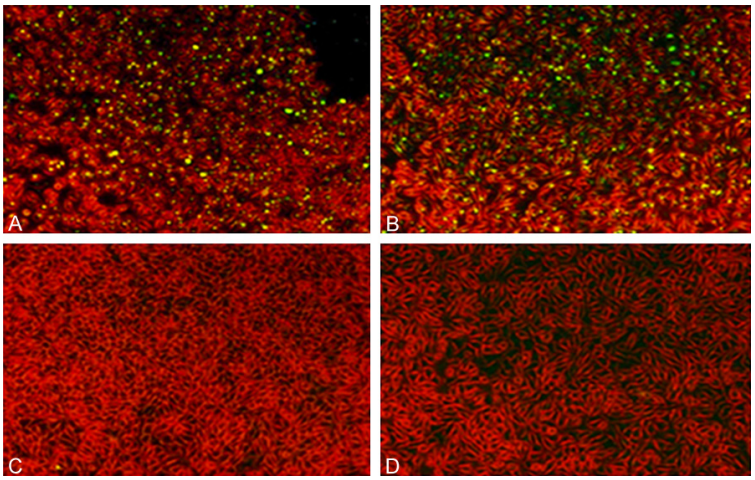

Figure 1. Intracellular CF488A-labeled proteins by green fluorescence. Different intensity of green fluorescence was showed CF488A-labeled TAT-tCNTF (A), TAT (B) and rhCNTF (C) in SH-SY5Y cells. The distribution of CF488A-labeled TAT-tCNTF was displayed with the pretreatment of SH-SY5Y with AMI (D). Green fluorescence intracellular represented the protein entered the cells.

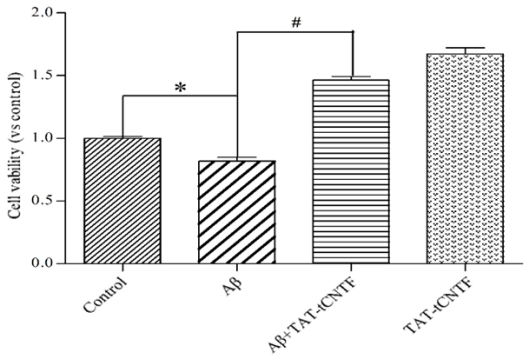

Figure 2. Cell viability of hippocampal neurons was determined via the CCK-8 kits. The cell viability in control group, $*P < 0.05$ vs Aβ injury group, $^{\#}P < 0.05$ vs Aβ+TAT-tCNTF group. Values are expressed as mean ± SD, n=6.

analysis using Bonfferoni t test. A value of $P < 0.05$ was considered statistically significant.

Results

TAT-tCNTF enters cells by macropinocytosis

To detect the transmembrane mechanism of TAT-tCNTF, SH-SY5Y cells were pretreated with AMI, a macropinocytosis inhibitor, before addition of CF-488A-labeled TAT-tCNTF. Without AMI pretreatment, considerable green fluorescence was directly observed by fluorescence microscopy in the CF-488A-labeled TAT-tCNTF (**Figure 1A**) and TAT (**Figure 1B**) groups. Fluorescence was hardly observed in the CF-488A-labeled rhCNTF group (**Figure 1C**). Thus, it is reasonable to assume that TAT-tCNTF can traverse biological membranes and has a similar transmembrane tendency as TAT. Further, TAT-tCNTF is more readily facilitated than rhCNTF. Next, we pretreated SH-SY5Y cells with AMI (**Figure 1D**), and barely observed any green CF-488A-TAT-tCNTF fluorescence. Thus, AMI as a macropinocytosis inhibitor can prevent TAT-tCNTF from entering cells. Additionally, TAT-tCNTF likely crosses biological membranes via macropinocytosis. A more detailed mechanism will be further considered.

TAT-tCNTF promotes cell growth of primary hippocampal neurons injured by Aβ in vitro

To determine the therapeutic action of TAT-tCNTF in cells damaged by Aβ, cell viability of primary hippocampal neurons were examined by CCK-8 (**Figure 2**). Aβ reduced the cell viability of hippocampal neurons (*vs.* control, $P < 0.05$), while TAT-tCNTF increased cell viability (*vs.* Aβ, $P < 0.05$). This indicates that TAT-tCNTF accelerates growth and resists Aβ damage in hippocampal neurons.

Aβ reduces cell proliferation in mouse brain

To observe the effect of Aβ on cell proliferation in vivo model, we performed immunohistochemistry experiments using Ki67. Compared with controls, we observed a significant decrease in the ratio of Ki67-positive cells to total cells in the mouse hippocampus after Aβ injection (i.c.v.), as well as in the DG, CA1, and CA3 regions ($P < 0.05$) (**Figure 3**). This suggests that Aβ reduces cell proliferation in the mouse hippocampus.

TAT-tCNTF maintains hippocampal neuronal survival in Aβ-injured mice

Immunohistochemistry experiments using the marker, anti-NeuN, were performed (**Figure 4A** and **4B**). The ratio of NeuN-positive cells to total cells significantly decreased in Aβ groups compared with control groups in the DG ($P <$

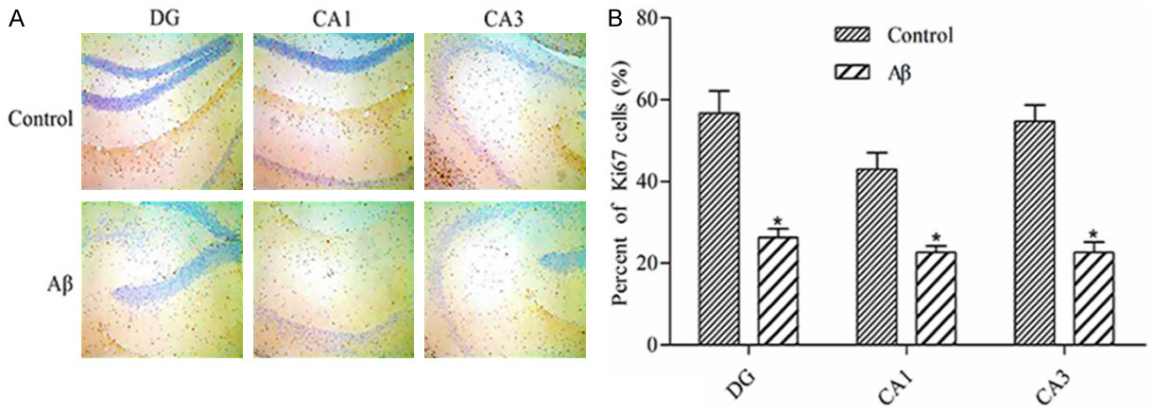

Figure 3. A: Cell proliferation in the hippocampus with treatment of Aβ. Ki67-positive cells were detected by Ki67 immunoreactivity. B: The percent of Ki67-positive cells in the regions of DG, CA1 and CA3 after Aβ injection, $*P < 0.05$ vs control group. Values are expressed as mean ± SD, n=3.

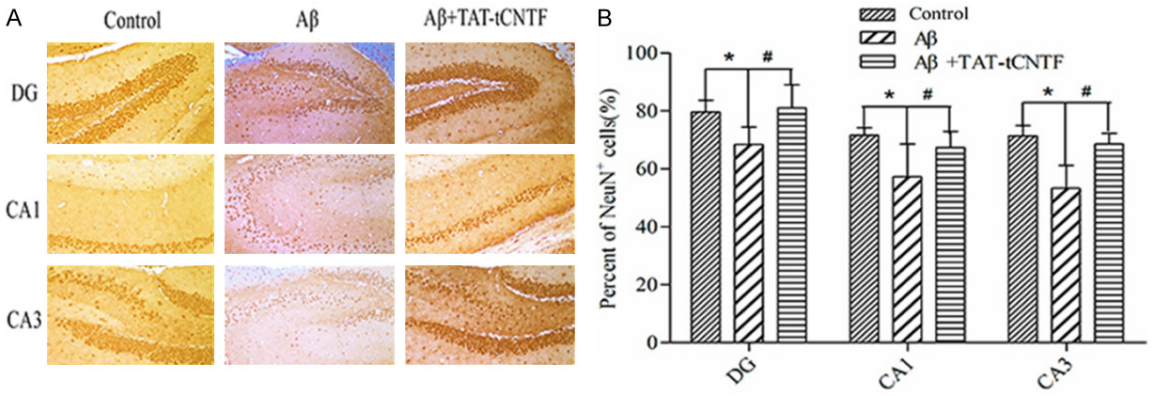

Figure 4. NeuN-positive cells were detected by the immunohistochemistry and photographed by microscope. $NeuN^+$ cells in DG, CA1 and CA3 of the hippocampus decreased when treated with Aβ. But $NeuN^+$ cells in the three regions significantly increased after the treatment of TAT-tCNTF (A). The percent of $NeuN^+$ cells in the regions of DG, CA1 and CA3, $*P < 0.05$ vs Aβ injury group, $^{\#}P < 0.05$ vs Aβ+TAT-tCNTF group (B). Values are expressed as mean ± SD, n=3.

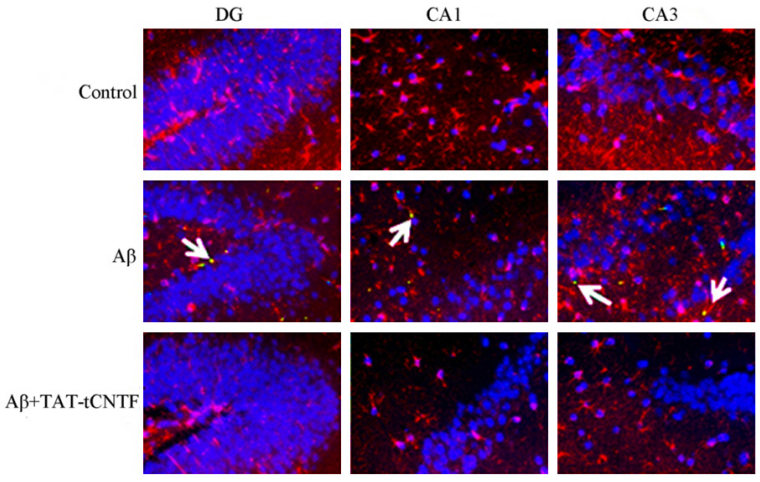

Figure 5. Micrograph montages of BrdU and GFAP immunofluorescence for GFAP astrocytes. Blue represented the DAPI, green did the BrdU and red did the GFAP. And the white arrow meant the BrdU and GFAP-double positive cells.

0.05), CA1 ($P < 0.05$), and CA3 ($P < 0.05$) regions. However, compared with the Aβ group, the ratio of NeuN-positive cells increased after treatment with TAT-tCNTF in the DG ($P < 0.05$), CA1 ($P < 0.05$), and CA3 ($P < 0.05$) hippocampal regions. These findings support our *in vitro* results and confirm that TAT-tCNTF promotes neuronal survival in the hippocampus of mouse brain.

TAT-tCNTF reduces reactive astrogliosis in the hippocampus of Aβ-injured mice

To observe the effect of TAT-tCNTF on Aβ-induced astrogli-

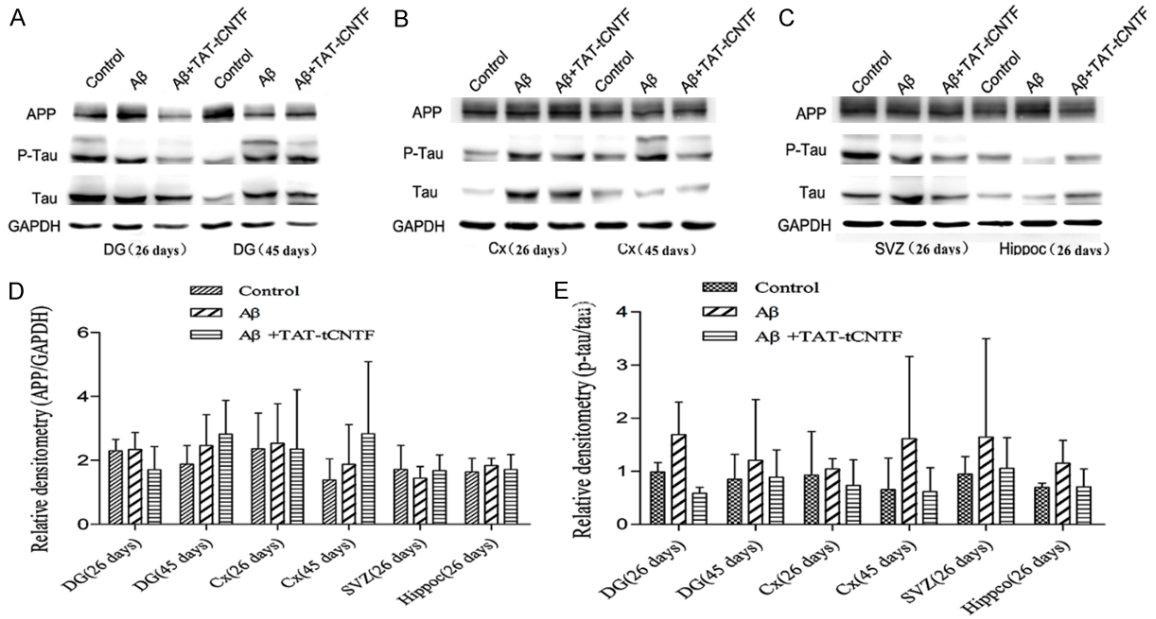

Figure 6. Western blots analyzed protein expressions of APP, tau and p-tau in different regions of brains of mice and at different times. Protein expression of DG (A) and Cx (B) on the 26th day and 45th day of the animal experiments, and SVZ and Hippoc (C) on the 26th day and 45th day of the hippocampus, respectively. The column chart shows the relative expression of APP/GAPDH (D) and p-tau/tau (E). Values are expressed as mean ± SD, n=6.

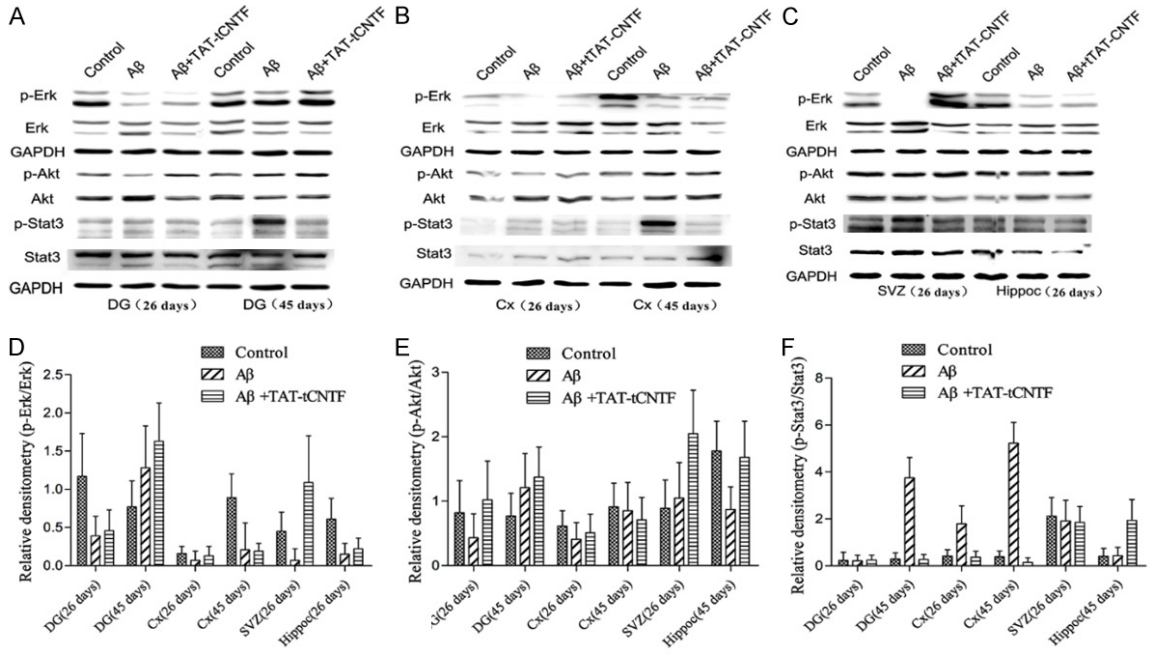

Figure 7. Expression of phosphorylated Erk to Erk, phosphorylated Akt to Akt and phosphorylated Stat3 to Stat3 of the CNTFRα pathway related proteins. Protein expression in DG (A) and Cx (B) on the 26th and the 45th day of the animal experiment, and SVZ and Hippoc (C) on the 26th day are displayed in micrographs. Phosphorylated Erk in the Cx and SVZ regions on the 26th day (D) and phosphorylated Akt in DG and Cx on the 26th day (E) were expressed more in the TAT-tCNTF group than those in Aβ group. However, the Stat3 pathway (F) showed no differences.

osis, we performed dual-label immunofluorescence of brain tissue slices, pairing anti-BrdU with the astrocytic marker, anti-GFAP (**Figure 5**). BrdU-positive cells are a direct indicator of

slow cell proliferation. As predicted, BrdU-positive cells were observed in the hippocampal DG, CA1, and CA3 regions in Aβ-treated mouse brain. However, BrdU-positive cells were rarely observed in the control and TAT-tCNTF groups on the 45th day. Our previous result demonstrated that TAT-tCNTF promotes cell growth, even in neurons. Here, we show that the number of both BrdU-positive and GFAP-positive cells in the mouse hippocampus is significantly reduced in the TAT-tCNTF group compared with the Aβ group. Furthermore, co-localization of anti-BrdU and anti-GFAP confirms that TAT-tCNTF decreases Aβ-induced reactive astrogliosis in these hippocampal regions.

TAT-tCNTF reduces tau phosphorylation in mouse brain

To investigate the mechanism of TAT-tCNTF on Aβ injury, we performed quantitative Western blot analysis for p-tau and tau. Protein expression of p-tau and tau significantly increased in the DG, Cortex (Cx), Subventricular Zone (SVZ) and other hippocampal regions on the 26th and 45th days after Aβ injection (i.c.v.). Tau hyperphosphorylation reflects NFT formation, indicating that Aβ has led to NFT formation. After TAT-tCNTF injection (i.p.) to Aβ-injured mice, tau phosphorylation decreased compared with before therapy (**Figure 6**). This observation appeared simultaneously in the DG and Cx on both experimental days, and also in the SVZ and hippocampus on the 26th day. Interestingly, this suggests that amyloid deposits are generated from APP via γ-secretase. However, APP protein expression showed almost no change in mouse brain before or after TAT-tCNTF therapy, even in the DG, Cx, SVZ, and other hippocampal regions on the 26th and 45th days after first administration. Hence, these results show that Aβ does not cause APP activation via upstream pathways.

TAT-tCNTF exerts its pharmacological effects by activating the Erk and Akt pathways

To investigate the mechanism by which TAT-tCNTF exerts its therapeutic effect in Aβ-injured mice, we analyzed CNTF signaling molecules (including Erk, Akt, and Stat3) by Western blot analysis (**Figure 7**). Compared with the Aβ group, TAT-tCNTF increased expression of p-Erk in the Cx and SVZ on the 26th day (**Figure 7D**), as well as Akt in the DG and Cx on the 26th day (**Figure 7E**). These findings show that TAT-tCNTF activates the Erk and Akt pathways. However, TAT-tCNTF barely influenced p-Stat3 (**Figure 7F**), suggesting that TAT-tCNTF does not activate the Stat3 pathway.

Discussion

Here, we show that TAT-tCNTF can cross biomembranes via macropinocytosis *in vitro*. Furthermore, TAT-tCNTF was also able to pass through the BBB of mice and enter the brain. TAT-tCNTF showed neuroprotective activity in Aβ-injured models. Indeed, previously we have shown that TAT-CNTF contributes to improved learning capacity and memory in Aβ-injured mice. While here, we show (at least in part) the molecular mechanism of pharmacological action involves the anti-Aβ signaling pathways of Erk and Akt.

In AD, amyloid deposition caused by Aβ is known as a common pathophysiological mechanism that leads to neuronal damage and necrosis. In this study, we examined the capacity of TAT-tCNTF to cross biomembranes and protect against neuronal injury using *in vitro* and *in vivo* models. First, TAT-tCNTF (and TAT) entered SH-SY5Y cells *in vitro*, which was not observed with rhCNTF treatment. Consequently, it is reasonable to suggest that this transmembrane characteristic of TAT-tCNTF is attributable to TAT. Accordingly, to determine if TAT-tCNTF could enter cells by macropinocytosis, we performed an endocytosis inhibition assay using AMI. Indeed, our results support the former proposition. While, these *in vivo* results further corroborate our finding that TAT-tCNTF can penetrate biomembranes.

Using an injured model of primary hippocampal neurons, we found that TAT-tCNTF increases cell viability. We then performed further studies on the therapeutic action of TAT-tCNTF using an *in vivo* model of Aβ-induced amyloid deposition. Mouse brain sections were obtained and then were investigated by immunohistochemistry. In accordance with our previous results, Ki67-positive cells in the hippocampus (including the DG, CA1, and CA3 regions) decreased markedly after Aβ injection. Consistent with *in vitro* result, Aβ treatment caused the decrease of cell prolif-

eration. These results suggested that Aβ injury models are feasible for our study.

Aβ plaques are surrounded by activated astrocytes, which produce reactive oxygen and nitrogen species that may contribute to AD pathogenesis [35]. Correlational research has shown that the occurrence of astrocytosis antedates amyloid plaque deposition in the brain of Alzheimer APPs, in the transgenic mouse [36]. Here, we found that after i.c.v. injection of Aβ, astrocyte proliferation was induced in mouse brain, but markedly decreased with TAT-tCNTF therapy. These results are identical with those reported, illustrating that TAT-tCNTF is able to relieve reactive astrogliosis and enhance brain cell proliferation [37-39].

Our mouse experiments show that TAT-tCNTF significantly reduces tau phosphorylation in the brain, while APP expression does not change. The appropriate data fully illustrates this point, specifically, that TAT-tCNTF reduces Aβ injury not via affecting upstream pathways, but by reducing levels of excessive p-tau protein and further reducing NFTs.

In addition to showing a pharmacological effect, we turned to identifying the pharmacological mechanism of TAT-tCNTF, as it is virtually unknown. To date, its pharmacological action has been shown to be mediated via its receptor, CNTFRα [40]. Nevertheless, novel information on the intracellular signaling pathway of TAT-tCNTF was obtained. Our Western blot results show that TAT-tCNTF increases Erk phosphorylation and p-Akt/Akt expression. In contrast, Stat3 phosphorylation showed no significant change throughout our experiment. This shows that after binding to CNTFRα, TAT-tCNTF activates Erk and Akt signal transduction pathways, rather than Stat3. Nevertheless, previous studies have indicated that activation of the JAK2/STAT3 signaling pathway is induced by CNTF to prevent neuronal cell death after nerve injury [31, 41, 42]. In addition to JAK2/STAT3, the PI3K/Akt and MEK/ERK signaling pathways were also previously implicated in CNTF-induced neurite outgrowth, with JAK2/STAT3 and PI3K/Akt being major activators in promoting neuronal survival due to CNTF [43]. This conflicts with this conclusion, and it was speculated that the discrepancy is due to TAT and CNTF receptor distribution. The CNTF receptor is principally located in hypothalamic nuclei, as a member of the interleukin-6 (IL-6) type cytokine family [44-46]. Fusion of the TAT protein transduction domain has been shown to change rhCNTF distribution within the central nervous system, resulting in insufficient dosage in the hippocampal region [47, 48].

Taken together, our results support the view that TAT-tCNTF protects against amyloid deposition caused by Aβ (which is the main histopathological characteristic of AD), both *in vitro* and *in vivo*, by promoting Erk and Akt pro-survival signaling pathways. Thus, TAT-tCNTF may be of potential use against AD, although further investigations are required.

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Disclosure of conflict of interest

None.

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