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Transducible P11-CNTF rescues the learning and memory impairments induced by amyloid-beta peptide in mice

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ABSTRACT

Alzheimer's disease is a progressive brain disorder with the loss of memory and other intellectual abilities. Amyloid species and neurofibrillary tangles are the prime suspects in damaging and killing nerve cells. Abnormal accumulation of Amyloid-beta peptide (Aβ) may cause synaptic dysfunction and degeneration of neurons. Drugs that can prevent its formation and accumulation or stimulate its clearance might ultimately be of therapeutic benefit. Ciliary neurotrophic factor (CNTF), a neurotrophic cytokine, promotes the survival of various neurons in brain. However, the blood-brain barrier hinders the systemic delivery of CNTF to brain. Recently the 11-amino acid of protein transduction domain TAT has successfully assisted the delivery of many macromolecules to treat preclinical models of human disease. The present study aimed to evaluate whether P11-CNTF fusion protein (P11-CNTF) is protective against the Aβ25-35-induced dementia in mice. Immunofluorescence experiments showed that P11 effectively carried CNTF to the SH-SY5Y cells in vitro, and to the brains of mice in vivo. The learning and memory impairments of mice induced by Aβ were substantially rescued by supplement with the P11-CNTF. Furthermore, mRNAs of enzymes involved in the Aβ metabolism, e.g. neprilysin (NEP), endothelin-converting enzyme 1 (ECE-1) and insulin degrading enzyme (IDE), increased in the P11-CNTF treated dementia mice, accompanied by the proliferation of nestin- and choline acetyltransferase (ChAT)-positive cells in hippocampus. It implies that the delivery of P11-CNTF may be a novel treatment for Alzheimer's disease.

1. Introduction

Alzheimer's disease is a progressive and fatal neurodegenerative disorder manifested by cognitive and learning and memory deterioration, progressive impairments of daily living activities, and a variety of neuropsychiatric symptoms and behavioral disturbances. The characteristic features of Alzheimer's brains are the presence of senile plaques and the formation of neurofibrillary tangles which are believed to responsible for the neuronal loss and the degeneration of the cholinergic system. The abnormal accumulation of extracellular amyloid-beta peptide (Aβ) and the intracellular neurofibrillary tangles causes the degeneration of neurons in brains (Mattson, 2004).

Alzheimer's disease now affects millions of the aging population worldwide and will affect millions more in the next 20 years. However, there are not yet strictly causal therapies available. Most of the current clinical treatments for Alzheimer's disease are largely symptomatic, including the use of cholinesterase inhibitors to improve the cognitive ability and psychotropic drugs to modify the patients' behaviors (Francis et al., 2005). The *N*-methyl-D-aspartate (NMDA) receptor antagonist Memanatine and the anti-amyloid immunotherapy were involved in the strategies for Alzheimer's disease (Schenk, 2002) to manage symptoms and delay the onset of symptoms. However, all of them do cause side effects. Therefore, a therapy that not only prevents the development of symptoms but also inhibits the formation of plaques and tangles and enhances the functions of the survival neuronal cell is needed badly.

Ciliary neurotrophic factor (CNTF) is a cytokine with neurotrophic and differentiation-inducing effects across a broad spectrum of peripheral and central nervous system (CNS) cells. CNTF enhances the survival of sensory neurons, motor neurons, cerebral neurons and hippocampal neurons (Skaper et al., 1986; Ip et al., 1991; Sendtner et al., 1992; Larkfors et al., 1994). It is promising for the treatment of neurodegenerative diseases. However, the blood-brain barrier hinders the systemic delivery of CNTF while its therapeutic potential for CNS diseases has been clear for sometime. Poor permeability of most proteins across cell membranes compromises their potential as therapeutic reagents. Recently, it has been shown that the protein transduction domains (PTDs) have the ability to be internalized into the cell from the external environment even carrying a variety of large, biologically-active molecules. The undecapeptide YGRKKRRQRRR (P11) is an efficient transduction domain (TAT 47–57) (Schwarze et al., 1999; Vives et al., 1997) without the neurotoxicity of the full-

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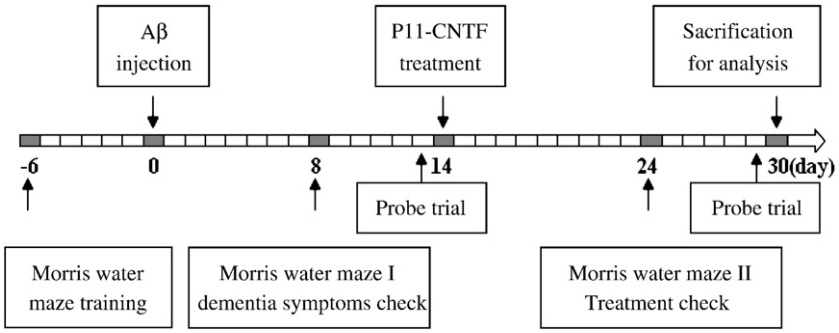

Fig. 1. Experimental schedule for P11-CNTF treatment of the Aβ-induced dementia model mice.

length TAT of 86 amino acids (Nath et al., 1996). TAT 47–57 has been shown to deliver various macromolecules passing the cell membranes and blood-brain barrier of mammals (Tan et al., 2007; Kilic et al., 2003; Dietz et al., 2002). In this paper, a fusion protein P11-CNTF was constructed for delivery into brain. And whether learning and memory deficits induced by Aβ could be prevented with the supplement of P11-CNTF was investigated.

2. Materials and methods

2.1. Cloning and expression of CNTF and P11-CNTF genes

A gene encoding the truncated form (15 amino acids at the C terminus and 14 amino acids at the N terminus were deleted) of the natural human CNTF was constructed in order to make the expressed protein more stable and effective in therapy than the native CNTF (Panayotatos et al., 1993). The truncated CNTF gene was amplified by recombinant PCR from normal human genome, and subcloned into pBV220 vectors. In order to deliver the exogenous CNTF protein into the brains of mammals in an invasive way, a vector bearing the gene of the membrane-permeable undecapeptide P11 (pBV220-P11) was used. The truncated-CNTF gene was then inserted into the plasmid to construct the expression vector (pBV220-P11-CNTF). The nucleotide sequences of the truncated CNTF gene were analyzed for correctness by Sangon (Shanghai, China). The plasmids were then transformed into *E. coli* BL21 (DE3) respectively. The expressed CNTF and P11-CNTF were purified by superdex-50 chromatography. Protein concentrations were determined according to Bradford assay. Western blot was performed to identify the specificity of CNTF and P11-CNTF.

2.2. In vitro experiments

2.2.1. SH-SY5Y cells culture

Human neuroblastoma SH-SY5Y cells were incubated in the Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM Glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2/ 95% air at 37 °C (Tanaka et al., 1995).

2.2.2. Immunofluorescence analysis to examine the transmembrane efficacy

SH-SY5Y cells of 80–90% confluence were exposed to 1 μM CNTF or P11-CNTF for 1 h. PBS was used instead as control. After 1 h treatments, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and then washed with PBS. The fixed cells were mixed with 0.3% Triton X-100 in PBS for 10 min, blocked with 1% BSA/PBS for 1 h at room temperature and incubated with the anti-human CNTF monoclonal (Santa cluz)at 4 °C overnight. After washing three times with PBS, the secondary antibody goat anti-mouse IgG-FITC was added to the cells for 1 h at 37 °C, followed by three washings of PBS and once of H_2O. The cells were examined under the Olympus IX70 fluorescence microscope.

2.2.3. Flow cytometry

When SH-SY5Y cells reached 50% confluence, Aβ25-35 (Sigma-Aldrich) (dissolved in DMEM incubated at 37 °C for 7d to aggregate) was diluted to cell culture medium to the final concentration of 10 μM for 24 h culture to induce apoptosis. And then 1 μM CNTF or P11-CNTF was added to cells for 24 h culture. Apoptosis assay was performed using the Apoptosis Assay Kit (Beckman Coulter) on flow cytometer following the manufacturer's instructions. Emission wave lengths of 518 nm (annexin V-FITC) and 617 nm (PI) were used to identify the populations of the viable cells (annexin V−PI−), the early apoptotic cells (annexin V+PI−), the necrotic cells (annexin V−PI+), and the late apoptotic cells (annexin V+PI+). The collected events per sample were 10,000.

2.2.4. Transmission electron microscopy

10 μM Aβ25-35 with or without 1 μM P11-CNTF was incubated at 37 °C for 7d. Then 5 μl of each reaction mixture was applied to the carbon-coated collodium film on a 200 mesh copper grid, negatively stained with 2% (w/v) uranyl acetate, and viewed on a Philips TECNAI 10 transmission electron microscope operating at 80 kV.

2.3. In vivo experiments

2.3.1. Animals

C57BL/6 male mice, aged 8 weeks, weighing 20±2 g, were group-housed and maintained on a 12 h light/dark cycle (07:00–19:00) enviroment with *ad libitum* access to standard food and water. Animal studies were approved by the Animal Care and Use Committee, Ministry of Science and Technology, China. The experimental procedures were approved by the local Committee on Animal Care and Use.

The Aβ-induced dementia model was generated according to a previously described protocol (Maurice et al., 1996). In brief, the Aβ25-35 was dissolved in normal saline to 1 mg/ml, incubated at 37 °C for 7 d to transform to the aggregated phase, and then stored at −70 °C ready for use. The aggregated Aβ of 3 μl was administered intracerebroventricularlly (i.c.v.) to each mouse anesthetized with pentobarbital sodium using a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long (Hamilton). The needle was inserted 2 mm behind the connecting line of

Table 1
Primer pairs used in RT-PCR

Genes	Primers sequence(5′ to 3′)	Products
NEP	Forward GTCATTCCCGAGACCAGTTCC Reverse CGGTCTTCTGGTTTAGTTGTAG	595 bp
ECE-1	Forward GCCATCAACTGGTTACCCTTTC Reverse GTTGGGTGAAGAGCGGGTATC	751 bp
IDE	Forward ATGTGCGGGTGGCAATAGTTT Reverse CAGTCATCAGCAAGCGGAGGT	651 bp
GAPDH	Forward GCAGTGGCAAAGTGGAGATT Reverse GGTCCTCAGTGTAGCCCAAG	768 bp