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Intranasal nerve growth factor attenuates tau phosphorylation in brain after traumatic brain injury in rats

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ABSTRACT

Traumatic brain injury (TBI) is a considerable cause of mild cognitive impairment and dementia. Intranasal administration of nerve growth factor (NGF) has previously been found to improve cognitive function after TBI, but the mechanism remains unclear. This study aimed to investigate the effects of intranasal NGF on the tau hyperphosphorylation following TBI. A modified Feeney's weight-drop model was used to induce TBI. Rats were randomly divided into control group, TBI group, TBI + NGF group, TBI + PDTC group and TBI + IL-1ra group. Rats in TBI + NGF group were administered with NGF (5 μg/d) for 3 d before surgery. Hyperphosphorylated tau protein was remarkable in the peri-contusional cortex area with TBI. Both western blotting and immunostaining results displayed intranasal pretreatment of NGF significantly reduced tau phosphorylation. To evaluate the underlying mechanism, the levels of glycogen synthase kinase 3β (GSK-3β), interleukin-1β (IL-1β), and the DNA binding activity of nuclear factor-κB (NF-κB) were assayed. NGF markedly inhibited GSK-3β. NGF also reduced TBI-induced elevation of IL-1β and NF-κB DNA binding activity. Furthermore, PDTC and IL-1ra were injected to prove a potential signaling pathway among NF-κB, IL-1β and GSK-3β. Taken together, these findings demonstrated that intranasal NGF could effectively attenuate the hyperphosphorylation of tau after TBI, which might involve an integrated signaling pathway related to NF-κB.

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1. Introduction

Traumatic brain injury (TBI) is a considerable cause of disability and death worldwide [1]. There are more than 10 million people suffering from it every year [2], and nearly 80% of these patients have cognitive deficit [3]. Alzheimer's disease (AD), the most common form of cognitive deficit induced by TBI [4], interferes with the life of patients and causes a huge burden to the society. To resolve this problem, scientists are engaged in looking for an effective therapeutic tool in decades.

A lot of evidence suggests that nerve growth factor (NGF) is a promising treatment to prevent or slow-down cognitive deficit in AD [5,6]. Administration of NGF into the brain gives rise to attenuation of memory loss [7,8]. Nevertheless, the clinical application of NGF is restricted due to its poor permeability through the blood–brain barrier (BBB). Our lab has demonstrated that intranasal delivery, a noninvasive and convenient method, successfully targets NGF to the central nervous system [9], bypassing the BBB and minimizing systemic exposure [10,11]. On this basis, our team has done a series of research about the effect of NGF on TBI rats. We found that intranasal delivery of NGF, started at 6 h after TBI, attenuates the brain edema [12]. Identified by Nissl staining, more surviving neurons were observed in NGF treated rats [13]. These effects improved the long-term cognitive outcome, which confirmed by the Morris water maze and beam walk [14]. However, the underlying molecular mechanism remains unclear.

Amyloid hypothesis and tau hypothesis are the classic pathogenesis of AD. The overproduction of amyloid-β leads to the deposit of amyloid plaque, which correlates with cognitive deficit. Tian et al. have already observed that intranasal delivery of NGF ameliorated β-amyloid deposition in TBI rats [14]. Tau is phosphorylated on 2–3 amino acid residues in the healthy brain [15]. Yet in pathological conditions, tau becomes abnormally hyperphosphorylated [16,17], and is aggregated to neurofibrillary tangles [18], which is resistant to degradation and induction of conformational changes [19]. In this study, we aimed to investigate whether intranasal delivery of NGF rescued the hyperphosphorylation of tau in TBI rats and address the underlying signaling pathway.

2. Materials and methods

2.1. Animals

All protocols were conformed to the Guide for the Care and Use of Laboratory Animals by National Institutes of Health (NIH Publications

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no. 80-23) and approved by the Jinling Hospital Animal Welfare Committee. Every effort was made to minimize the number and sufferings of the animals. We used 280–310 g adult male Sprague–Dawley rats (Experimental Animal Center of Jinling Hospital, Jiangsu Province, China). All rats were acclimated to standard laboratory conditions, at a room temperature of 24 ± 1 °C and humidity of 60 ± 10%, on a 12-hour light/dark cycle with access to food and water ad libitum.

2.2. Antibodies

Anti-total tau (Tau-5) (1:5000, Millipore, Billerica, MA, USA); anti-tau (phosphor S396) (1:1000, Abcam, Cambridge, MA, USA); anti-total GSK-3β (1:1000, CST, Danvers, MA, USA); anti-GSK-3β (phosphor S9) (1:1000, Abcam, Cambridge, MA, USA); and anti-GAPDH (1:1000, CST, Danvers, MA, USA).

2.3. Induction of TBI model

A modification of the Feeney's weight-drop model was used [20], in which there is a steel rod weighing 40 g with a 4 mm flat end to fall onto a piston resting on the dura from a height of 25 cm to produce a standardized parietal contusion. Rats were anesthetized with sodium pentobarbital (20%, 40 mg/kg) intraperitoneally, and then placed in a stereotactic frame. A craniotomy was made by a 5 mm trephine drill over the right anterior parietal cortex centered 1 mm posterior to bregma and 2 mm lateral to the midline, leaving the dura mater intact. The piston was allowed to compress the tissue at a maximum of 5 mm and the depth of brain deformation was set at 3 mm. The craniotomy was sealed with bone wax immediately after contusion, and the scalp was closed with interrupted 6-0 silk sutures. After surgery, rats were left to recover on a warm pad until thermoregulation and the righting reflex was re-established. Then they were returned to their cages with free food and water. The rectal temperature was monitored and was kept at 37 ± 0.5 °C throughout the experiment. The control group was subjected to identical anesthetic and a craniotomy without cortical injury.

2.4. Experimental protocols

132 rats were randomly divided into five groups: (A) the control group (n = 42), (B) the TBI + vehicle group (n = 42), (C) the TBI + NGF group (n = 36), (D) the TBI + PDTC group (n = 6), and (E) the TBI + IL-1ra group (n = 6). This study contained two parts: 1) to observe the effect of NGF on phosphorylated tau and the change of some protein, as glycogen synthase kinase 3β (GSK-3β), interleukin-1β (IL-1β), and the DNA binding activity of nuclear factor-κB (NF-κB); and 2) to investigate the latent relation of these proteins above. In part 1, NGF was dosed once daily beginning 3 d before TBI. For the TBI group, only PBS was received in same manner. The control group received nothing. After TBI surgical, the rat was executed at 1 d, 3 d and 7 d respectively. In part 2, the relation of GSK-3β, IL-1β and NF-κB was investigated through intracerebroventricular injection of PDTC (inhibitor of NF-κB, Sigma, 10 μg dissolved in 10 μl PBS) and IL-1ra (inhibitor of IL-1β, R&D Systems, 1 μg dissolved in 0.1 M PBS). Before allowing the piston to compress, rats with craniotomy were given intracerebroventricular injection by using a Hamilton syringe. The needle was lowered into the right lateral cerebral ventricle (3.4 mm below dura) and the drug delivered over 1 min. The TBI group was received PBS in same manner, and the control group received nothing. At 1 d after TBI, brain tissues were obtained for testing.

2.5. Intranasal administration

NGF was dissolved in PBS to a final concentration of 0.1 mg/ml. A modified protocol was used for delivering [21]. After anesthesia (1% mebumal sodium, 40 mg/kg, i.p.), rats were placed supinely, with the head in upright position, then received 10 times for each nostril application of 5 μl drops, alternating the nostrils with a lapse of 2 min. The nostrils were kept open during the procedures. The protocol was repeated daily. Before surgical, rats in T + NGF group received NGF (5 μg/d) intranasally for 3 d. The T group received only PBS in same manner, and the control group received nothing.

2.6. Western blotting

Western blotting was conducted as we previously described [22,23]. The levels of tau and GSK-3β were evaluated by western blotting at 1 d, 3 d and 7 d after TBI (six rats of each group were involved at each time point). The tissue from the peri-contusional cortex were homogenized in radio immunoprecipitation assay (RIPA) buffer, and then centrifuged at 13,000 *g* (4 °C) for 10 min. The supernatant was collected. Protein concentrations were determined using the Bradford method. An equal volume of 6× sodium dodecyl sulfate (SDS) sample buffer was added, and the samples were then boiled for 3 min. Samples (100 μg per lane) were subjected to separate by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, CA, U.S.A.), and then transferred to 0.22 μm polyvinylidene difluoride (PVDF) membranes using a Trans-Blot semi-dry system (Bio-Rad, CA, U.S.A.). The membranes were blocked with 5% nonfat dry milk in saline buffer for 2 h at room temperature and then incubated overnight at 4 °C with the primary antibodies. The membranes were washed by PBST for 10 min and incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. The members were developed using a chemiluminescence kit (Pierce, U.S.A.) and were exposed to film. The bands on the film were scanned and analyzed with Image J.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The preparation prior to detection was as previously described in western blotting. The protein level of IL-1β was quantified by ELISA kits specific for rats according to the manufacturers' instructions (Diaclone, France). The protein content in the brain samples was expressed as pg per milligram total protein.

2.8. Quantity real-time-polymerase chain reaction (qRT-PCR)

The level of IL-1β mRNA expression was determined by qRT-PCR. Total RNA was extracted (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions and the cDNA was synthesized using Reverse Transcription System (Promega Corporation, Madison, WI, USA). qRT-PCR was performed on a Stratagene M×3000P (Agilent Technologies, CA, U.S.A.) using a SYBR Premix Ex Taq RT-PCR kit (TaKaRa Biotechnology, Dalian, China). Primers were as follows: IL-1β: (FP: 5-TCGCTAGGGTCACAAGAAA-3; RP: 5-ATCAGAGGCAAGGAGGAA ACAC-3); β-actin: (FP: 5-GACGTTGACATCCGTAAAGACC-3; RP: 5-TGCT AGGAGCCAGGGCAGTA-3). The level of IL-1β gene expression was quantified by a cDNA standard curve and data were normalized to β-actin. Data were expressed as fold change.

2.9. Nuclear protein extract and EMSA

Nuclear protein extraction was performed according to the method we described previously [24]. Protein concentrations were determined using the Bradford method. EMSA was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, WI, USA). Consensus oligonucleotide probe (5-AGTTGAGGGGACTTTCCCAGGG-3) was end labeled with [γ-^{32}P]-ATP (Free Biotech, Beijing, China) with T4-polynucleotide kinase. Nuclear protein (20 μg) was preincubated in a total volume of 20 μl in a binding buffer, consisting of 10 mmol/L Tris–HCl (pH 7.5), 1 mmol/L $MgCl_2$, 0.5 mmol/L NaCl, 4% glycerol, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, and 2 μg poly dI-dC for 20 min at room temperature. After addition of the 1 μl ^{32}P-labeled oligonucleotide

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