Research report

Interfering effect and mechanism of neuregulin on experimental dementia model in rats

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ABSTRACT

Objective: To investigate the effect of neuregulin 1β (NRG1β) on the neuronal apoptosis and the expressions of Bcl-2 and Bax proteins in experimental dementia model rats. Methods: Thirty adult healthy male Wistar rats were randomly divided into control group, model group and treated group consisting of 10 rats, respectively. The experimental dementia models were established by injecting beta-amyloid protein 1–40 (Aβ1–40) stereotactically into the left lateral ventricle, and treated by injecting NRG1β into right lateral ventricle. The cognitive capacity of rats was evaluated with Y-electric maze. The neuronal apoptosis was counted by TUNEL assay. The expressions of Bcl-2 and Bax were determined with immunohistochemistry assay and double immunofluorescence labeling. Results: The cognitive ability in model group rats decreased, along with the number of neuronal apoptosis and the expressions of Bcl-2 and Bax increased significantly than those in control group (P<0.05). After treatment with NRG1β, the cognitive ability of rats improved, the number of neuronal apoptosis reduced and the expression of Bcl-2 increased significantly than those in model group (P<0.05). Conclusion: NRG1β could inhibit neuronal apoptosis by regulating the expressions of Bcl-2/Bax to improve the capacity of learning and memory in experimental dementia rats.

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1. Introduction

Alzheimer’s disease (AD) is the most widespread neurodegenerative disease worldwide [1]. AD accounts for at least 60% of all dementia diagnosed clinically. The major pathological hallmarks of AD are the loss of neurons, occurrence of senile plaques (SPs) as well as neurofibrillary tangles (NFTs) [2]. AD is considered a multifactorial disease [3,4], and the mechanism of neuronal apoptosis in the hippocampus is of importance [5,6]. Although the clinical symptoms of AD are frequently diagnosed in older age, the degenerative process probably starts many years before the clinical onset of the disease [7,8]. Since the diagnosis and therapy of AD is limited, there is no medication to treat or control the disease currently. Neuregulin1β (NRG1β), an excitomotor of tyrosine kinase receptor (erbB) family, possessed many important regulative effects in the development of nervous system, adjusting the proliferation, migration, differentiation and survival of various cells, and taking part in the formation process of synapse [9]. Previous reports have demonstrated that NRG1β might participate in the neuroprotection during the brain ischemia-reperfusion injury [10]. Recent researches indicated that NRG1β attenuated cerebral ischemia-reperfusion injury via inhibiting apoptosis and upregulating aquaporin-4 (AQP-4) [11], regulating the expressions of signal transducer and activator of transcription (STAT3) to activate JAK/STAT signal transduction pathway [12], inhibiting the activation of matrix metalloproteinase-9 (MMP-9) and development of inflammation [13], as well as inhibiting the mitochondrial apoptotic pathway via balancing the activity of X-linked inhibitor of apoptosis protein (XIAP) and the second mitochondrial derived activator of caspases (Smac) proteins to avoid the irreversible neuron death in brain tissue of ischemia/reperfusion rat [14], and thus play a neuroprotective effect on cerebral ischemia-reperfusion damage. In this experiment, experimental dementia rats models were induced by intracerebroventricular microinjection of Aβ1–40,
so to explore concrete neuroprotective mechanism of NRG1β from neuronal apoptosis and its gene regulation in experimental dementia rats.

2. Materials and methods

2.1. Experimental animals

The total of 40 adult male Wistar rats, weight 200–220 g, SPF grade, were granted by Experimental Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 2007/0010). The local legislation for ethics of experiment on animals and guidelines for the care and use of laboratory animals were followed in all animal procedures. All animals were adapt the laboratory environment, allowed free access to food and water in room temperature (23 ± 2 °C) and humidity-controlled housing with natural illumination for a week.

2.2. Learning and memory detection

Spontaneous alternation behavior in a Y-electric maze was assessed as a spatial working memory task [15, 16]. The Y-electric maze apparatus (Chinese Institute of Medical and Pharmacy) consisted of three identical arms. Each rat was placed at the end of one fixed arm and allowed to move freely through the maze during a 3-min session. Rats were initially placed at the end of one arm and allowed to adapt to the light signal for 60 s. The rats were then exposed to light signals from the two arms, followed by electric stimulation for 2 s at 60 V, 10 times per second. The correct response was defined if the rats immediately escaped to the safe area. Subsequent to safe escape, the lights continued to turn on and off for an additional 1 min for consolidation of memory, which indicated the end of one exercise. The rat was then shocked in the former safe area to go on a new test, so on and so forth, to alter places of safe areas.

Test of learning capacity: Rats were given 10 trials per day for 7 consecutive days, so as to test the number of electric shocks for right reaction (9/10); the number of trials stood for learning capability. Ruled out slow rats.

Test of memory reconsolidation abilities: The Y-maze test went on 24 h after the learning test, so as to reach 9/10 standard. A stood for the number of times of right reaction (RR); A/10 implied ability of retentive memory, and the higher score, the better memory.

2.3. Establishment of animal models

Beta-amyloid protein 1–40 (Aβ1–40) (Sigma Company, USA) was diluted into 20 g/L injection by the application of 0.1 mol/L PBS sodium, and incubated for 72 h at 37 °C to be changed into colloidal state Aβ1–40, then stored at 4°C to be used.

Total of 30 selected rats which own learning and memory abilities were randomly divided into control group, model group and treated group consisting of 10 rats in each group. All surgical procedures were conducted under aseptic conditions, and every effort was made to minimize animal suffering and to reduce the number of animals used. All rats were anesthetized intraperitoneally by 100 g/L chloral hydrate at the dosage of 300 mg/kg, according to Nabhenshima [17–19]. Rats were anesthetized and positioned in a stereotaxic frame (Huanghua Agricultural Machinery Works of Shanghai, Jiangwan-1). Under sterile conditions and with the use of a stereotaxical technique, skin incision was made along the midline of the cranial, the puncture point was produced in the skull (AP = 0.8 mm, ML = 1.1 mm, DV = 3.6 mm), and 5 μL of Aβ1–40 was injected into the left lateral ventricle with a microsyringe at a rate of 1 μL/min. Retaining needle for 5 minutes after the injection was to make sure Aβ1–40 distributed equally and dispersedly. Withdraw microsyringe slowly, repaired with dental cement, applied gentamicin to the skin incision, and then closed the incision. The same volume PBS (0.1 μmol/L) was injected into corresponding position of the control group at the same time.

2.4. Treatment methods

Recombinant human NRG1β (purity ≥ 97%, R&D Systems, Inc., Catalog number: 396-HB) was diluted into 1 g/L water-solution using 0.1 mol/L phosphate buffered solution (PBS) in advance. Seven days after the experiment, memories were tested by the Y-electric maze. Then, in accordance with 5 ml/kg Aβ1–40 solution (PBS) in advance. Seven days after the experiment, memories were tested. (PBS) in advance. Seven days after the experiment, memories were tested. (PBS) in advance. Seven days after the experiment, memories were tested.

2.5. Evaluating index

2.5.1. Abilities of learning and memory

The abilities of learning and memory of rats was evaluated using the Y-electric maze 7 days after treatment with NRG1β.

2.5.2. Sample collection and preparation of paraffin sections

2.5.3. Assessment of apoptosis by TUNEL assay

To observe brain damage, terminal deoxynucleotidyl transference-mediated biotinylated deoxyuridine triphosphates nick end labeling technique (TUNEL) was performed to detect neurocyte apoptosis according to the protocol of TUNEL Detection System (Wuhan Boster Biochemical Techniques Co., Ltd., China). Coronal paraffin sections added D/Nase I at a dose of 1 μg/ml were regarded as positive control sample.

2.5.4. Immunohistochemistry assay

A rabbit anti-rat Bcl-2 and Bax multilcon antibody (Wuhan Boster Biochemical Techniques Co., Ltd., China) was used at a titer of 1:100 to detect the activity of Bcl-2 and Bax in brain tissue. All procedure was strictly performed in accordance with the manufacture directions. Paraffin sections as described above were dewaxed by xylene, hydrated with gradient alcohol, repaired antigen in a microwave oven, blocked non-specific binding sites with 3% immunized normal goat serum for 20 min, and then probed with primary antibody anti-Bcl-2 and Bax (diluted 1:100 in PBS) for 2 h at 37°C, biotin-conjugated secondary antibody (1:100) and SABC in PBS for 20 min, respectively. Covered with waterborne mount and finally detected directly under a light microscopy. Negative control slides added 0.1 mol/L PBS (containing 1:100 blocking serum) instead of primary antibody has no immunological reaction. Immunoreactivity was visualized under a 400-fold light microscope; four views were detected randomly in hippocampus of the confined areas from 4 serial slices in each rat. The absorbance (A) value of each view was determined with LEICA Qwin image processing and analysis system (Leica Company).

2.5.5. Double immunofluorescence labeling

Double immunofluorescence labeling for Bcl-2 and Bax proteins was performed to assess the associations between these reactions. Before immunofluorescence labeling, four serial sections from the same tissue were reacted with primary antibody of Bcl-2 and Bax, and further stained by different agents, respectively. For example, using laser excitation of 488 nm and reception of 510–530 nm wavelength, it appears yellow-green light posterior to SABC-FITC addition, while with excitation of 568 nm and reception of 600–650 nm, it shows red color after SABC-CY3 accession. According to the shade of the coloration, Bcl-2 (SABC-FITC) was defined as the first colorating antibody, and Bax (SABC-CY3) as the second colorating antibody.

Double fluorescence labeling steps: paraffin sections as described above were dewaxed by xylene, hydrated with gradient alcohol, repaired antigen in a microwave oven, blocked non-specific binding sites for 20 min, and then probed with primary antibody anti-Bcl-2 (diluted 1:200 in PBS) for 2 h at 37°C, biotin-conjugated secondary antibody (1:100) and SABC-FITC (1:100) for 30 min at 37°C, respectively. The sample was added with anti-Bax (1:200), the secondary antibody IgG (1:100), SABC-CY3 (1:100), PBS, successfully covered with waterborne mount and finally detected directly under a fluorescence microscopy. All slides were carried out under a dark circumstance throughout the operation and washed fully in PBS so as to avoid excessive fluorescence residual or degeneration.

2.6. Statistical analysis

SPSS11.5 software was used for statistical analysis. Data were expressed as mean ± standard error (x ± s). Multi-group comparison was made by analysis of variance (ANOVA) and Student’s test, and two-group comparison by t-test. Values were considered to be significant when P is less than 0.05.

3. Results

3.1. The learning and memory abilities of rats

The number of trials were increasing after building the model, and the different was significant between before and after the injection (P < 0.05), which meant learning memory ability decreased; but there was no evident difference in the control group (P > 0.05). After
**Table 1**
The average learning scores evaluated with Y-electric maze of rats (X±s).

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Before modeled</th>
<th>After modeled (before treated)</th>
<th>After treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>8</td>
<td>60.24±4.46</td>
<td>59.13±7.52</td>
<td>60.18±1.69</td>
</tr>
<tr>
<td>Model group</td>
<td>7</td>
<td>58.14±2.52</td>
<td>79.84±6.46(^a,b)</td>
<td>81.22±3.92(^b)</td>
</tr>
<tr>
<td>Treated group</td>
<td>7</td>
<td>56.56±9.63</td>
<td>81.31±3.31(^a,b)</td>
<td>68.54±8.86(^b,c)</td>
</tr>
</tbody>
</table>

\(^a\) P<0.05 vs control model.
\(^b\) P<0.05 vs control group.
\(^c\) P<0.05 vs model group.

**Table 2**
The average memory scores evaluated with Y-electric maze of rats (X±s).

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Before modeled</th>
<th>After modeled (before treated)</th>
<th>After treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>8</td>
<td>8.1±1.6</td>
<td>7.9±2.2</td>
<td>8.0±2.3</td>
</tr>
<tr>
<td>Model group</td>
<td>7</td>
<td>7.8±1.2</td>
<td>4.8±3.1(^a,b)</td>
<td>4.6±1.6(^b)</td>
</tr>
<tr>
<td>Treated group</td>
<td>7</td>
<td>7.5±5.2</td>
<td>5.1±2.5(^a,b)</td>
<td>7.1±1.1(^b)</td>
</tr>
</tbody>
</table>

\(^a\) P<0.05 vs control model.
\(^b\) P<0.05 vs control group.
\(^c\) P<0.05 vs model group.

NRG1β treatment for 7 days, the trial numbers of treated group were less than the model group (P<0.05), as meant learning memory ability of treated group increased significantly, however, the trial numbers were more than control group (P<0.05). Shown as Tables 1 and 2.

### 3.2. Apoptosis

There were a number of apoptosis positive cells and absorbance (A) control, model, and treated groups, while the number and absorbance (A) of apoptotic cell in model group (22.4±4.37 and 0.46±0.17) and treated group (13.6±3.62 and 0.34±0.13) more than those in control group (5.3±2.12 and 0.14±0.06)(P<0.05), as well as that in treated group less than that in model group (P<0.05). Shown as Table 3 and Fig. 1.

### 3.3. Immunohistochemical assay

Using a light microscopy, the Bcl-2 and Bax positive cells had irregular shapes and yellow (Figs. 2 and 3) signals and scattered in the hippocampus. There were a few of Bcl-2 and Bax positive cells could be seen in hippocampus in control group. The number of positive cells and the expression Bcl-2 and Bax proteins increased significantly, as well as the heavier A value in model groups (P<0.05). As expected, the positive cells and the A values of Bcl-2 protein in treated group were significantly higher, while Bax protein lower than those in model group (P<0.05). Shown as Table 4 and Figs. 2 and 3.

### 3.4. Double immunofluorescence labeling

Based on immunofluorescent double labeling, we found that almost all the Bcl-2 positive fluorescent signals were co-located in the Bax positive cells in hippocampus. In the treated group with NRG1β, the results demonstrate that exogenous NRG1β could enhance the Bcl-2 and reduce the Bax expressions than those in the model group. Shown as Fig. 4.

### 4. Discussion

#### 4.1. AD and Aβ

The deposition of amyloid β-protein (Aβ) is the major reason for the formation of senile plaques (SPs) and it might be the common pathway of Alzheimer’s disease caused by all factors [20]. Amyloid β-protein (Aβ) is composed by 39–43 amino acid residues formed in the process of modifying amyloid precursor protein (APP) by various cutting methods. The deposition of condensed Aβ in parenchyma starts pathological cascade, results in the formation of neurofibrillary tangles (NFTs), the damage of synapse and neuraxton, leads to neurocyte denaturation and apoptosis. Amyloid precursor protein (APP) is widely in the cells of all tissues throughout the body, and it is transmembrane glycoprotein with transmembrane receptor protein structure. It mainly locates on neuron synapses, and its functions are unclear until now [21]. Studies suggest that lack of APP may result in enhancement of synapse transmission [22]. APP is normally hydrolyzed by α-secretase into soluble secreted APP (β-APP), and it stimulates cells proliferation, promotes adhesion between cells and matrix and protects neurons from the damage caused by excitatory toxin and oxidative stress reaction. β-APP is hydrolyzed into soluble Aβ outside the membrane or at transmembrane location by β-secretase and γ-secretase, and there are usually two types of Aβ (Aβ\(_{40}\) and Aβ\(_{42}\)). Aβ has a strong neurotoxicity, and may lead to huge amounts of neurons death rapidly [23,24]. Besides excessive production of Aβ caused by APP gene mutation, the metabolic disorder of Aβ clearance and degradation is an essential reason for the deposition of Aβ. Aβ may inhibit the activity of high molecular weight protease in brain which will bring about the failure degradation of Aβ and a vicious circle of deposition [25]. More and more evidences have shown that amyloid β1–40 (Aβ1–40) causes neurons damage mainly by apoptotic pathway [26]. Aβ1–40 will lead to oxidative stress and Ca\(^{2+}\) overloading, and activate intra-cellular apoptins and damage mitochondria at the same time, which will result in the disorder of energy supply system, cells dysfunction and neurons apoptosis [27].

Bcl-2 family is important regulators in apoptosis mechanism, and Bcl-2 and Bax will play the role in the form of homodimer or heterodimer. The increase expression of Bax protein will form homodimer to induce apoptosis. Because of cell self-protection mechanism, the expression of Bcl-2 protein will be increased in response which will lead to part of Bax homodimer separation. Heterodimer will be formed by separated Bax homodimer and Bcl-2 protein, and that will inhibit apoptosis [28]. Studies of Meng [29] has shown that TUNEL positive cells exist in temporal cortex brain of both AD patients and non AD patients, but the number of temporal lobe neurons in AD patients is significantly decreased, the relative amount of TUNEL positive cells is significantly increased. The reason for apoptosis of temporal lobe neurons in AD patients is the increase of Bax, caspase expression which is related to...
apoptosis cascade reaction and the decrease in expression of Bcl-2 expression with the function of anti-apoptosis, both of which promote apoptosis further. In this experiment, injecting \( \text{A} \beta_1-40 \) into the lateral ventricle of rats, the expression of Bax protein promoting apoptosis is dominant, and it leads to apoptosis of neurons in specific parts of the brain and significant injuries of learning and memory of rats. The rats perform cognitive dysfunction like AD behaviors. This is consistent with previous reports.

4.2. NRG and AD

Researches have proved that in the target region with basal forebrain cholinergic innervation (such as cerebral cortex and hippocampus), there is high concentration expression of NGFmRNA. The expression of NGFmRNA may synthesize nerve growth factor (NGF) which can be taken into neuraxon of basal forebrain cholinergic innervation, transported to cell body reversely. NGF may induce a series of intracellular information transmission, and finally lead to a series of biological effects including expression of functional protease and growth of neurite in neurons. All of these play an important part in the survival of neurons and neuraxon regeneration [30]. The neuregulins are a family of multipotent growth factors that includes acetylcholine receptor inducing activities (ARIAs), glial growth factors (GGFs), heregulins and neu differentiation factors (NDFs). Neuregulins effects appear to be mediated by interaction with a class of tyrosine kinase receptors related to the epidermal growth factor (EGF) receptor which includes erbB2, erbB3 and erbB4 [31]. In the rat brain, NRG-1, erbB2 and erbB4 are localized in various areas of the cerebral cortex, hippocampus and cholinergic nucleus in the diencephalon primarily in neuronal cell bodies [32]. ErbB4 expression was not seen until 3 day following the injury, suggesting that NRG-1/erbB4 signaling was associated with a delayed neuroprotective or injury promoting response. However, in each case, the induction of NRG-1 and erbBs was localized to cells at the edge of the lesion (penumbra) suggesting a role for NRG-1 and erbBs in neural protection and repair [10]. Xu et al. [9] reported that cerebral ischemia rats treated with either vehicle or NRG1β before MCAO, extensive TUNEL staining was found in the nuclei of cells within the ischemic cortical and subcortical areas after reperfusion 24 h. Ischemia-induced TUNEL staining was abolished by NRG1β treatment in the areas within the cortical penumbra. NRG1β-treated tissues showed no TUNEL-positive cells in the cortex. In the striatum, TUNEL-positive cells were seen, although the appeared fewer in number and were lightly stained after NRG1β.
administration. A large number of these TUNEL-positive cells were co-labeled with antibodies against MAP-2 and neuron specific enolase indicating that neurons were protected from apoptosis by NRG1B although many of the TUNEL-positive cells were not neurons.

After the treatment of injecting NRG1B into ventricle, neurons apoptosis is decreased, the positive expression of Bcl-2 is increased while that of Bax is decreased, and Bcl-2/Bax ratio is increased. This suggests that NRG1B might play a role in anti-apoptosis by increasing the expression of Bcl-2, decreasing the expression of Bax, increasing the heterodimer produced by Bcl-2 and Bax, thus reduce the apoptosis caused by amyloid and improve the functions of learning and memory of rats.

5. Conclusion

NRG1B could inhibit neuronal apoptosis by regulating the expressions of Bcl-2/Bax to improve the capacity of learning and memory in experimental dementia rats.

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