

NR2B-Containing NMDA Receptors Expression and Their Relationship to Apoptosis in Hippocampus of Alzheimer's Disease-Like Rats

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Abstract Although studies have shown that excitotoxicity mediated by N-methyl-D-aspartate receptors (NMDARs, NR) plays a prominent role in Alzheimer's disease (AD), the precise expression patterns of NMDARs and their relationship to apoptosis in AD have not been clearly established. In this study, we used A β (A β) 1-40 and AlCl₃ to establish AD rat model. The behavioral changes were detected by morris water maze and step-down test. The hippocampal amyloid deposition and pathological changes were determined by congo red and hematoxylin-eosin staining. Immunohistochemistry was used to detect expression of NR1, NR2A and NR2B, and TUNEL staining was used to detect apoptosis. Results showed that water maze testing escape latency of AD-like rats was prolonged significantly. Reaction time, basal number of errors, and number of errors

of step-down test were increased significantly; latency period of step-down test was shortened significantly in AD-like rats. Amyloid substance deposition and obvious damage changes could be seen in hippocampus of AD-like rats. These results suggested that AD rat model could be successfully established by A β 1-40 and AlCl₃. Results also showed that expression of NR1 and NR2B were significantly increased, but expression of NR2A had no significant change, in AD-like rat hippocampus. Meanwhile, apoptotic cells were significantly increased in AD-like rat hippocampus, especially in CA1 subfield and followed by dentate gyrus and CA3 subfield. These results implied that NR2B-, not NR2A-, containing NMDARs showed pathological high expression in AD-like rat hippocampus. This pathological high expression with apoptosis and selective vulnerability of hippocampus might be exist a specific relationship.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and also is the most common form of dementia, with more than 20 million cases worldwide [1, 2]. AD is characterized by dementia that typically begins with subtle and poorly recognized failure of memory and slowly becomes more severe and, eventually, incapacitating [3]. Progressive neurodegeneration in AD is initially characterized by synaptic damage accompanied by neuronal loss, especially in the hippocampus [4]. Typical neuropathological changes of AD are formation of amyloid-beta (A β)-containing senile plaques (SP) and neurofibrillary tangles (NFT) composed of hyperphospho-

phorylated tau [4, 5]. Among many hypotheses on pathogenesis of AD, the amyloid cascade hypothesis always lies in the core, and continues to dominate AD literature and grant applications [6]. Meanwhile, studies have also reported that Aluminium (Al) is a neurotoxic metal and Al exposure may be a factor in aetiology of various neurodegenerative diseases such as AD [7, 8]. Al causes accumulation of amyloid-beta protein and tau protein in experimental animals, and Al induces neuronal apoptosis *in vivo* as well as *in vitro* [9]. Although the animal models of Al exposure can cause behavioral changes of experimental animals, the experiment cycle often is very long and the main neural pathological changes of experimental animals can not be seen. In view of this, the present study used A β 1-40 and AlCl₃ double intervention methods to establish an ideal and practical AD rat model. In order to deeper research animal models, etiology, pathogenesis and screening related treatment drugs of AD provide theoretical foundation and experimental platforms.

N-methyl-D-aspartate receptors (NMDARs, NR) are key molecules involved in physiological and pathophysiological brain processes such as synaptic plasticity and excitotoxicity [10]. AD is characterized by decreased synapse density in hippocampus and neocortex, and synapse loss is the strongest anatomical correlate of the degree of clinical impairment [11]. Recent experimental evidence suggests that amyloid-beta disturbs NMDA receptor-dependent long-term potentiation (LTP) induction in CA1 and DG both *in vivo* and *in vitro* [12]. Using two-photon Ca²⁺ imaging in a mouse model of AD *in vivo*, study found that although a decrease in neuronal function was seen in 29% of layer 2/3 cortical neurons, 21% of neurons displayed an unexpected increase in the frequency of spontaneous Ca²⁺ transients. These hyperactive neurons were found exclusively near the plaques of amyloid beta-depositing [13]. It suggest that AD may be associated with activity of these hyperactive neurons. Neuronal function regulates NMDA receptor levels in the cell membrane, however, in AD, little is known on whether the two main dimeric NMDA receptor subtypes in forebrain, NR1/NR2A and NR1/NR2B, are regulated in a similar fashion [14]. As these differ considerably in their electrophysiological properties, the NR2A/NR2B ratio affects the neurons' reaction to NMDA receptor activation [15]. Although these studies have shown that excitotoxicity mediated by NMDA receptors plays a prominent role in pathogenesis of AD, the precise expression patterns of NMDA receptors and their relationship to apoptosis in AD have not been clearly established. The present study will use immunohistochemistry to detect expression of NR1, NR2A and NR2B; use TUNEL staining to detect apoptosis in hippocampus of AD-like rats, and further to explore the relationship between expression of NMDA receptors and apoptosis.

Experimental Procedures

Main Reagents and Apparatus

Abeta1-40, AlCl₃ and congo red were purchased from Sigma-Aldrich (USA). The product numbers of Abeta1-40 and AlCl₃ are A4473 and 563919, respectively. Both of them can be dissolved in the corresponding solvent. Goat polyclonal anti-NR1, NR2A and NR2B antibody were purchased from Santa Cruz (CA). Secondary antibodies used in our experiment was rabbit anti-goat IgG, which was purchased from Abcam Ltd (UK). TUNEL *in situ* cell death detection kit was obtained from Roche (Germany). Diaminobenzidine, and 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were obtained from Promega (Madison, WI, USA). The morris water maze system was purchased from Actimetrics (USA). Micro-camera system was purchased from Olympus Corporation (Japan). Image Analysis Software were obtained from Media Cybernetics (USA).

Animal Training, Screening and Grouping

Healthy male aged Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) were trained and screened by morris water maze system. The training was executed 4 times daily for 5 days. Facing the pool wall, rats were put into water from four different points. Meanwhile, the time (escape latency) of rats finding platform was recorded. If the rats could not find platform in 2 min, they would be tracted to platform by experimenters, and staying for 20 s. The rats always could not find platform in 2 min would be eliminated. After training and screening, rats were randomly divided into AD group, vehicle group and normal group, which group have 20 rats.

Animal Model Establishing

In this study, AD rat animal model was established by one-time unilateral intracerebroventricular injection of A β 1-40 and continuous intraperitoneal injection of AlCl₃ every other day for 4 weeks. Referring to 'The Rat Brain in Stereotaxic Coordinates' written by Paxinos and Watson [16], stereotaxic coordinates (anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma) of lateral ventricle were selected. After the animals were anesthetized, 30 μ l A β 1-40 (0.5 mg/ml) was slowly injected into lateral ventricle. After 5 days, 3% AlCl₃ with 100 mg/kg doses were continuous injected into the peritoneal cavity (the animals were not anesthetized) every other day for 4 weeks. The rats were allowed free access to food and water, and were housed in a room with a light–dark cycle

of 12 h that was maintained at a constant temperature of 23–25°C and a humidity of 55%. The protocols for animal use are approved by the local legislation for ethics of experiments on animals. After the treatment in different time points, we mainly through observing animals' behavioral changes and behavioral experiments to evaluate whether the animals are dementia.

Behavior Analysis

Morris water maze was used to test rats' learning and memory capacity of spatial orientation. Experiment was carried out 4 times daily for 5 days. Facing pool wall, rats were put into water from four different points. Meanwhile, the time (escape latency) of rats finding the platform in 2 min was recorded. After 5 days of morris water maze test, step-down test was used to detect rats' function of learning and memory. Rats were put into reaction chamber free activities 5 min for familiar with environment. Then, the copper grid power (0.25 mA, alternating current) was connected. The "copper grid power" was connected to a small transformer which can adjust the voltage and current, the transformer was connected to daily alternating current. In order to avoid shock, normal reaction of rats was looking for safety platform. The Reaction time (from rats subjected shock to first jumped on safety platform) and basal number of errors (number of rats jumped off security platform in 5 min) were recorded, and they were regarded as learning scores of rats. After 24 h, above experiment was repeated. The latency period (time of rats first jumped off security platform) and number of errors (number of rats subjected shock in 5 min) were recorded, and they were regarded as memory scores of rats. If the rats on security platform residence time more than 5 min, their latency period were regarded as 5 min.

Perfusion Fixation and Slices Preparation

After behavior analysis and anesthesia, rats were perfused quickly with 4% paraformaldehyde by intubations through heart to ascending aorta. Brain tissue containing hippocampus was removed to carry out paraffin embedding. Consecutive coronal brain slices with a thickness of 6 μ m were made from embedded brain tissue.

Histology Analysis

Congo red staining was used to detect amyloid deposition in rats hippocampus. The brain slices were deparaffinized with xylene and rehydrated by gradient ethanol, followed by washing. Then slices were stained in congo red working solution for 20 min. After washing, slices were differentiated quickly in alkaline alcohol solution. After washing,

slices were counterstained with hematoxylin for 5 min. After washing, slices were dehydrated through 95% alcohol and 100% alcohol, and were cleared in xylene and sealed with neutral balsam. H-E staining was used to determine pathologic changes in rats hippocampus. The brain slices were deparaffinized with xylene and rehydrated by gradient ethanol, followed by washing. Then, slices were stained with hematoxylin and eosin (their concentrations are all 0.5%), and were examined with light microscopy.

Immunohistochemistry

After dewaxing, rehydration and washing, antigen retrieval was operated in ethylenediaminetetraacetic acid (EDTA) buffer solution using microwave, then brain slices were transferred to 3% hydrogen peroxide for 10 min to block

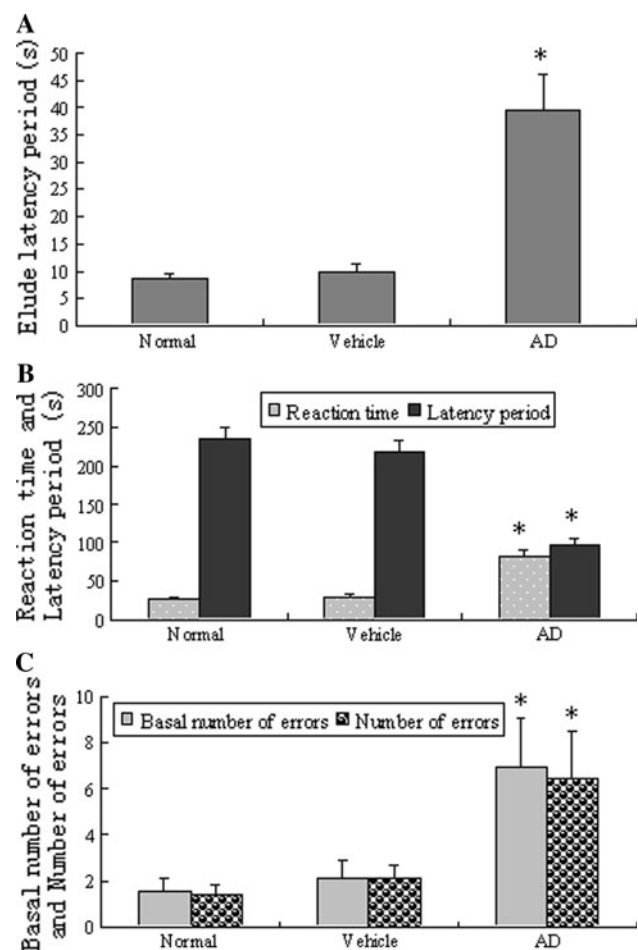


Fig. 1 Behavior analysis of control and Alzheimer's disease (AD)-like rats. Comparison of elude latency period in each group by morris water maze testing (a). Comparison of reaction time, basal number of errors, latency period and number of errors in each group by step-down testing (b, c). Data were shown as mean \pm SD, $n = 15$. In a, b and c, asterisk indicates significantly different to vehicle or normal ($p < 0.05$)

endogenous peroxidase. After washing, bovine serum albumin was added to brain slices (37°C, 1 h), then primary antibodies (1: 100) were loaded to brain slices (4°C, 48 h). After washing, corresponding secondary antibodies (1:200) were loaded to brain slices (37°C, 30 min). Again washing, horseradish peroxidase was added to brain slices (37°C, 30 min). After washing, the slices were developed color in peroxidase substrate.

TUNEL Staining

TUNEL staining was performed using an alkaline phosphatase-conjugated in situ apoptosis detection kit according to the manufacturer's protocol with minor modifications. After dewaxing and rehydration, brain slices were treated with protease K (20 µg/ml) for 15 min at room temperature. Then slices were incubated with reaction buffer containing TdT enzyme (37°C, 1 h). After washing with stop/wash buffer, slices were treated with anti-digoxigenin conjugate for 30 min at room temperature, and subsequently developed color in alkaline phosphatase substrate.

Statistical Analyses

Values are expressed as mean \pm standard deviation (SD). The intensity of cell staining was measured by gray-scale using LEICA Qwin image analyzer. Statistical analysis of the results were performed using one-way analysis of the variance (ANOVA), multiple comparisons between groups were then performed by post hoc test with the method of

Student–Newman–Keuls (S–N–K). In all cases, p values <0.05 were considered significant.

Results

Ethological Performance of AD-like Rats

During the model making, the weight, diet, activity and explore behaviors of AD-like rats were all progressive decline, and the hair of AD-like rats was also gradually becoming sparse and mixed and disorderly. The normal rats and control rats had no obvious abnormal manifestation. Compared with control group rats, the elude latency period by morris water maze testing of model group rats was significantly longer (Fig. 1a); the reaction time, basal number of errors, and number of errors by step-down testing of model group rats were increased significantly (Fig. 1b, c); the latency period by step-down testing of model group rats was shortened significantly (Fig. 1b).

Histologic Changes in the Hippocampus of AD-like Rats

Congo red staining, a specific method, was used to detect the amyloid substance deposition. By congo red staining, the specific orange sediments could be seen in hippocampus of AD-like rats (Fig. 2Ac, d). It suggested that the hippocampus of model group rats had amyloid protein deposit. Meanwhile, no specific orange sediments could be seen in hippocampus of control groups (Fig. 2Aa, b).

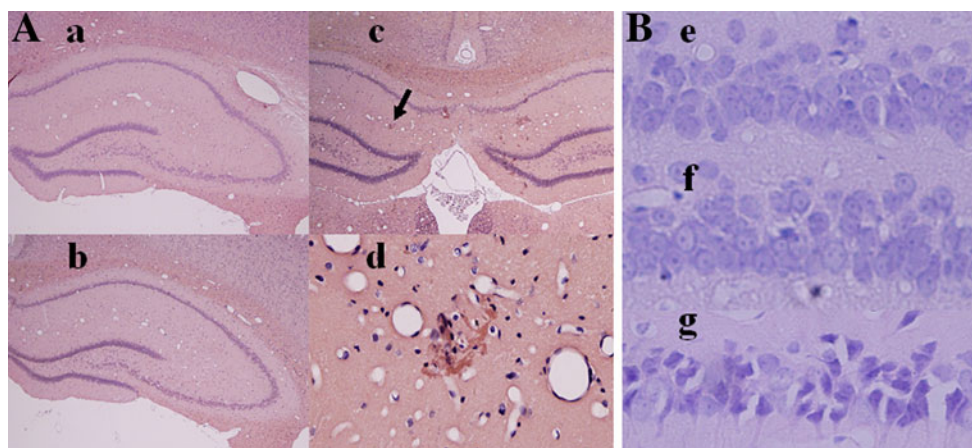


Fig. 2 Histology analysis of control and Alzheimer's disease (AD)-like rats. Congo Red staining of amyloid protein deposits in hippocampus of rats (A). No amyloid protein deposit in hippocampus of normal group (a) and vehicle group (b). The amyloid protein deposit can be seen obviously in hippocampus of AD group (c, d), and d is local amplification of black arrow in c. H-E staining of pathologic

changes in CA1 region of rats hippocampus (B). There are no obvious pathologic changes in normal group (e) and vehicle group (f). However, the significantly different of pathologic changes to control group can be seen in AD group (g) ($p < 0.05$). In a, b and c, magnification is $\times 40$. In d, e, f and g, magnification is $\times 400$

The results of H-E staining showed that, comparing with control groups (Fig. 2Be, f), the obvious damage changes in morphology and reduction of cells number could be seen in hippocampal CA1 region of model group rats (Fig. 2Bg) ($p < 0.05$).

Expressions of NR1, NR2A and NR2B in the Hippocampus of AD-like Rats

Comparing with control groups (Fig. 3Aa, c, e, 3B), the expression of NR1 was significantly increased in hippocampus of AD-like rats (Fig. 3Ab, d, f, B). Comparing with control groups (Fig. 3Cg, i, k, D), the expression of NR 2B was also significantly increased in hippocampus of AD-like rats (Fig. 3Ch, j, l, D). Comparison between CA1, CA3 and DG subfields, the increased scopes of NR1 and NR2B expression had differences, but no statistical significance. Comparing with control groups, the expression of NR2A had no significant changes in hippocampal different subfields of AD-like rats ($p > 0.05$). The colouration of immunohistochemical reaction positive cells was mainly in cytomembrane and showed claybank, and cytoplasm dyeing was very shallow, sometimes, the nucleolus could be seen (Fig. 3A, C).

Apoptosis in the Hippocampus of AD-like Rats

Comparing with control groups (Fig. 4Aa, c, e, B), the apoptosis positive cells were significantly increased in hippocampus of AD-like rats, especially in CA1 subfield and followed by dentate gyrus and CA3 subfield (Fig. 4Ab, d, f, B). The nucleus of apoptosis positive cells showed mazarine, and their shape was irregular, some pyknosis and some dissociation (Fig. 4Ab, d, f).

Discussion

The all existed Alzheimer's disease (AD) animal models have certain limitations, therefore, to establish an ideal and practical AD animal models is still one of the key problems [2]. Transgenic animal models of AD is too expensive and complex, so it is difficult to be widely spread. Administration of A β 1-40 can produce morphological changes, but behavioral changes are not obvious. Administration of AlCl₃ can cause behavioral deficits, but the main neural pathological changes of AD can not be duplicated. So we explored to establish a new AD rat model with A β 1-40 and AlCl₃. Beta amyloid protein cascade hypothesis of AD has been recognized. It suggested that generation and accumulation of abeta was the core of AD pathogenesis, and oxidative stress, excitotoxicity, formation of SP and NTF, as well as cell death cascade activation were all

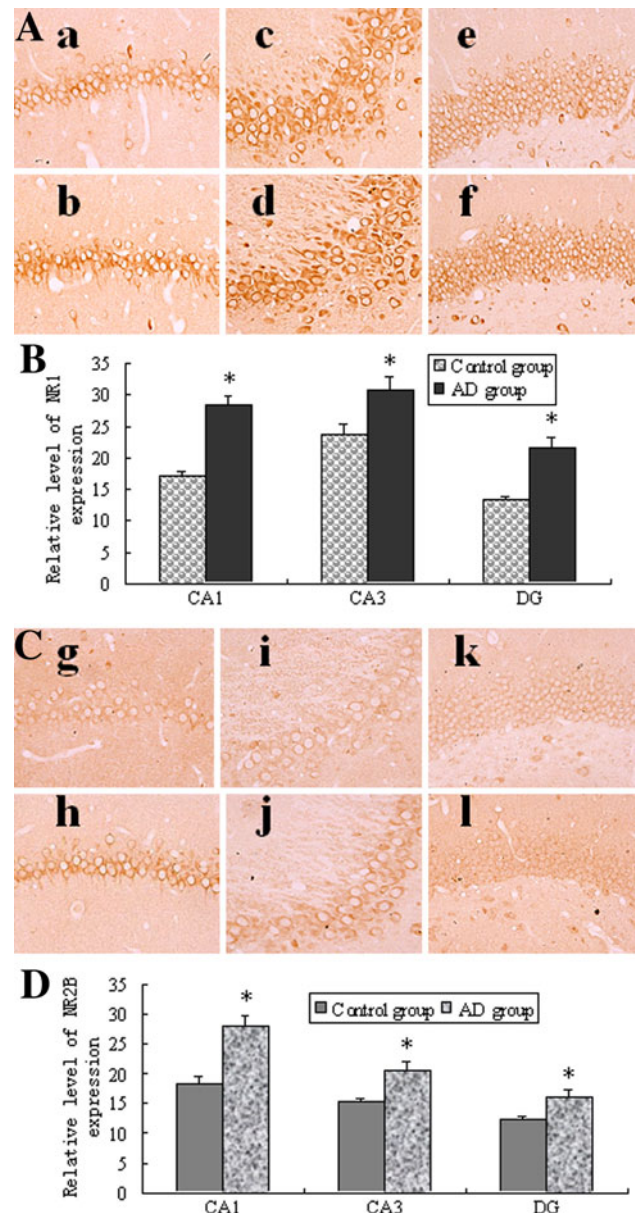
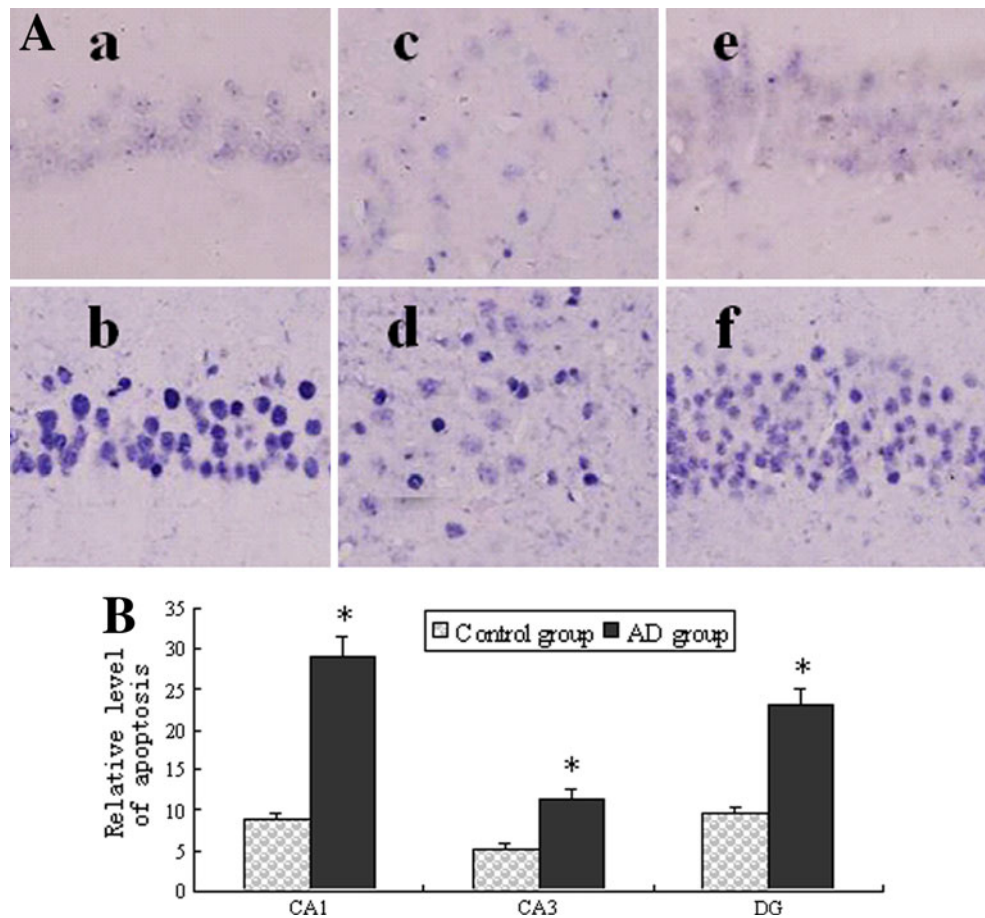


Fig. 3 Immunohistochemistry of NR1 and NR2B in hippocampus of control and Alzheimer's disease (AD)-like rats. Immunohistochemical photomicrographs showing NR1 (A) and NR2B (C) immunopositive cells in different subfields of rat hippocampus (DAB staining, $\times 100$). a, b, g and h: CA1 subfield; c, d, i and j: CA3 subfield; e, f, k and l: dentate gyrus. a, c, e, g, i and k: control group; b, d, f, h, j and l: AD group. Semi-quantitative analysis of expression levels of NR1 (B) and NR2B (D) in different subfields of rat hippocampus of control and AD groups. Photomicrographs were scanned and the intensity were determined by gray value measurements. Data were obtained from five independent animals in each experimental group, and the results of a typical experiment are presented. Asterisk indicates significantly different to control groups ($p < 0.05$) both in B and D

regarded as downstream events [1]. Aluminum has been associated with several neurodegenerative diseases, in particular, AD [9]. Aluminium can cause lipid peroxidation and free radical increase through damaging mitochondria,

Fig. 4 TUNEL staining of apoptosis in hippocampus of control and Alzheimer's disease (AD)-like rats. TUNEL reactive photomicrographs showing the apoptosis positive cells in different subfields of rat hippocampus (A) (BCIP/NBT staining, $\times 100$). a, b: CA1 subfield; c, d: CA3 subfield; e, f: dentate gyrus. a, c, e: control group; b, d, f: AD group. Semi-quantitative analysis of apoptosis level in different subfields of rat hippocampus of control and AD groups (B). Photomicrographs were scanned and the intensity were determined by gray value measurements. Data were shown as mean \pm SD, $n = 5$. Asterisk indicates significantly different to control groups ($p < 0.05$)



and affect the form and maintain of long-term potentiation (LTP) through damaging related receptors and ion channels [17]. Aluminum may play crucial roles as a cross-linker in β -amyloid oligomerization, and it can cause accumulation of amyloid-beta protein in experimental animals [18]. For these reasons, in this study, we first to establish an AD rat model with β -amyloid protein-40 ($A\beta 1-40$) and $AlCl_3$.

The methods of morris water maze test and step-down test were two classical behavioral experiments reflecting function of learning and memory [19, 20]. Their results had obvious consistency and both showed that learning and memory function of AD-like rats suffered significant damage. From angle of behavior, the results suggested that the AD rat model intervened by $A\beta 1-40$ and $AlCl_3$ was successful. Congo red staining is a frequently-used specific method to detect amyloid material deposition in tissue [21]. Its results showed that amyloid substance deposition could be seen in hippocampus of AD-like rats. The generation and accumulation of abeta is a central part of the AD pathogenesis [22]. It implied that beta amyloid protein deposits might be the upstream events that occurred before hippocampal cells injury and behavioral changes. The results of H-E staining showed that obvious damage changes in morphology and reduction of cells number could be seen in

hippocampus of AD-like rats. This was inevitable results of $A\beta 1-40$ and $AlCl_3$ intervention, meanwhile, also was an important reason of cognitive impairment of learning and memory of AD-like rats. In a word, results of above-mentioned four experiments suggested that an ideal and practical AD rat model could be successfully established by $A\beta 1-40$ and $AlCl_3$. The main merits of this model were not only can produce the morphological changes, but also can cause behavioral deficits of experimental animals, as well as easily to be spreaded for use.

Although reports have showed that apoptosis is a main form of AD-like cell death, the exact mechanism of AD-like cell death is not clear [23]. Increase of cytoplasm Ca^{2+} content was thought to be the initiate factors of apoptosis, and the prerequisites of activation endogenous endonuclease and degradation DNA [24, 25]. Moreover, pathological cytoplasm Ca^{2+} overload was mainly mediated by NMDA receptors [26]. Therefore, we speculate that NR1, NR2A and NR2B acting as functional subunit and regulative subunits of NMDA receptors, respectively, their expression in hippocampus of AD-like rats may be exist some inherent connection with apoptosis.

Our results showed that high expression of NR1 and NR2B, and apoptosis in hippocampus of AD-like rats had remarkable

consistency. We thought pathological high expression of NR1 and NR2B might contribute to apoptosis. High expression of NR1 caused Ca^{2+} channels excessive open and cytoplasm Ca^{2+} overload [27]. Overloaded Ca^{2+} might combine with calmodulin (CaM) and caused excessive phosphorylation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). The phosphorylated CaMKII, in turn, acted on NR1 and promoted its channels open, thus constituted positive feedback loop and formed vicious circle. Meanwhile, overloaded Ca^{2+} might also mediate excitotoxicity injury and produce lots of free radicals, and caused oxidative stress and oxidative damage, and eventually caused apoptosis through mitochondria pathways [28]. High expression of NR2B was likely to change structure, function and components of NMDA receptor complex, made ion channels permeability exceptional increasing, caused Ca^{2+} overload and participated in excitotoxicity injury mediated by NMDA receptors [29]. Meanwhile, in NMDA receptor complex, NR2B was main substrate of phosphokinase, and it was more likely to happen phosphorylation in brain injury [30]. We thought pathological high expression's NR2B was likely to happen abnormal phosphorylation. The abnormal phosphorylated NR2B might combine with CaMKII, which was regarded as molecular switches of learning and memory, and locked it in a activated state of can't be phosphatase reversal, and caused CaMKII in a lasting phosphorylation state [31]. This state would intensify Ca^{2+} internal flow and cytoplasm Ca^{2+} overload, further induced NR2B phosphorylation, thereby formed vicious circles, and promoted cells injury.

Comparison of apoptosis positive cells between CA1 and DG had differences but no statistical significance, and compared with CA3, both had significant difference. Literature reported that obvious space memory disorders could be seen in AD transgenic mice, at the same time, LTP of hippocampal CA1 and DG subfield was damaged significantly [12], which was consistent with our results. LTP inhibition of CA1 and DG was obviously relevant with their cell apoptosis. As a basic structure of learning and memory, hippocampus had higher sensitivity and highly selective to AD, and had basic characteristics of AD lesions [32]. In hippocampal formation, CA1 subfield was functional areas of most close relations with learning and memory, and also was a selective vulnerability of AD [33]. This was demonstrated by our studies, and our results could provide a reasonable explanation for selective vulnerability phenomenon, which might be relevant with pathological high expression of NR1 and NR2B. Our results also implied that DG might be another selective vulnerability of AD-like rats, but CA3 had relatively tolerability [34]. This conclusion has not been reported in related literature, its specific reason and mechanism needed to be further research.

Definite roles of NR2B and NR2A in LTP and LTD still existed larger dispute and was not clear, and different roles

of NR2B and NR2A in learning and memory was also rare reports in the literature. The expression of NR2B and NR2A in hippocampus of AD-like rats and their specific relationship with learning and memory function obstacle have not been reported in the literature. Our results showed that expression of NR2A was no significant changes in hippocampus of AD-like rats. It indicated that, in learning and memory function obstacle of AD-like rats, NR1/NR2B type NMDA receptors, not NR1/NR2A type NMDA receptors, exerted important roles. Previous studies showed that main type of NMDA receptors in synaptic plasticity, LTP and central nervous degenerative disease was NR1/NR2B, rather than NR1/NR2A [35]. It strongly implied the reliability of our results.

Taken together, the current studies provided information for the first time that pathological high expressions of NR1 and NR2B in hippocampus of AD-like rats were probably the main reason of apoptosis. The high expressions of NR1 and NR2B, and apoptosis were probably the important reason of selective vulnerability of CA1 and DG, and relatively tolerability of CA3 in hippocampus of AD-like rats. In hippocampal apoptosis and dysfunction of AD-like rats, NR1/NR2B type NMDA receptors, not NR1/NR2A type NMDA receptors, exerted important roles. Our results also bring a new insight on the prevention and treatment of clinical Alzheimer's disease, as well as the research and development of specific new drugs aiming at NR2B-containing NMDA receptors.

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