Original Article

Effects of Exposure to Aluminum on Long-term Potentiation and AMPA Receptor Subunits in Rats *in vivo*[*]

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Abstract

Objective To explore the effects of exposure to aluminum (Al) on long-term potentiation (LTP) and AMPA receptor subunits in rats *in vivo*.

Methods Different dosages of aluminum-maltolate complex [$Al(mal)_3$] were given to rats via acute intracerebroventricular (i.c.v.) injection and subchronic intraperitoneal (i.p.) injection. Following Al exposure, the hippocampal LTP were recorded by field potentiation technique *in vivo* and the expression of AMPAR subunit proteins (GluR1 and GluR2) in both total and membrane-enriched extracts from the CA1 area of rat hippocampus were detected by Western blot assay.

Results Acute Al treatment produced dose-dependent suppression of LTP in the rat hippocampus and dose-dependent decreases of $GluR_1$ and $GluR_2$ in membrane extracts; however, no similar changes were found in the total cell extracts, which suggests decreased trafficking of AMPA receptor subunits from intracellular pools to synaptic sites in the hippocampus. The dose-dependent suppressive effects on LTP and the expression of AMPA receptor subunits both in the membrane and in total extracts were found after subchronic Al treatment, indicating a decrease in AMPA receptor subunit trafficking from intracellular pools to synaptic sites and an additional reduction in the expression of the subunits.

Conclusion $Al(mal)_3$ obviously and dose-dependently suppressed LTP in the rat hippocampal CA1 region *in vivo*, and this suppression may be related to both trafficking and decreases in the expression of AMPA receptor subunit proteins. However, the mechanisms underlying these observations need further investigation.

Key words: Aluminum-maltolate complex; Long-term potentiation; AMPA receptor; Hippocampus; *in vivo*

Biomed Environ Sci, 2014; 27(2):77-84 *doi: 10.3967/bes2014.006* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Aluminum (Al) is the most abundant metal in the Earth's crust, yet it has no known physiological function in biological systems. Recently, Al's involvement in various cognitive dysfunctions and neuronal diseases such as dialysis encephalopathy[1], Parkinson dementia of Guam[2-3] and Alzheimer's disease[4-5] has been recognized. With the exception of dialysis encephalopathy, the hypothesis that Al is a causative agent in neurodegenerative diseases is still

[*]This work was supported by the Natural Science Foundation of China (NSFC, 30972512 and 81202182) and the Research Foundation for the Doctoral Program of Higher Education (20121417110002).

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Received: July 16, 2013; Accepted: September 7, 2013

controversial.

To characterize the neurotoxicological effects of Al, we performed experiments measuring cognitive abilities, such as synaptic plasticity (a measure of neurodevelopment, learning, and memory). The most widely studied example of synaptic plasticity is long-term potentiation (LTP) in the hippocampus. Hippocampal LTP is an activity-dependent increase in synaptic efficiency, and its impairment is closely associated with deficits in behavioral learning and memory[5-7]. As a neuronal model of synaptic plasticity[8], hippocampal LTP has been widely used in experiments aiming at elucidating the synaptic mechanisms involved in the loss of learning and memory. However, whether and how Al affects hippocampal LTP *in vivo* is still an open and controversial question. For example, some *in vivo* and *in vitro* studies have reported that Al impairs LTP in the hippocampus[9-12], whereas Gilbert and Shafer have concluded that Al has no effect on LTP *in vitro*[13].

Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors (AMPAR) are multimeric proteins composed of GluR1, GluR2, GluR3, and GluR4 subunits that mediate the majority of fast excitatory transmission in the central nervous system[14-16]. The trafficking of GluR1 and GluR2 from intracellular pools to synaptic sites alters synaptic strength and has been recognized as a central mechanism of LTP[17-18]. Since Al has been reported affecting LTP, may it be related to AMPAR? However, few studies on the effects of Al on AMPARs have been performed. Surprisingly, in a previous study, we observed decreased expression of glutamate receptors, including AMPARs, in the hippocampus after subchronic Al exposure in rats[19].

On the basis of this previous study of the effects of Al on AMPARs and the possible involvement of AMPARs in LTP, the present study was aimed to investigate the effects of acute and subchronic treatment with different doses of aluminum-maltolate complex [$Al(mal)_3$] on LTP and the total and membrane levels of the GluR1 and GluR2 AMPAR subunits in the rat hippocampus. Here, a possible link between LTP dysfunction and AMPAR expression following Al exposure was also explored.

MATERIALS AND METHODS

$Al(mal)_3$ Preparation

Aluminum-maltolate complex [$Al(mal)_3$] was prepared according to the procedure described in previous publications[20-21]. $AlCl_3 \cdot 6H_2O$ (Sigma Chemical Co., MO, USA) and maltolate (Sigma Chemical Co., MO, USA) were dissolved in distilled water and phosphate buffered saline (PBS), respectively. $Al(mal)_3$ was freshly prepared for each experiment by mixing these solutions in equal volumes, adjusting the pH to 7.4 with NaOH, and filtering with 0.22 μmol/L syringe filters.

Animal Models and $Al(mal)_3$ Treatments

The experiments were performed on 48 adult male rats (the Laboratory Animal Center, Shanxi Medical University, Taiyuan, China) that were kept under constant temperature and humidity conditions with free access to food and water. The rats were randomly divided into four groups (6 rats /group): a control group, a low-dose group, a medium-dose group and a high-dose group in both the acute and subchronic Al treatment procedures. In the acute treatment procedure, the rats (weight 220-250 g) received one dose of 5 μL of saline (control group) or a single dose of $Al(mal)_3$ (2.43, 12.15, or 60.75 μg Al) over 5 min via intracerebroventricular (i.c.v.) injection. For the subchronic administration procedure, the rats (weight 180-200 g) received saline (control group) or $Al(mal)_3$ (0.41, 0.82, or 12.43 mg/kg) via intraperitoneal (i.p.) injection for 8 weeks. After electrophysiological measurements were performed, rats were decapitated and the hippocampus was rapidly removed for immunoblot analyses and stored at -80 °C until use.

The present study was approved by the Ethics Committee for Animal Studies of Shanxi Medical University. All efforts were made to minimize the number of animals used and their suffering.

Electrophysiological Measurements

Surgical Procedure Following Al exposure, extracellular electrophysiological recordings from the CA1 area of the rat hippocampus were conducted. The surgical procedure was performed according to the methods of Zhang[22]. Rat was anesthetized with an intraperitoneal (i.p.) injection of 1.5 g/kg urethane (Sigma Chemical Co., MO, USA), and a supplementary injection (0.2-0.3 g/kg) was given if necessary to ensure satisfactory anesthesia. The animal was fixed in a stereotaxic apparatus (Narishige Group, Japan). Body temperature was monitored throughout the experiment, and a heating pad was utilized to maintain the animal's temperature at 37.5±0.5 °C. Part of the scalp was

removed, and two small holes were drilled in the skull at the appropriate positions for inserting stimulating and recording electrodes; for the acute Al treatment experiment, another small hole was drilled to introduce a guide cannula (1.3 mm posterior to bregma, 0.8 mm lateral to the midline and 4.0 mm below the dura mater) for i.c.v. injection. A bipolar stimulating electrode (FHC, Ino, USA) was inserted into the Schaffer collateral commissural pathway (4.2 mm posterior to bregma, 3.8 mm lateral to the midline) to induce LTP, and a monopolar recording electrode (FHC, Ino, USA) was positioned in the stratum radiatum of area CA1 (3.8 mm posterior to bregma, 2.9 mm lateral to the midline) to record field excitatory postsynaptic potentials (fEPSPs). The correct placement of electrodes in the CA1 region was confirmed primarily by electrophysiological criteria (field potential orientation) and postmortem histological analysis of paraffin-embedded tissues. The isolated pulse stimulator (2 100) and AC/DC differential amplifier (3 000) were created by A-M Systems, Inc., USA. The high-speed data acquisition system (Micro 1 401) was produced by CED, UK.

Electrophysiological Recordings The amplitude of the baseline fEPSP was chosen as 50% of the maximum fEPSP amplitude that was found by adjusting the stimulation pulse intensity. Baseline fEPSP recording was monitored for at least 30 min before induction of LTP to ensure the steady state of synaptic responses. LTP was induced with a high-frequency stimulus (HFS) protocol composed of a train of 20 pulses at 200 Hz that was repeated three times at an interval of 30 s. Test stimuli were applied again after HFS for 1 h to monitor changes in the amplitudes of the fEPSPs. The average value of the fEPSP amplitude over the 30 min of baseline recording was defined as 100%, and all recorded fEPSPs were standardized to this baseline value.

Biochemical Analysis

Production of Total and Membrane-enriched Protein Extracts The GluR1 and GluR2 AMPAR subunits were analyzed separately in total and membrane-enriched protein extracts using the Tissue Protein Extraction Kit (CoWin Biotech Co., China) and Membrane Protein Extraction Reagent Kit (Thermo Fisher Scientific Inc., USA).

Hippocampal tissue was homogenized in ice-cold Tissue Protein Extraction Reagent (containing a protease inhibitor mixture) provided by the kit and kept on ice for 20 min. The tissue homogenate was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was obtained and used as the total hippocampal protein extract measured by BCA (bicinchoninic acid) assay (CoWin Biotech Co., China) to determine protein concentration and stored at -80 °C until use.

To obtain the membrane-enriched protein fraction, the hippocampus was homogenized in ice-cold TBS (0.025 mol/L Tris, 0.15 mol/L NaCl; pH 7.2, containing 0.1 mol/L PMSF). The homogenate was centrifuged at 1 000 g for 5 min at 4 °C. The pellet was resuspended in 150 μL Reagent A after discarding the supernatant and incubated for 10 min at room temperature with occasional vortexing. Four hundred fifty microliters of diluted Reagent C (obtained by diluting 2 parts Reagent C with 1 part Reagent B) was added to each tube of lysed cells, and the tubes were incubated on ice for 30 min then centrifuged at 10 000 g for 3 min at 4 °C. The supernatant was transferred to a new tube, incubated for 10 min in a 37 °C water bath, and centrifuged at room temperature for 2 min at 10 000 g to isolate the hydrophobic fraction (which contained the membrane protein of interest). The hydrophilic phase (i.e., the top layer, which was used as the membrane-enriched hippocampal protein extract) was removed carefully from the hydrophobic protein phase (bottom layer) as quickly as possible and saved in a new tube. Protein concentrations were measured by BCA assay (CoWin Biotech Co., China) and stored at -80 °C until use.

Western Blotting Samples were diluted in an equal volume of loading buffer (0.125 mol/L Tri/HCl, pH 6.8, 4.6% SDS, 20% glycerin, 10% β-mercaptoethanol and 0.1% bromophenol blue) and denatured at 95 °C for 5 min. Approximately 30 μg of protein was loaded in each well and separated in 10% SDS-polyacrylamide electrophoresis gels. The proteins were transferred onto nitrocellulose membranes. The membranes were saturated and blocked with 5% fat-free milk at 37 °C for 1 h and incubated overnight at 4 °C in one of the following primary antibodies, which were diluted in 5% fat-free dried milk in PBS: rabbit anti-GluR1 and mouse anti- GluR2 (1:1 000, Millipore, USA), mouse anti-β-tubulin (the protein loading control, 1:3 000, CoWin Biotech Co., China). Blots were washed four times for 1 h in PBST at room temperature and then incubated for 90 min in one of the following HRP-conjugated antibodies, which were diluted in fat-free dried milk in PBS: anti-rabbit IgG (1:2 000, CoWin Biotech Co. China) for detection of GluR1 or

anti-mouse IgG (1:2 000, CoWin Biotech Co. China) for detection of GluR2 and tubulin. After four times washes for 1 h in PBST, immunolabeled protein bands were detected using the ECL Western blot detection kit (CoWin Biotech Co. China). Graphs of blots were obtained in the linear range of detection and were quantified for the level of specific induction by scanning laser densitometry. All experiments were independently performed in triplicate, and the average was used for comparison.

Data Analysis

All values are represented as the mean±SD. Comparisons between groups were made with one-way analyses of variance. Probabilities less than 0.05 were considered significant. All data analyses were performed with the SPSS 12.0 for Windows statistical analysis software (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of Al Exposure on LTP in vivo

The Acute Toxic Effects of Al To investigate the effects of acute Al exposure on LTP and whether the effects were dose dependent, three dosages (2.43, 12.15, and 60.75 μg of Al) of $Al(mal)_3$ were given i.c.v. in this experiment. As shown in Figure 1(A), three concentrations of Al were given for the same exposure time (30 min), and these doses significantly suppressed LTP; i.e., increasing Al concentrations produced greater decreased in LTP. The histograms shown in Figure 1 (B) summarize the effects of different concentrations of Al on LTP at three time points. The fEPSP amplitudes of the control group (n=6) were 219%±15%, 196%±22%, and 189%±19% at 1, 30, and 60 min after HFS, respectively. The average fEPSP amplitudes of the 2.43 μg Al group (n=6) were 162%±17% at 30 min and 155%±9% at 60 min after HFS, which represented a slight but statistically significant decrease compared to the control group (P<0.05); these values dropped to 178%±24%, 151%±14%, and 142%±11% in the 12.15 μg Al group (n=6, P<0.05) and further decreased to 155%±7%, 132%±16%, and 123%±6% in the 60.75 μg Al group (n=6, P<0.05). Notably, the suppression of LTP by Al was apparent between the Al and control groups (P<0.05), and further suppression was apparent with increasing Al concentrations.

The Subchronic Toxic Effects of Al To observe whether similar suppressive effects were present in the subchronic experiment, three dosages (0.41, 0.82, 12.43 mg/kg Al) of $Al(mal)_3$ were injected i.p. for 8 weeks, and LTP was examined under the same test conditions. As shown in Figure 2 (A, B), similar dose-dependent depressions of LTP were found in the subchronic experiment. However, unlike the acute experiment, the low-dose Al group (0.41 mg/kg, n=6) was not significantly different than the control group (n=6, P>0.05). In further contrast to the acute experiment, the three dosages did not induce significant effects at 1 min. The average fEPSPs amplitudes of the 0.82 mg/kg Al (n=6, P<0.05) and 12.43 mg/kg Al groups (n=6, P<0.05) were significantly suppressed at 30 and 60 min after HFS.

Effects of Al Exposure on AMPA Receptor Subunit Protein Expression

The Acute Toxic Effects of Al GluR1 and GluR2 levels were measured in the total and membrane-enriched protein extracts after acute Al treatment. As shown in Figure 3 (A, C), none of the three dosages (2.43, 12.15, and 60.75 μg of Al) of $Al(mal)_3$ induced significant changes in GluR1 (F=0.410, P>0.05) or GluR2 (F=2.264, P>0.05) in the total extracts. However, as shown in Figure 3 (B, D), signifi-cant decreases in GluR1 (F=4.446, P<0.05) and GluR2 (F=5.001, P<0.05) were found in the membrane-enriched extracts. With increasing Al concentrations, the expressions of GluR1 and GluR2 proteins gradually decreased. Compared with the control group, the GluR1 and GluR2 levels of 12.15 μg Al group and the 60.75 μg Al group were significantly decreased (P<0.05).

The Subchronic Toxic Effects of Al In contrast to the acute experiment, subchronic Al treatment caused dose-dependent decreases in GluR1 and GluR2 that were not restricted to the membrane extracts [F(GluR1)=3.348, P<0.05; F(GluR2)=3.447, P<0.05)] (Figure 4, B and D) but were present in the total extracts [F(GluR1)=4.416, P<0.05; F(GluR2)= 2.898, P<0.05)] (Figure 4, A and C). Compared with the control group, the GluR-1 level of the 1.23 mg/kg Al group and the GluR-2 levels of the 0.82 mg/kg Al and 1.23 mg/kg Al groups decreased gradually in the total extracts (P<0.05). Similarly, in the membrane-enriched extracts, the GluR-1 and GluR-2 levels of the 0.82 mg/kg Al and 1.23 mg/kg Al groups also decreased gradually (P<0.05).

DISCUSSION

The present study investigated the effects of

different doses of Al on hippocampus LTP *in vivo* after acute and subchronic Al exposure. We found that both acute and subchronic pretreatment with Al significantly suppressed LTP in a dose-dependent manner. These results suggest that Al suppresses hippocampus LTP by impairing the induction and maintenance of hippocampus LTP. The present *in vivo* findings are consistent with our previous report[20], which found that Al suppressed *in vivo* LTP and damaged spatial learning and memory capacities. Additionally, our report confirms results from other laboratories: rats exhibit significant impairments in spatial memory abilities and inhibitions of population spike (PS) amplitudes in the CA1 area after they are given drinking water containing three different concentrations of $AlCl_3$ for three months[23]. Moreover, microinjection of Al into CA3 blocks *in vitro* LTP[24]. We propose that the suppressive effects of Al on hippocampus LTP should eventually influence behavioral performance and should be even more strongly related to impairments in learning and memory.

To explore the possible link between the disruptions of LTP and AMPARs after Al exposure, we assayed AMPAR subunits in both total and membrane-enriched extracts from rat hippocampus after LTP measurements to determine whether changes in receptor subunit trafficking between intracellular and synaptic sites in neurons were present. Immunoblot assays demonstrated that acute Al treatment produced dose-dependent decreases of GluR1 and GluR2 in membrane extracts

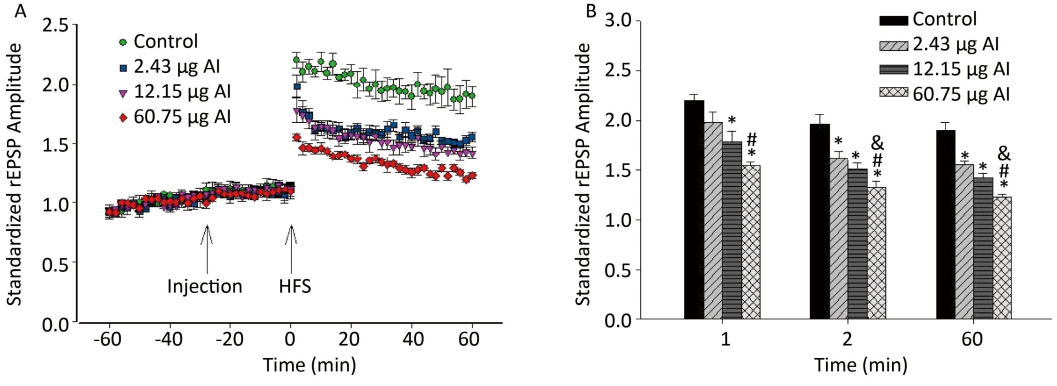

Figure 1. Effects of acute Al exposure on LTP in rats. (A) Acute Al exposure resulted in a dose-dependent depression in HFS-induced LTP. (B) The effects of different concentrations of Al on LTP at three time points (1 min, 30 min, and 60 min). *: *P*<0.05 *vs* control group, #: *P*<0.05 *vs* the 2.43 μg group, &: *P*<0.05 *vs* the 12.15 μg group.

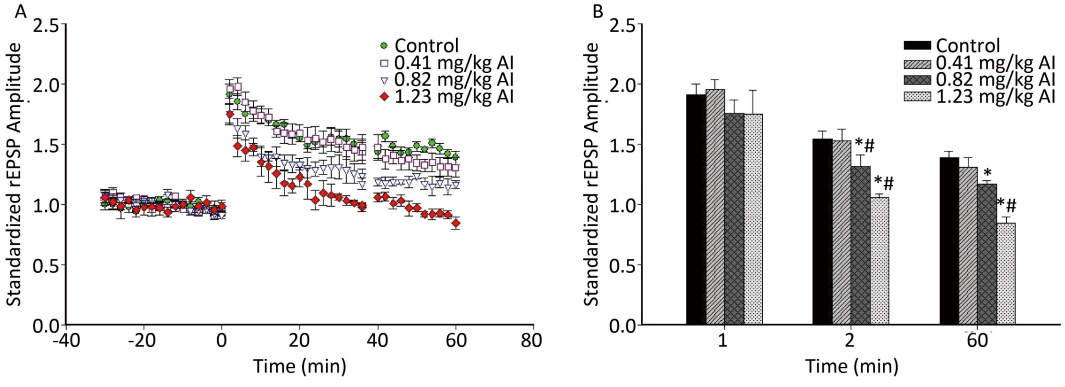

Figure 2. Effects of subchronic Al exposure on LTP in rats. (A) Subchronic Al exposure dose-dependently depressed HFS-induced LTP. (B) The effects of different concentrations of Al on LTP at three time points (1 min, 30 min, and 60 min). *: *P*<0.05 *vs* the control group, #: *P*<0.05 *vs* 0.41 mg/kg group.

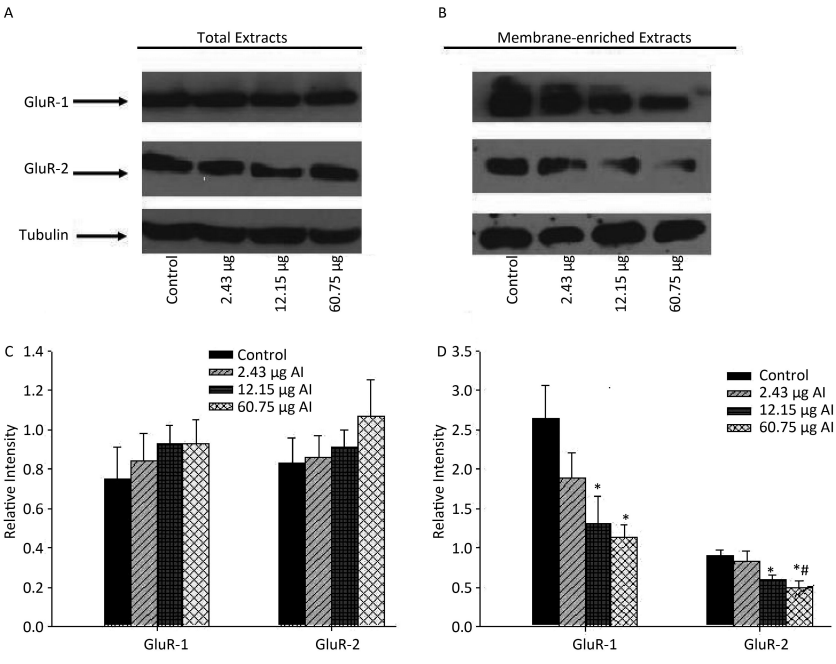

Figure 3. Expression of AMPA receptor subunits in the hippocampi of rats acutely exposed to Al. Acute Al treatment produced a dose-dependent decrease of GluR1 and GluR2 in membrane extracts (B, D), but similar changes were not found in the total extracts (A, C). *: *P*<0.05 *vs* the control group, #: *P*<0.05 *vs* the 2.43 μg group.

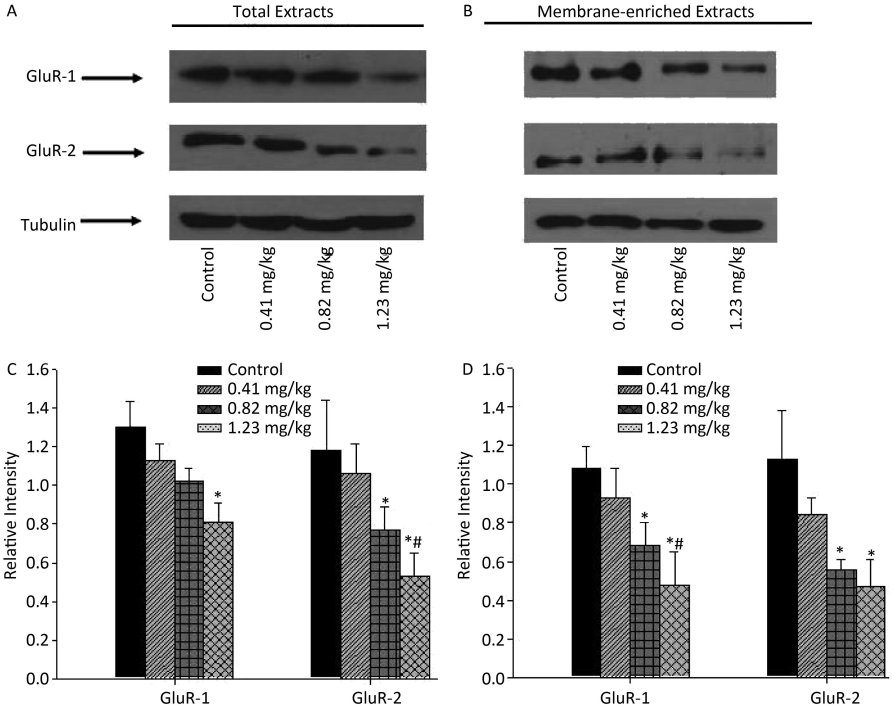

Figure 4. Expression of AMPA receptor subunits in the hippocampus of rats subchronically exposed to Al. Subchronic Al treatment dose-dependently decreased GluR1 and GluR2 in membrane extracts (B, D) and in total extracts (A, C). *: *P*<0.05 *vs* the control group, #: *P*<0.5 *vs* the 0.41 mg/kg group.

but not in total extracts, suggesting that the trafficking of AMPA receptor subunits from intracellular pools to synaptic sites during HFS-induced LTP was suppressed. Interestingly, in the subchronic experiment, this effect was accompanied by a similar decrease in the total amounts of protein; thus, under these conditions, it is likely that receptor proteins are being degraded, which results in consequent decreases of proteins in the synapses. However, the mechanisms of this effect are unclear.

The mechanism responsible for AMPAR trafficking between intracellular and synaptic sites in neurons is not fully elucidated. One important aspect of this mechanism is the phosphorylation site of the AMPARs; this site not only modulates synaptic efficiency but may also be involved in the dynamic regulation of receptor trafficking[25]. The GluR1 subunit has been shown to have two distinct phosphorylation sites, Ser831, which is phosphorylated by CaMKII and PKC, and Ser845, which is phosphorylated by PKA[26]. Two sites of phosphorylation by PKC have been identified in the GluR2 subunit, and they are located at Ser863 and Ser880[27]. In addition, AMPAR trafficking has been functionally coupled to a variety of signal transduction pathways that include Src-family, MAPK and PI3-K signaling[28-29].

Wang B. et al.[12] have reported that chronic exposure to Al significantly decreases PKC and MAPK activities and reduces ERK1/2 and CaMKII expression levels in the hippocampus, which attenuates the population spike (PS) amplitude of LTP in the hippocampal CA1 region and causes impaired memory in rats. Others have proposed that Al toxicity in plants could be mediated through the MAPK signal transduction pathway[30]. In addition, Mori A. has reported that Al selectively inhibits DC expression by PI3-K signaling[31]. The above-mentioned modifications to the kinase may help to explain the effects of Al treatment on LTP and AMPAR trafficking identified in the present study. Thus, AMPARs appear to be a new perspective mechanism by which LTP is impaired by Al.

In addition, Al resulted in remarkable lose of body weight and brain weight of rats in the subchronic experiment[19], which means that other functions, such as, digestion, metabolism and energy consumption, may also be affected by subchronic exposure to Al and these changes may indirectly affect LTP and AMPA receptors.

In summary, we provide strong evidence that Al suppresses LTP in the CA1 region of rat hippocampus in a dose-dependent manner *in vivo*, and this effect may be related to impaired trafficking and reduced expression of AMPA receptor subunit proteins. These findings suggest that the modulation of AMPARs may be a new perspective on toxic effect of Al.

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