




# The Role of PKC in Regulating NMDARs in Aluminum-Induced Learning and Memory Impairment in Rats

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## Abstract

Aluminum is a widespread environmental neurotoxicant that can induce Alzheimer's disease (AD)-like damage, such as neuronal injury and impairment of learning and memory. Several studies have shown that aluminum could reduce the synaptic plasticity, but its molecular mechanism remains unclear. In this study, rats were treated with aluminum maltol (Al(mal)<sub>3</sub>) to establish a toxic animal model and PMA was used to interfere with the expression of PKC. The Morris water maze and open field test were used to investigate the behavioral changes of the rats. Western blotting and RT-PCR were used to detect the expression levels of NMDAR subunits, PKC and CaMKII. The results showed that Al(mal)<sub>3</sub> damaged learning and memory function and reduced anxiety in rats. During this process, the expression of PKC was downregulated and it inhibited the expression of NMDARs through the phosphorylation of CaMKII.

**Keywords** Aluminum · *N*-methyl-D-aspartate receptor · Protein kinase C · CaMKII · Learning and memory

## Abbreviations

Al(mal) <sub>3</sub>	Aluminum maltol
PKC	Protein kinase C
NMDAR	<i>N</i> -methyl-D-aspartic acid receptor
LTP	Long-term potentiation

## Introduction

Aluminum (Al) is the most abundant metal element in the Earth's crust and mainly exists in nature as aluminosilicate ore. However, it does not have any known beneficial effects on biological systems (Exley 2009; Kiss 2013; Shaw et al. 2013). During the past 200 years, the rapid development of the Al industry has made occupational aluminum exposure a serious occupational health problem (Bondy 2010). Additionally, because of its excellent properties, Al is widely used in daily life and medications, and it can enter and accumulate in the human body through food, water, medicines, vaccines, and cosmetics (Krewski et al. 2007). Al ions have a high charge and a small ion radius which makes it easy for Al ions to cross the blood–brain barrier (BBB) and accumulate in different areas of the brain (Tomljenovic 2011), thereby damaging brain tissue.

Since the nineteenth century, the toxicity of Al has attracted much attention from neurotoxicologists (Wenting et al. 2014). Research has estimated that adults directly or indirectly intake approximately 3–12 mg of Al per day (Shaw et al. 2014). Long-term Al exposure causes Al to accumulate in the brain, bones, muscles, kidneys, and other organs, thereby causing neurodegenerative diseases (Colomina and Peris-Sampedro 2017). Epidemiological studies have shown that areas with a higher burden of Al in

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drinking water had a higher incidence of Alzheimer's disease (AD) (Krewski et al. 2007). Animal experiments have shown that excessive intake of Al could damage the learning and memory ability of rats or mice and cause changes in learning and memory-related proteins (Farhat et al. 2017; Thippeswamy et al. 2013). Scholars have proposed that Al might be involved in glutamate-mediated nerve damage and cause neurotransmitter changes (Matyja 2000). Al is widely exposed to the population and has certain neurotoxicity, which makes the study of Al neurotoxicity of great public health significance.

*N*-methyl-D-aspartate receptor (NMDAR) is the most important excitatory glutamate ionotropic receptor in the central nervous system which is highly expressed in the cerebral cortex and hippocampus and plays a vital role in neurodevelopment, learning and memory, sensory perception, and synaptic plasticity (Kumar 2015). Synaptic plasticity is divided into long-term potentiation (LTP) and long-term inhibition (LTD), which are considered as electrophysiological manifestation of possible mechanisms of learning and memory. NMDARs and other ionotropic receptors (AMPA and KAs) play an important role in the regulation of synaptic plasticity. Functional NMDARs are composed of NMDAR1 and one or more NMDAR2 subunits. With increasing age, the expression of NMDAR1 and NMDAR2 decreases (Mota et al. 2014), and the overexpression of NMDAR2B improves the synaptic plasticity and memory ability of aged mice (Brim et al. 2013; Cao et al. 2007). Burnashev found that LTP in the hippocampus of NMDAR1 knockout mice was significantly reduced, and the escape latency was significantly prolonged in a water maze experiment (Burnashev and Szepetowski 2015). NMDAR is necessary for the induction of LTP, and studies have found that NMDAR antagonists could inhibit the occurrence of LTP (Villarreal et al. 2002). These findings suggest that there is an inevitable connection between NMDARs and neural function, and changes in NMDAR expression cause learning and memory dysfunction.

Studies have shown that NMDAR is dynamically regulated, and its subunit composition and number change with increasing brain activity; so it plays an important role in NMDAR-dependent brain function (Bu et al. 2015; Hunt and Castillo 2012). This regulation of NMDAR is mainly dependent on the PKC–Src signaling pathway (Horak et al. 2014). Protein kinase C (PKC) is a serine/threonine protein kinase involved in the formation of synaptic plasticity and LTP. Li HB found that PKC could relieve the blocking effect of  $Mg^{2+}$  on NMDAR channels, increase  $Ca^{2+}$  influx, and cause changes in synaptic plasticity (Li et al. 2011). It was found that the tyrosine kinase Src could phosphorylate NMDARs and promote their further opening (Weilinger et al. 2016; Yang et al. 2012). PKC promoted the transfer of NMDARs to the cell membrane through interactions with

NMDAR-related proteins (Yan et al. 2011). PKC first causes the autophosphorylation of CaMKII, thereby increasing the interaction between CaMKII and NMDAR2, which is transported to the postsynaptic membrane in the form of the NMDAR2–CaMKII complex, thus playing a role in the formation of LTP. In summary, PKC significantly increases the opening of NMDAR channels through the PKC–Src signaling pathway. PKC can phosphorylate CaMKII to promote the binding of CaMKII and NMDAR2 to the membrane.

Therefore, we speculated that the learning and memory impairment of Al-exposed rats might be related to the regulation of NMDAR expression by PKC, and the phosphorylation of CaMKII is involved in this process. In this study, the Morris water maze was used to test the learning and memory ability of Al-exposed rats, and an open field test was used to evaluate their anxiety and tension. NMDARs were detected by western blotting and PCR. PKC and CaMKII, which are involved in synaptic plasticity-related pathways, were measured to explore whether PKC and CaMKII play a role in learning and memory impairment caused by Al.

## Materials and Methods

### Laboratory Animals

Seventy 2-month-old healthy male Sprague–Dawley (SD) rats without specific pathogens were provided by the Experimental Animal Center of the Chinese Academy of Military Medical Sciences (Experimental Animal Production License Number: SCXK (Army) 2012-0004), weighing 180–220 g. Rats were raised in a barrier environment that met the requirements of GB14925-2010 "Laboratory Animal—Requirements of environment and housing facilities." During the entire Al exposure period, the rats were fed standard fodder and water ad libitum. The temperature was controlled at 22–24 °C, and the relative humidity was 40–60% with day and night natural rhythm lighting. This study was reviewed and approved by the Medical Animal Ethics Committee of Shanxi Medical University.

### Al(mal)<sub>3</sub> and PMA Preparation

AlCl<sub>3</sub> (Sigma-Aldrich, USA) was dissolved in normal saline to final concentrations of 8 mM, 32 mM, and 128 mM. Maltol (Sigma-Aldrich, USA) was dissolved in PBS to final concentrations of 24 mM, 96 mM, and 384 mM. AlCl<sub>3</sub> and maltol solutions were mixed freshly when used in equal volumes to make Al(mal)<sub>3</sub> solutions with concentrations of 4 mM, 16 mM, and 64 mM. The pH of the Al(mal)<sub>3</sub> solution was adjusted to 7.4 with NaOH and filtered with a 0.22 mm filter membrane. The PKC agonist PMA (Sigma-Aldrich,

USA) was dissolved in normal saline to a final concentration of 0.2  $\mu\text{M}$ .

## Surgery and Treatments

After 1 week of adaptive feeding, the rats were modeled by surgery. After the rats were anesthetized with 10% chloral hydrate, they were fixed in the prone position on a three-dimensional brain stereotaxic apparatus (RWD, China). The fur was cut to fully expose the skull. Starting from the fontanelle, 0.8 mm backward, and 1–1.5 mm laterally, the skull was drilled with a bone drill, and a cannula was placed. Screws were placed at 3 points on the skull, and dental cement was fixed. One week after the operation, the rats were divided randomly into 7 groups of 10 according to body weight: operation control group (lateral ventricle intubation only), saline control group (injected with 0.9% normal saline),  $\text{Al}(\text{mal})_3$  exposure groups (4 mM, 16 mM, and 64 mM  $\text{Al}(\text{mal})_3$ , 5  $\mu\text{L}$ ), PMA (0.2  $\mu\text{M}$ , 5  $\mu\text{L}$ ), and  $\text{Al}(\text{mal})_3$  + PMA (64 mM/5  $\mu\text{L}$   $\text{Al}(\text{mal})_3$  + 0.2  $\mu\text{M}$ /5  $\mu\text{L}$  PMA). The prepared 5  $\mu\text{L}$  solution was slowly injected into the lateral ventricle with a microsyringe within 5 min for continuous injection for 7 days.

## Morris Water Maze

The experimental device was mainly composed of two parts: a water maze and an automatic image monitoring system. The water maze was a circular pool with a diameter of 130 cm. There was a platform with a diameter of 10 cm that was 2 cm below the water surface, and the water temperature was controlled at 22–24 °C. The four directional points S, W, N, and E were marked on the side of the pool, which divided the pool into four quadrants, namely, NW, NE, SW, and SE. When the rat entered the water maze, the upper camera monitored and recorded the rat's swimming track. When the rat found the platform and stayed for 10 s or failed to find the platform within 120 s, the computer automatically stopped recording.

The day before the experiment, the rats were put into the pool to swim freely for 120 s to adapt to the environment. In the 5-day positioning navigation experiment, the rats were trained 4 times a day, entering the water from different entry points (S, SE, NW, W). The platform was placed in the target quadrant (NE), and the time for each rat to find the platform was recorded. Then, the average value was calculated, which was the escape latency period of the rat on that day. If the rat did not find the platform within 120 s, it could be guided to the platform and held in place for 10 s. At this time, the escape latency was 120 s. On the second day after the positioning navigation experiment, the platform was removed, and the rat entered the water at the point (SW) farthest from the target quadrant. The camera recorded the swimming

trajectory within 120 s and analyzed the first time arriving at the platform, the target quadrant dwell time, and the number of crossing the platform.

## Open Field Test

The experimental device consisted of an open field reaction box (70 × 70 cm with 40 cm walls) and an automatic monitoring and acquisition system. The rat was placed in the center of the open field, and at the same time, the camera began to record the trajectory of the rat in the box for a total of 10 min. Time in the center area (30 × 30 cm), number of rearing (standing frequency of hind limbs in rats), and number of grooming (quick cleaning action of the front legs toward the face or body) were analyzed and quantified by a video monitoring system. After each rat was finished, the apparatus was cleaned with 70% alcohol to avoid the remaining urine, feces, and odor from affecting the next test.

## Western Blotting

Total proteins were extracted from the hippocampus of rats with a tissue protein extraction reagent (CWBIO, China) and quantified with a BCA protein Assay Kit (CWBIO, China) according to the instructions. The samples were added to 5 × loading buffer at a ratio of 4:1 and boiled in water for 5 min. Forty-five micrograms of each sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred to a polyvinylidene difluoride membrane (PVDF). The membrane was blocked with 5% skimmed milk powder at room temperature for 2 h and then incubated with a specific primary antibody (GAPDH, 1:5000, CWBIO, China; Tublin, 1:5000, CWBIO, China; NMDAR1, 1:2000, Abcam, UK; NMDAR2A, 1:1000, Abcam, UK; NMDAR2B, 1:1000, CST, USA; PKC, 1:2000, Abcam, UK; CaMKII, 1:8000, Abcam, UK; p-CaMKII (Thr286), CST, USA) at 4 °C overnight. After rinsing 4 times with PBST, the membrane was incubated with the corresponding secondary antibody at 37 °C for 2 h. After rinsing with PBST, ECL-enhanced luminescent solution was developed. A gel electrophoresis image analysis system (Bio-Rad, USA) was used to detect the gray value of the protein, and the optical density ratio of the target protein to the internal reference protein was calculated (Quantity One-4.6.5 software).

## Quantitative Real-Time PCR

Total RNA was extracted from the hippocampus of rats using TRIzol reagent (CWBIO, China) according to the instructions. RNA concentration and purity were detected by a UV spectrophotometer (Eppendorf, Germany). Complementary DNA (cDNA) was synthesized using Prime

Script™ RT Master Mix (Takara, Japan) in a 10 µL reaction containing 500 ng of the total RNA, and the reaction system was as follows: 4 °C; 37 °C 15 min, 85 °C 5 s, 4 °C. To detect gene expression levels, cDNAs were amplified using specific primers (Table 1). Real-time PCR was performed using SYBR® Premix ExTaq™ II (Takara, Japan) on an Applied Biosystems 7500 system (ABI, USA) with 1.6 µL cDNA in a 20 µL reaction. PCR amplification conditions were as follows: 95 °C 30 s, 1 cycle; 95 °C 5 s, 60 °C 30 s, 40 cycles.  $\beta$ -actin was used as an endogenous control. The relative messenger RNA (mRNA) expression level was calculated according to the  $2^{-\Delta\Delta C_t}$  method.

## Statistical Analysis

SPSS 22.0 was used for statistical analysis of the data, and all data are expressed as the mean  $\pm$  standard deviation. One-way analysis of variance was used for comparisons between multiple groups. For pairwise comparisons, the SNK method was used when the variance was uniform, and the Dunnett T3 test was applied for uneven variance. Repeated measurement analysis of variance was used for repeated measures data. A  $p$  value of  $<0.05$  was considered statistically significant.

## Results

### Investigation of Acute Al-Induced Neurotoxicity in Rats

#### Al Exposure Damages the Learning and Memory Function of Rats

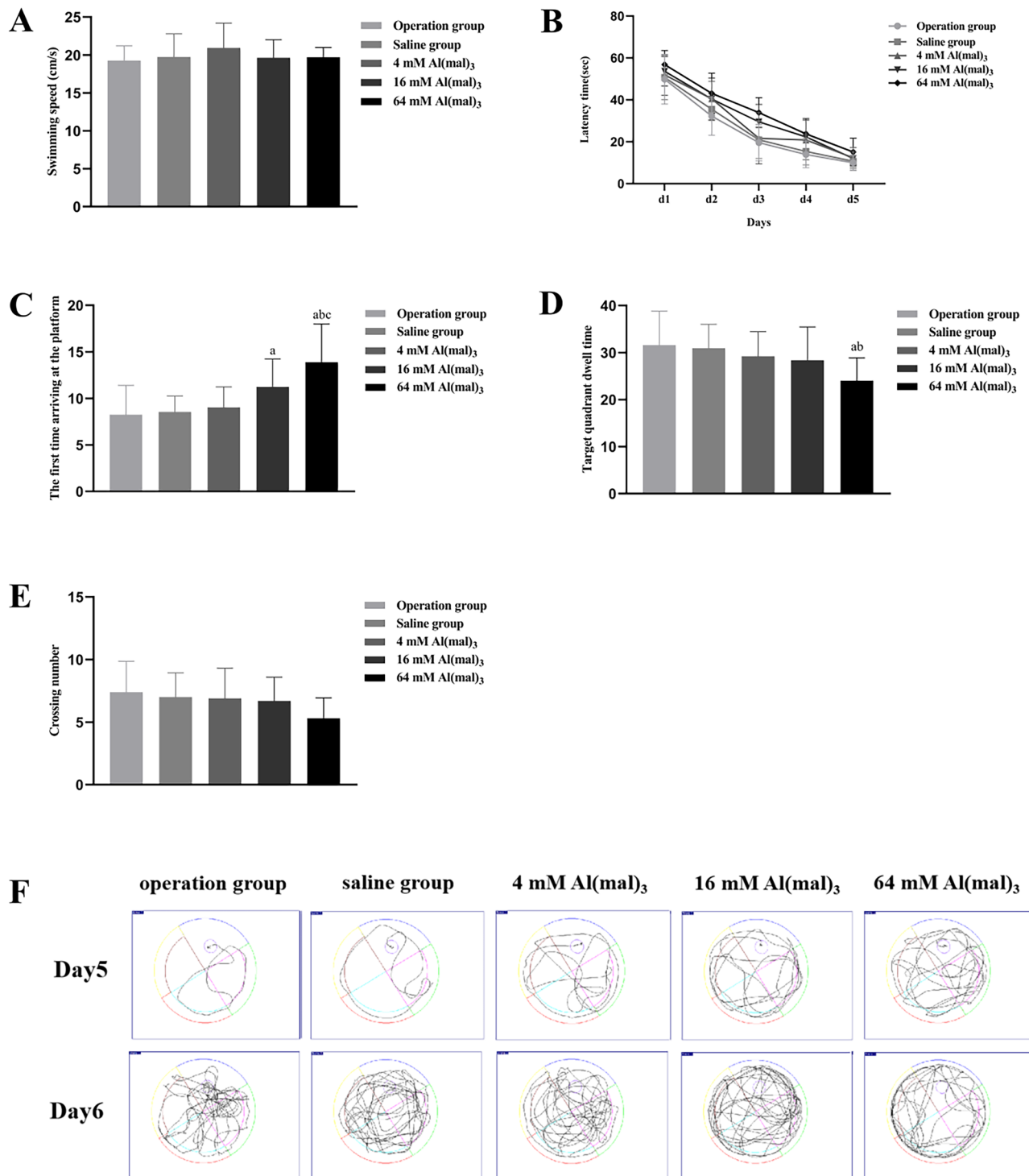
In this experiment, the swimming speeds of the rats in each group were similar, and there was no statistical difference, which ruled out the possibility of lateral ventricle surgery and aluminum exposure damaging the exercise ability of the rats (Fig. 1A). The positioning navigation experiment is mainly used to evaluate the spatial learning ability, and the index used is the escape latency period. As shown in Fig. 1B, as the number of training days increased, the escape latency period of rats in each group was gradually shortened, and the repeated measurement

analysis of variance results showed that the difference in the escape latency period between the five time points was statistically significant ( $F=281.362$ ,  $p<0.01$ ). In the daily training, the escape latency period of each group increased with the increase of the exposure dose, and there were statistical differences between the five groups ( $F=6.724$ ,  $p<0.01$ ). The results of multiple comparison analysis showed that from the second day, the escape latency period in each Al-exposed group was significantly longer than that of the operation control group, and the difference was statistically significant ( $p<0.01$ ). On the fifth day, the escape latency period in the 64 mM Al(mal)<sub>3</sub> group was significantly higher than that of the other groups, and there was no interaction between the exposure dose and the exposure time ( $F=0.692$ ,  $p=0.833$ ).

The space exploration experiment is mainly used to evaluate the spatial memory ability, and the indicators used are the first time arriving at the platform, the target quadrant dwell time, and the number of crossing the platform. As can be seen from Fig. 1C–E, with the increase of the exposure dose, the first time arriving at the platform was significantly prolonged ( $F=5.406$ ,  $p<0.01$ ), and the target quadrant dwell time was gradually shortened, and the difference was statistically significant ( $F=2.414$ ,  $p<0.05$ ). The first time arriving at the platform in the 64 mM Al(mal)<sub>3</sub> group was approximately 70% longer than that in the operation control group. The target quadrant dwell time of the 64 mM Al(mal)<sub>3</sub> group was reduced by 23.9% compared with the operation control group, and it was reduced by 21.7% compared with the 4 mM Al(mal)<sub>3</sub> group. Although there was no significant difference in the number of crossing the platform in each group, a downward trend was observed (Fig. 1E). Figure 1F shows that through training, the control group could quickly find the platform and the swimming path was clear. With increasing exposure dose, although the rats finally reached the platform, their swimming time was prolonged and the track was random. After the platform was removed on the sixth day, the number of times the rats crossed the platform gradually decreased as the exposure dose increased. The water maze results showed that acute lateral ventricle exposure to Al could damage the learning and memory ability of rats.

**Table 1** The specific primers used in RT-PCR

Gene	Primer sequences	
NMDAR1	F: 5'-GGACTGACTACCCGAATGTCCA-3'	R: 5'-GTAGACTCGCATCATCTCAAACCA-3'
NMDAR2A	F: 5'-GCTTGTGGTGATCGTGCTGAA-3'	R: 5'-AATGCTGAGGTGGTTGTCATCTG-3'
NMDAR2B	F: 5'-TGGCTATCCTGCAGCTGTTT-3'	R: 5'-TGGCTGCTCATCACCTCATT-3'
$\beta$ -actin	F: 5'-GGAGATTACTGCCCTGGCTCCTA-3'	R: 5'-GACTCATCGTACTCCTGCTTGCTG-3'



**Fig. 1** Morris water maze results of the operation group; saline group; and 4, 16, and 64 mM Al(mal)<sub>3</sub> groups. **A** Swimming speed of rats in each group. **B** Comparison of the mean escape latency period per day in the navigation experiment. **C** The first mean time arriving at the platform. **D** Water maze space exploration results. **E** Mean

crossing platform frequency. **F** Swimming track graphs of rats in each group on the fifth and sixth days. Compared with the operation group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the 4 mM Al(mal)<sub>3</sub> group, <sup>c</sup> $p < 0.05$

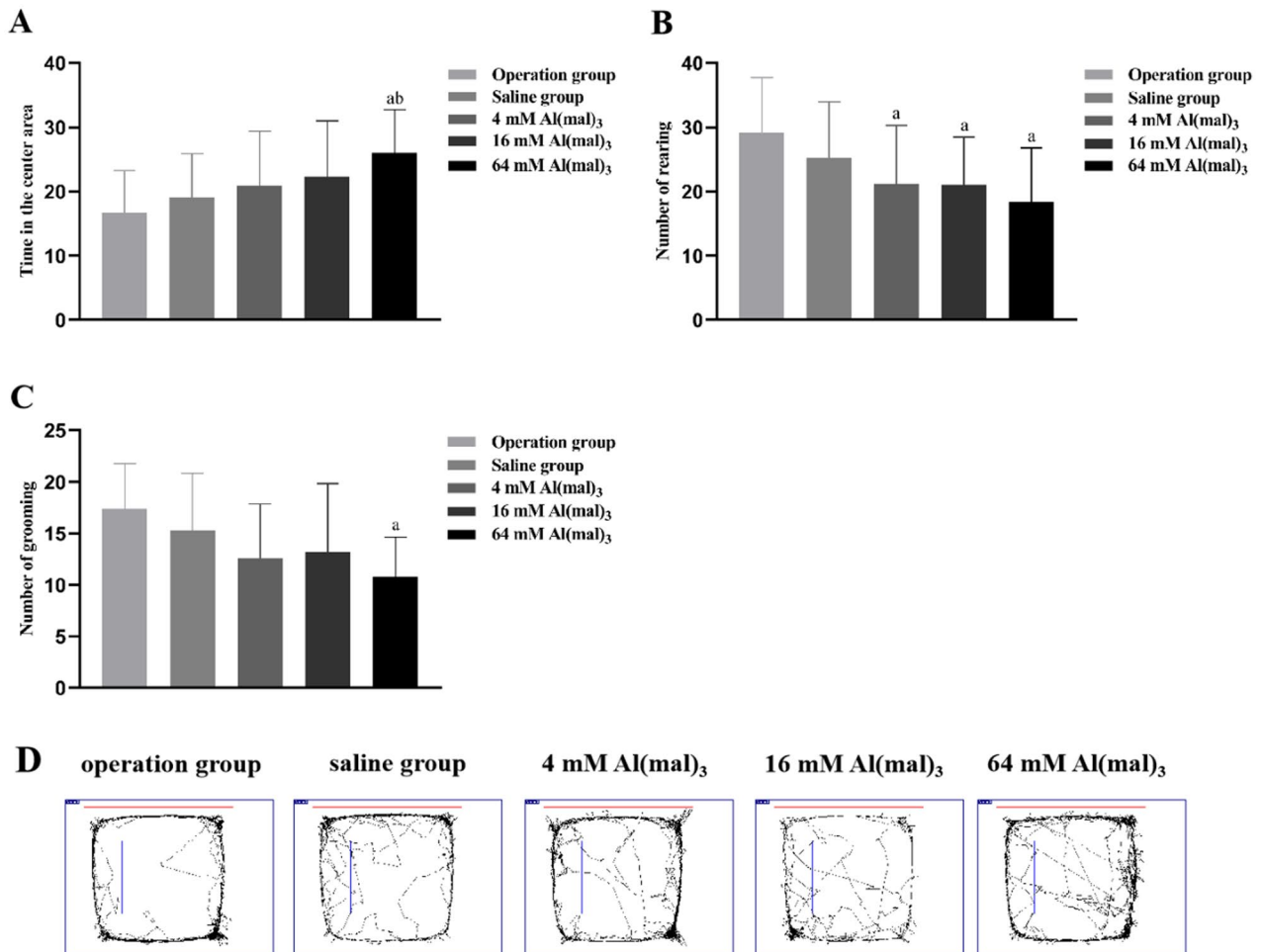
## Al Exposure Reduced Anxiety in Rats

The open field test is used to evaluate the anxiety and tension of animals in a novel environment. As shown in Fig. 2A, the time in the center area of rats in each group prolonged with the increase of the dose, and the 64 mM Al(mal)<sub>3</sub> group was 56.5% longer than the operation control group ( $p < 0.05$ ). With the increase of the exposure dose, the number of rearing and grooming decreased, and the 64 mM Al(mal)<sub>3</sub> group had decreases of 36.8% and 37.9%, respectively, compared with the operation control group ( $p < 0.05$ ) (Fig. 2B–C). The open field test results showed that Al exposure could make rats feel less nervous and anxious in a new environment.

## Effects of Al(mal)<sub>3</sub> on the Protein Expression of NMDARs, PKC, CaMKII, and p-CaMKII(Thr286) in the Hippocampus

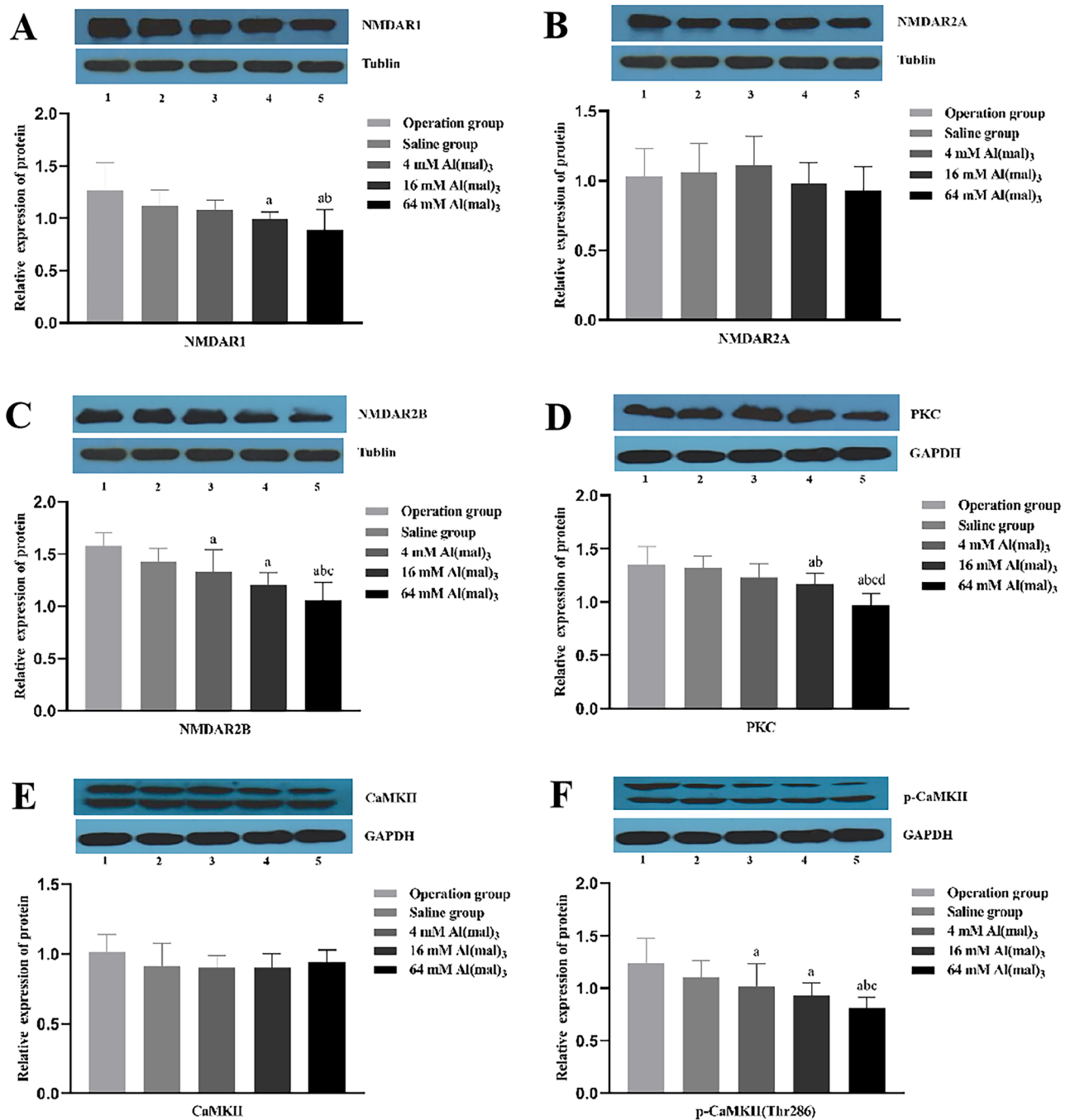
Western blotting is a protein detection technology that uses specific antibodies to detect a specific antigen. As shown in Fig. 3A–C, compared with the operation group, the protein expression of NMDAR1, NMDAR2A, and NMDAR2B in the saline group showed no significant difference ( $p > 0.05$ ). In the 16 mM Al(mal)<sub>3</sub> group, NMDAR1 and NMDAR2B protein expression decreased by 22% and 24.1%, respectively, compared with the operation control group ( $p < 0.05$ ). In the 64 mM Al(mal)<sub>3</sub> group, they decreased by 29.9% and 32.9% ( $p < 0.05$ ). Although the protein expression of NMDAR2A also showed a downward trend, the difference was not statistically significant ( $p = 0.537$ ). Each subunit of the NMDA receptor changed after aluminum exposure.

Next, we detected the relevant regulatory proteins of the NMDA receptors. As shown in Fig. 3D–F, the protein



**Fig. 2** The time in the center area (A), the number of rearing (B), and the number of grooming (C) in the open field test of rats in the operation group; saline group; and 4, 16, and 64 mM Al(mal)<sub>3</sub> groups. **D**

Trajectory diagram of each group of rats in the open field. Compared with the operation group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$



**Fig. 3** Changes in protein expression in rats exposed to Al(mal)<sub>3</sub>. **A** NMDAR1, **B** NMDAR2A, **C** NMDAR2B, **D** PKC, **E** CaMKII, **F** p-CaMKII (Thr286). Compared with the operation group, <sup>a</sup> $p < 0.05$ ;

compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the 4 mM Al(mal)<sub>3</sub> group, <sup>c</sup> $p < 0.05$ ; compared with the 16 mM Al(mal)<sub>3</sub> group, <sup>d</sup> $p < 0.05$

expression levels of PKC and p-CaMKII (Thr286) in the hippocampus of each group were significantly different ( $F = 8.980$ ,  $p < 0.001$ ;  $F = 5.290$ ,  $p = 0.003$ ). The expression level of PKC protein in the 16 mM Al(mal)<sub>3</sub> group was lower than that in the operation control group and saline group, and the expression level of p-CaMKII (Thr286) in the 4 and 16 mM Al(mal)<sub>3</sub> groups was lower

than that in the operation control group, which was statistically significant. Compared with the operation control group, the protein expression of PKC and p-CaMKII (Thr286) in the 64 mM Al(mal)<sub>3</sub> group decreased by 20.9% and 34.7% ( $p < 0.05$ ). There was no significant difference in the protein expression level of CaMKII in each group of rats.

## Effects of Al(mal)<sub>3</sub> on the mRNA Expression of NMDARs in the Hippocampus

The RT-PCR experiment results (Fig. 4) showed that with increasing Al exposure dose, the mRNA expression levels of NMDAR1 and NMDAR2A in the hippocampus of each group gradually decreased, and the differences were statistically significant ( $p < 0.05$ ). Compared with the operation control group, the NMDAR1 and NMDAR2A mRNA levels in the 64 mM Al(mal)<sub>3</sub> group decreased by 48% and 38%, respectively ( $p < 0.05$ ). NMDAR2B mRNA expression showed a downward trend, but the difference was not statistically significant ( $p > 0.05$ ).

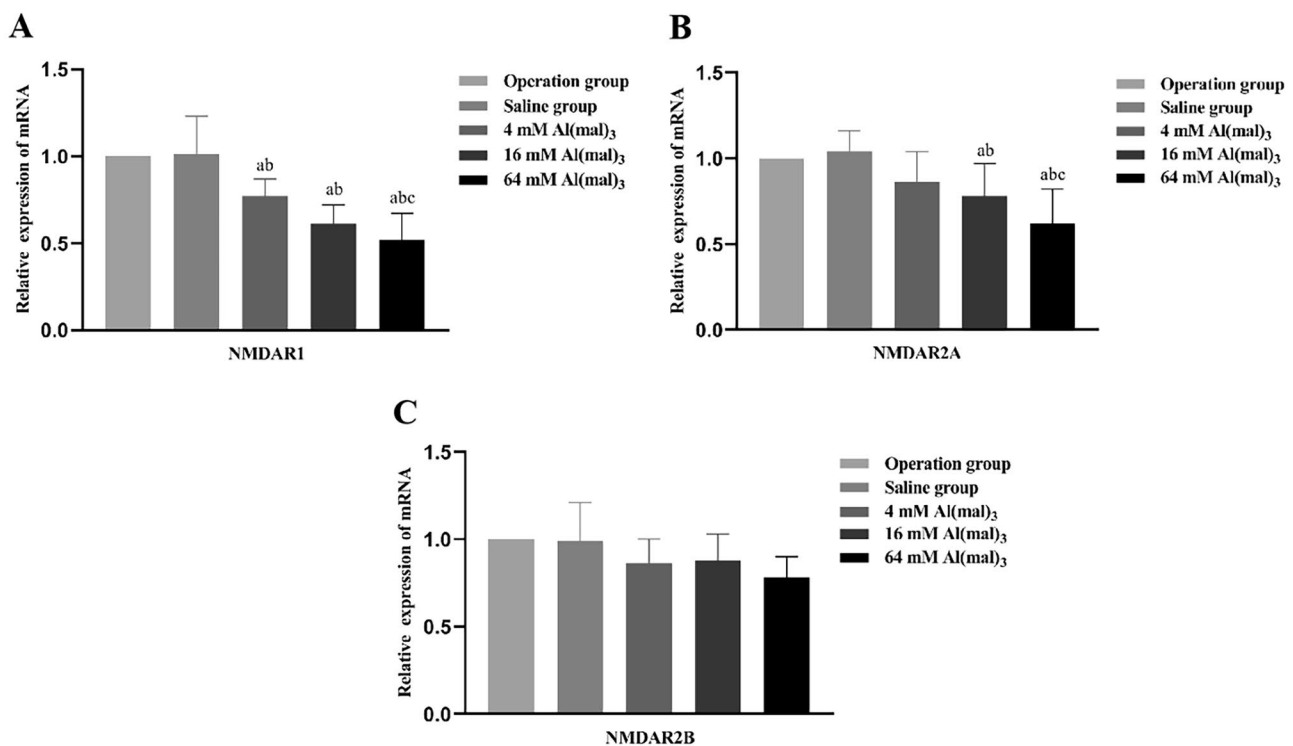
## PKC Regulates Al-Induced Neurotoxicity in Rats

### PKC Improved Learning and Memory Impairment Caused by Al

From the above results, it can be seen that acute Al exposure can cause a decrease in the level of NMDAR2B protein, but its mRNA level has no significant change, which reveals that the change of NMDAR2B does not occur in the transcription stage. Therefore, we speculate that the

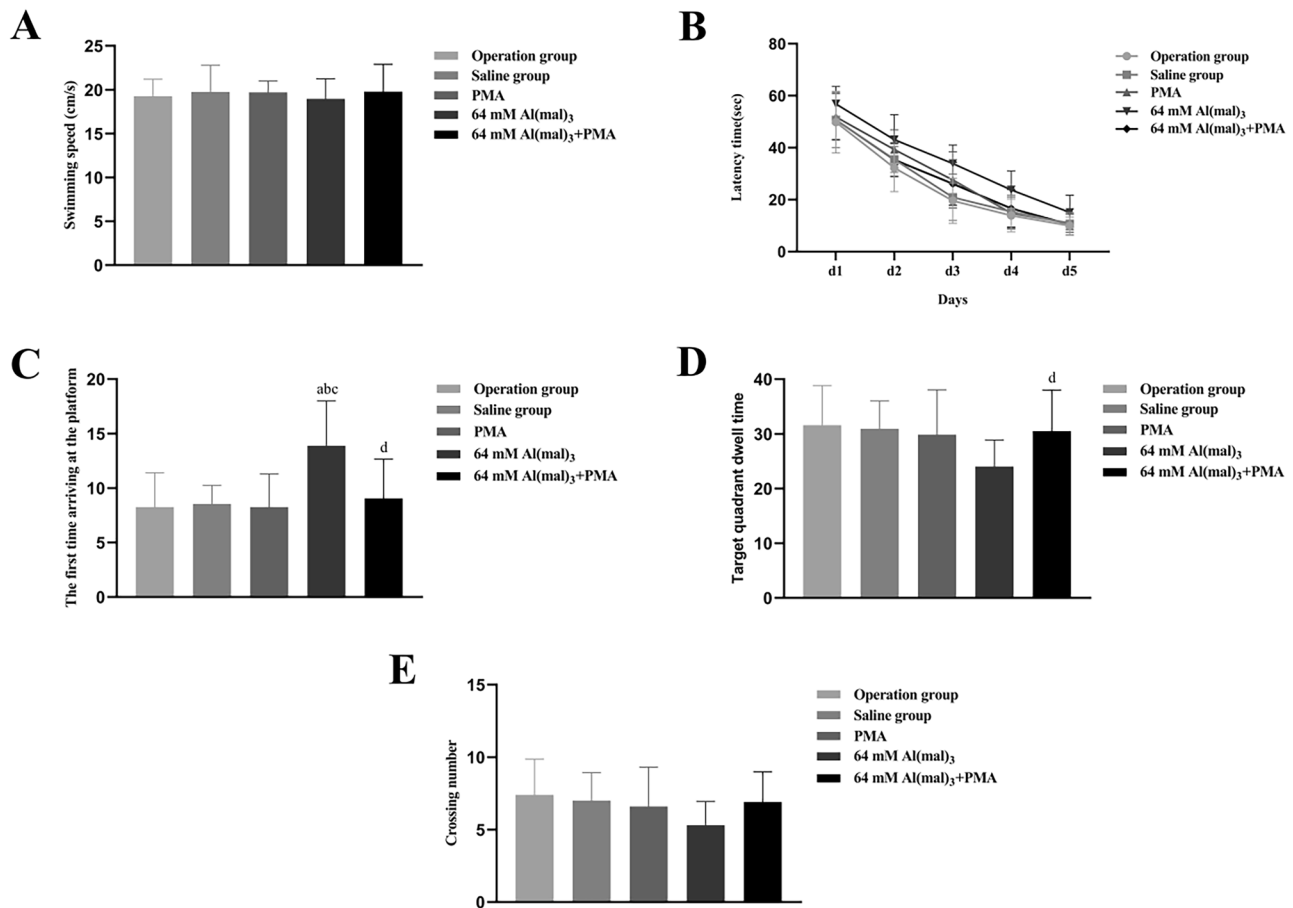
change in NMDA receptors may be caused by the modification of the interaction between proteins after transcription. PKC is an important protein kinase that plays an important role in the regulation of NMDA receptors. In this part, the PKC agonist PMA was used to treat rats to explore the main mechanism of PKC in Al-induced learning and memory impairment.

Similarly, there was no significant difference between the swimming speeds of rats in each group (Fig. 5A). In the positioning navigation experiment (Fig. 5B), there was no statistical difference in the escape latency period between the PMA group and the saline group ( $p < 0.05$ ), suggesting that PMA did not affect the learning and memory of the rats. It was significantly lower in the Al(mal)<sub>3</sub> + PMA group than that in the Al-exposed group on days 2, 4, and 5 ( $p < 0.05$ ), indicating that PMA can appropriately improve the learning and memory impairment caused by Al. The results of the space exploration experiments (Fig. 5C–E) showed that there was no statistical difference between the Al(mal)<sub>3</sub> + PMA group with the operation group and the saline group ( $p > 0.05$ ). While compared with the Al-exposed group, the first time arriving at the platform was reduced by 34.8% ( $p < 0.05$ ), and the target quadrant dwell time was increased by 26.9% ( $p < 0.05$ ) in the Al(mal)<sub>3</sub> + PMA group.



**Fig. 4** Changes in mRNA expression in rats exposed to Al(mal)<sub>3</sub>. **A** NMDAR1, **B** NMDAR2A, **C** NMDAR2B. Compared with the operation group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the 4 mM Al(mal)<sub>3</sub> group, <sup>c</sup> $p < 0.05$





**Fig. 5** Morris water maze results of the operation group, saline group, PMA group, 64 mM Al(mal)<sub>3</sub> group, and 64 mM Al(mal)<sub>3</sub>+PMA groups. **A** Swimming speed of rats in each group. **B** Comparison of the mean escape latency period per day in the navigation experiment. **C** The first mean time arriving at the platform. **D**

The water maze space exploration results. **E** Mean crossing platform frequency. Compared with the operation group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the PMA group, <sup>c</sup> $p < 0.05$ ; compared with the 64 mM Al(mal)<sub>3</sub> group, <sup>d</sup> $p < 0.05$

### PMA Did Not Improve the Anxiety Suppression Caused by Al

Pairwise comparison results showed that compared with the Al-exposed group, the Al(mal)<sub>3</sub>+PMA group had no significant difference in the time in the center area, number of rearing, and number of grooming ( $p > 0.05$ ) (Fig. 6).

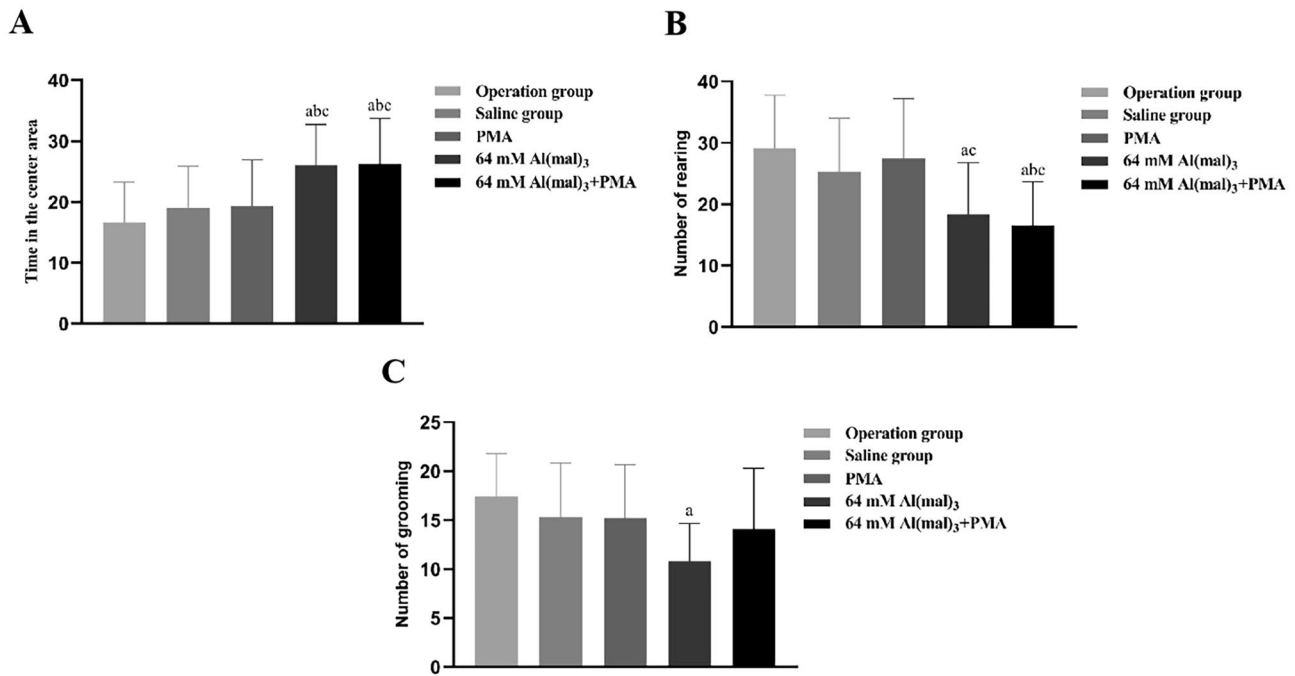
### Effects of PKC Activation on the Inhibitory Effect of Al(mal)<sub>3</sub> on the Protein Expression of p-CaMKII and NMDARs

In this experiment (Fig. 7), the protein expression level of NMDAR2A in each group did not change significantly ( $p > 0.05$ ), and the protein expression of NMDAR1 and NMDAR2B was statistically different between the groups ( $p < 0.05$ ). The use of PMA alone did not affect the subunits of the NMDA receptor. However, compared with the Al-exposed group, the expression levels of NMDAR1 and NMDAR2B in the Al(mal)<sub>3</sub>+PMA group increased by

34.9% and 34.3%, respectively ( $p < 0.05$ ). Western blotting results showed that after injection of the PKC agonist, PKC expression increased by 15.8%. Compared with the Al-exposed group, the PKC and p-CaMKII (Thr286) protein expression levels in the Al(mal)<sub>3</sub>+PMA group recovered by 23.5% and 40.2%, respectively ( $p < 0.05$ ).

## Discussion

Al was once considered a harmless metal element and was widely used in daily life and production. Food is an important source of Al, so many countries have evaluated Al exposure in their diets. The 2006 UK total diet study showed that the population dietary exposure to Al was 5.4 mg/day, the Spanish report estimated that the Al intake was 10.2 mg/day, and the Greek Al intake was 3.7 mg/day, and Hong Kong intake was 0.6 mg/kg bw/week, mainly from tea and fried dough sticks (Bratakos et al. 2012; Chen et al. 2014;



**Fig. 6** The time in the center area (A), the number of rearing (B), and the number of grooming (C) in the open field test of rats in the operation group, saline group, PMA group, 64 mM Al(mal)<sub>3</sub> group, and

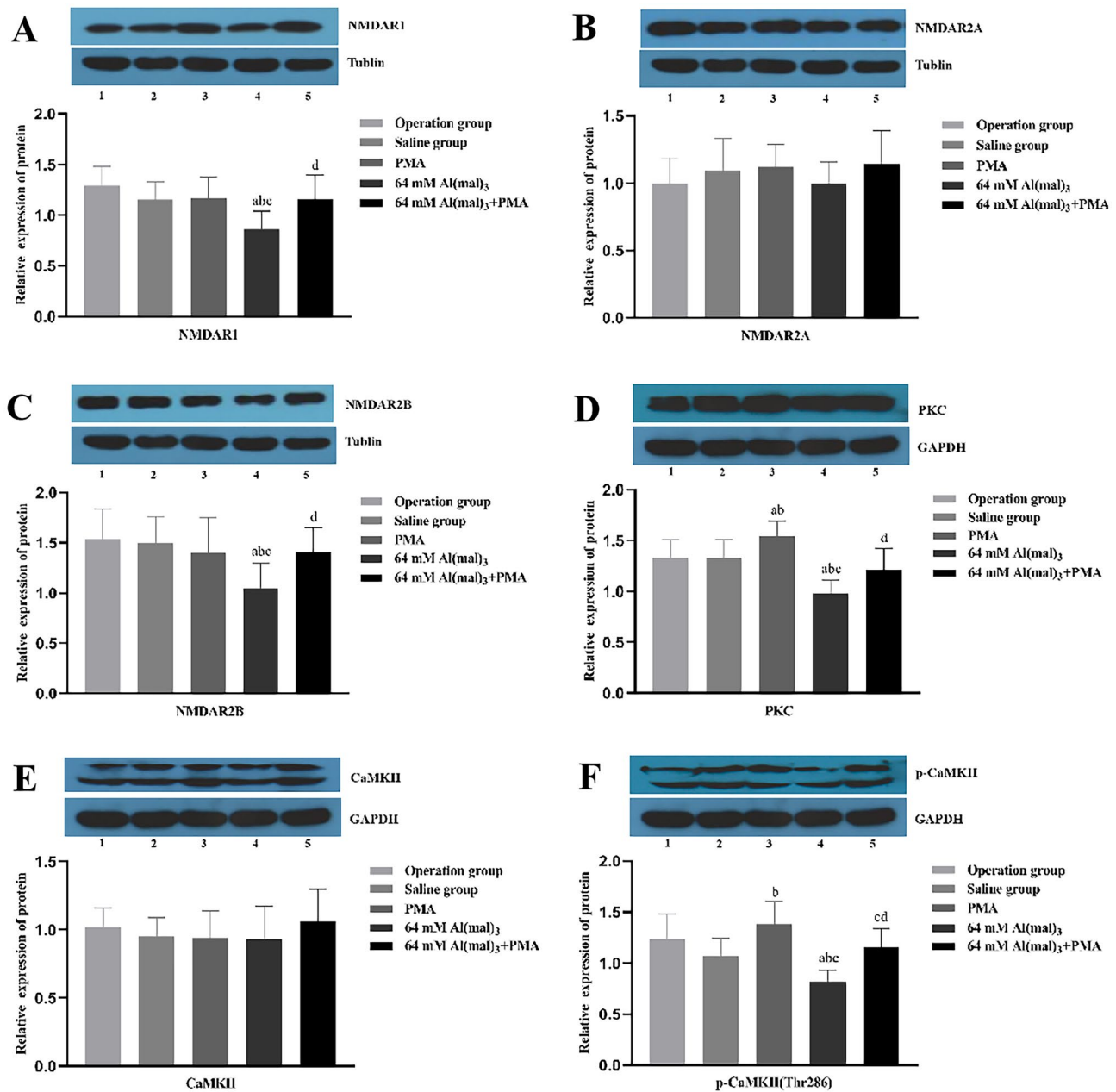
64 mM Al(mal)<sub>3</sub>+PMA group. Compared with the operation group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the PMA group, <sup>c</sup> $p < 0.05$

González-Weller et al. 2010; Rose et al. 2010). Since the neurotoxicity of Al was first reported, its neurotoxicity mechanism has attracted much attention. Epidemiology has shown that there is a certain correlation between Al content in drinking water and cognitive impairment, and occupational Al exposure also increases Al intake (Meyer-Baron et al. 2007). In vivo and in vitro experiments have confirmed that Al can enter the nervous system through the BBB, causing neurotoxicity such as decreased learning and memory and inattention. Paternain found that exposure to aluminum nitrate during pregnancy could cause weight loss in offspring and increase the incidence of visceral and bone deformities (Paternain et al. 1988). In this study, lateral ventricle injection was used to successfully establish a model of learning and memory impairment caused by Al in rats, and the mechanism of Al-induced synaptic plasticity damage was preliminarily studied.

Many studies have shown that, compared with AlCl<sub>3</sub>, Al(mal)<sub>3</sub> could induce stronger neurotoxicity (Kawahara et al. 2001; Lévesque et al. 2000; Zeng et al. 2012). It may be that in biological systems, Al is more likely to form complexes with organic lipophilic substances, so organic lipophilic aluminum salt is a medium for studying Al neurotoxicity (Campbell et al. 2001). The Morris water maze experiment is a classic experiment of choice for conducting behavioral research, especially exploring learning and memory functions (Barnhart et al. 2015; Vorhees and Williams

2006). This study showed that with the increase of the exposure dose, the escape latency period of rats gradually increased, the target quadrant dwell time decreased, and the first time arriving at the platform also increased significantly. The rats in the 64 mM Al(mal)<sub>3</sub> group showed significant impairment of learning and memory. Studies have shown that the escape latency period of rats in the 2 mg/mL and 3 mg/mL AlCl<sub>3</sub> groups was significantly prolonged and the number of crossing the platform was also significantly reduced after drinking Al for 12 weeks (Wang et al. 2014). After intraperitoneal injection of Al(mal)<sub>3</sub> for 60 days, the results of the water maze experiment showed that compared with the saline group, the learning and memory of rats in the middle- and high-dose Al-exposed groups was significantly impaired (Liang et al. 2012). These results were consistent with our study. The open field experiment is mainly used to detect animal exploration behavior and autonomous activity behavior. With the increase of the exposure dose, the time in the center area of rats gradually increased, the number of rearing and grooming decreased, and the difference was statistically significant, suggesting that acute lateral ventricle Al exposure could damage rats' exploration behaviors in new environments and reduce their anxiety, which is consistent with the results of subchronic Al exposure.

Synaptic plasticity damage is one of the neurotoxic mechanisms of Al. Synaptic plasticity is a cellular mechanism of learning and memory, and changes in activity-dependent



**Fig. 7** Changes in protein expression in Al-exposed rats after injection with PMA. **A** NMDAR1, **B** NMDAR2A, **C** NMDAR2B, **D** PKC, **E** CaMKII, **F** p-CaMKII (Thr286). Compared with the operation

group, <sup>a</sup> $p < 0.05$ ; compared with the saline group, <sup>b</sup> $p < 0.05$ ; compared with the PMA group, <sup>c</sup> $p < 0.05$ ; compared with the 64 mM Al(mal)<sub>3</sub> group, <sup>d</sup> $p < 0.05$

electricity and synaptic strength are closely related to LTP (Itoh et al. 2016). NMDARs play an important role in the occurrence of LTP, and the opening of their channels is the basis of learning and memory. The NR1/NR2 complex is widely distributed in the hippocampus, involved in learning and memory function, and can also cause excitotoxicity. It has been reported that the memory impairment of AD patients is not caused by A $\beta$  deposition or neurofibrillary tangles but by synaptic loss, in which NMDARs play a major

role (Terry et al. 1991). Studies have shown that the number and subunit composition of NMDARs in the postsynaptic membrane continue to change with the strength of synaptic activity. The release of glutamate activates AMPARs, leading to membrane depolarization. Ca<sup>2+</sup> enters the postsynaptic membrane through activated NMDARs, which initiates various metabolic pathways and finally affects synaptic strength. Therefore, AMPARs and NMDARs have different control mechanisms to ensure the appropriate number and

type of synaptic receptors on specific excitatory synapses (Bu et al. 2015; Horak et al. 2014). Increasing the expression of NMDARs could improve the symptoms of schizophrenia and improve memory function (Park et al. 2014), while inhibiting NMDARs in the hippocampus resulted in impairment of spatial learning and memory function and motor activity (Dai et al. 2015). The excitatory postsynaptic potential mediated by NMDARs decreased by 50–60% after stimulation with Schaeffer collateral in the hippocampus of aged rats (Kumar 2015; Lee et al. 2014). In this study, we first found that with increasing AI exposure dose, the protein expression levels of NMDAR1 and NMDAR2B decreased significantly, and there was no difference in NMDAR2A. However, RT-PCR results showed that the NMDAR2B gene level did not change significantly, indicating that the main reason for the decrease in NMDAR2B protein level was protein–protein interactions and multiple modifications. Therefore, we next focused on the regulation of NMDARs by PKC.

The regulation of NMDARs in neurons is the key to synaptic transmission and plasticity, and PKC can promote the regulation of NMDAR transport by interacting with NMDAR-related proteins (NAPs) on the cell surface (Yan et al. 2011). Immunofluorescence showed that PKC could increase the expression of NMDARs on the cell surface, and the PKC activator TPA could activate NMDAR channels (Lan et al. 2001; Lin et al. 2006). However, the transport of NMDARs to the membrane was not caused by direct phosphorylation of PKC. PKC promotes the transport of NMDARs through the autophosphorylation of CaMKII and through the phosphorylation of NMDARs through the tyrosine kinase Src (Yan et al. 2011). CaMKII is a very abundant brain protein concentrated in the postsynaptic compact (PSD) and a key molecule in the occurrence of LTP (Lisman et al. 2012). CaMKII knockout or mutant mice showed impaired learning ability and cognitive dysfunction (Bachstetter et al. 2014). The activation of CaMKII causes the autophosphorylation of T286, which promotes the effective binding of CaMKII and GluN2B and regulates the transport of NMDARs (Easton et al. 2011). At the same time, many studies have shown that the CaMKII/NMDAR complex is important not only for hippocampal LTP but also for the formation of hippocampal-dependent spatial learning and memory (Sanhueza and Lisman 2013; Stein et al. 2014). Increased synaptic activity leads to an increase in the CaMKII/NMDAR complex (Appleby et al. 2011). If the combination of CaMKII and GluN2B is destroyed, the LTP observed in the CA1 area is reduced by 50% by stimulating Shaffer collateral, and learning ability is also impaired in the water maze experiment (Halt et al. 2012). Sanhueza M found that the CaMKII/NMDAR complex was very important in maintaining synaptic strength (Sanhueza et al. 2011). PKC and

CaMKII play an essential role in regulating NMDARs. Therefore, this study detected the protein expression levels of PKC, CaMKII, and p-CaMKII (Thr286) in the rat hippocampus. The results showed that the protein levels of PKC and p-CaMKII (Thr286) decreased significantly as the exposure dose increased, suggesting that PKC was involved in AI-induced learning and memory impairment. To further explore its mechanism, this experiment used the PKC agonist PMA to treat rats. The results showed that PMA alone could not improve the learning and memory ability of rats, but it could improve the learning and memory impairment caused by AI. The results of western blotting showed that adding PMA to the AI treatment group could increase the expression levels of PKC, p-CaMKII (Thr286), and NMDAR subunits.

In this study, we found that acute lateral ventricle AI exposure could damage learning and memory in rats and cause changes in the expression of NMDARs, while the activation of NMDARs was involved in AD-related synaptic dysfunction. AD is the most common form of dementia and characterized by the accumulation of  $\beta$ -amyloid ( $A\beta$ ) and tau protein. Tau can cause abnormal phosphorylation of NMDARs (Ittner et al. 2010), and both  $A\beta$  and tau can change synaptic plasticity, leading to synapse loss, neurological dysfunction, and ultimately neuronal damage. Studies have shown that interference with synaptic transmission of glutamate might be the basis of the pathogenesis of AD (Rudy et al. 2015). An acute AI exposure rat model was used in this experiment; however, human exposure to AI is a chronic and long-lasting process. Therefore, the decreased expression of the NMDAR subunits PKC and p-CaMKII (Thr286) observed in the experiment needs to be further verified in other models, such as subchronic and chronic exposure models and even in the general population. In this experiment, it was observed that NMDAR2A had a decreasing trend, but there was no significant difference between the groups. This may be due to insufficient AI exposure time or dose, so the specific mechanism needs to be further studied with other models.

This study analyzed the effects of PKC on the expression of NMDAR subunits in AI-exposed rats, and the phosphorylation of CaMKII was involved in this process. We conducted a preliminary exploration of the mechanism of learning and memory impairment caused by AI. After AI enters nerve cells, it affects the translocation and activation of PKC, thereby reducing the expression and opening of NMDARs. It also reduces the influx of  $Ca^{2+}$  and the activation of CaMKII, thereby destroying the combination of NMDARs and CaMKII, further affecting the opening of NMDARs, and ultimately damaging learning and memory. NMDARs are also regulated by many other molecules, and their regulatory mechanism still requires further exploration.

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## Declarations

**Conflict of interest** The authors declare that there are no conflicts of interest.

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