

Original Article

Memantine improves spatial learning and memory of pentylenetetrazole-kindled rats with increasing Arc/Arg3.1 and CaMKII α expression

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Abstract: We supposed that over-excited pathological state might cause cognitive impairment by altering the expression of Arc/Arg3.1, CaMKII α and AMPA receptors. This study aimed to study whether epilepsy affected these molecules that were critical for learning and memory. Additionally, we applied memantine to PTZ-treated rats to investigate the effects of memantine on epileptic rats and the underlying mechanism. The results showed that water maze training was impaired by 5-14 seizures and treatment with memantine to PTZ treated rats improved performance. Our study suggested that the impaired spatial learning and memory in chronic epileptic rats may be related with the aberrant expression of Arc/Arg3.1, GluR1 and CaMKII α . Treatment with memantine could significantly rescue the spatial learning and memory function of the chronic epileptics rats kindled by pentylenetetrazol and attenuate the increase in GluR1 and decrease in Arc/Arg3.1, CaMKII α activity.

Keywords: Epilepsy, spatial learning and memory, Arc/Arg3.1, CaMKII α , AMPARs, memantine

Introduction

Epilepsy has adverse affect on mental development, cognition, and behavior, which may impair neuronal excitability, the release of neurotransmitter, enzymes, and factors critical for information processing and memory on some patients [1]. Some epileptic patients are at a significantly higher risk of impairments of cognitive function and behavioral abnormalities, where learning and memory deficits are the most common complaints [2].

Arc (activity-regulated cytoskeletal-associated protein)/Arg3.1 (activity-regulated gene 3.1 protein homolog) is an immediate early gene (IEG) that is rapidly and transiently induced in response to physiological and pathological stimuli in neurons. Plath et al. [3] demonstrated the critical role of Arc/Arg3.1 in the consolidation of enduring synaptic plasticity and memory storage. Arc/Arg3.1 knockout mice fail to form long-lasting memories for implicit and explicit learning tasks, despite intact short-term memory. They exhibit a biphasic alteration of hippocampal long-term potentiation in the dentate gyrus and area CA1 with an enhanced early and

absent late phase. Arc/Arg3.1 has been shown to interact with Calcium/calmodulin-dependent protein kinase II (CaMKII), which is the most abundant kinase in brain and critical for learning and memory [4-6] and, slightly increasing of Arc/Arg3.1 activity promotes CaMKII-dependent neurite outgrowth [7]. Chowdhury et al. [8] suggested that over expression of Arc/Arg3.1 could down-regulate surface expression of AMPA receptors by increasing the rate of AMPA receptor endocytosis through interaction with dynamin2 and endophilin3. DaSilva et al. [9] reported that Arc, AP-2 and AMPAR plays a vital role in the formation of learning and memory, by the bundled connection between Arc and AP-2 manages the AMPAR endocytosis which might be the crucial mechanistic link to explain how synaptic plasticity were managed by the activity-dependent expression of Arc. Evidently, convulsive action of corazol originating from suppression of GABA-ergic inhibition is realized through activation of glutamergic synaptic transmission [10].

Memantine acts as an antagonist of N-methyl-D-aspartate (NMDA) type receptors, which is thought to reduce abnormal activation of glutamate neurotransmission. Though Peltz G et al.

Memantine improves learning and memory of pentylenetetrazole-kindled rats

reported that treatment with memantine may cause epilepsy patients renal damage [11]. Meta-analysis suggested memantine could improve cognitive performance of AD. Pierson TM et al. speculated that high doses of memantine could better improve patients' seizure burden, but considering the pediatric safety guidelines of memantine, a dosage of ~0.5 mg/kg per day may don't have side effects [12]. 10 mg per day of memantine treatment, can significantly improve cognitive, memory, and the quality of life, in the mild to moderate cognitive impairment in patients with epilepsy, and was found to have a good security [13]. In another study, it was found that enhanced seizure-like activity can be rescued in an Angelman syndrome mouse model by genetically reducing the expression level of Arc, it was suggested that therapeutic interventions that reduce the level of Arc expression have the potential to reverse seizures [14]. A study of Arc expression in amygdala kindled rats suggested that arc mRNA expression may play a key role in synaptic reorganization in kindling [15].

Taken together, the current study sought to determine whether the cognitive impairment induced by epilepsy was associated with the aberrant expression of Arc/Arg3.1, CaMKII, and AMPAR. Moreover, we applied memantine to PTZ-kindled rats to investigate whether treatment with memantine counteracted the cognitive impairment and its potential mechanisms.

Materials and methods

This study was approved by the Institutional Review Board of our hospital, all animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Animals

Male Sprague-Dawley adult rats (200-250 g), provided by the Laboratory Animal Center of Second Hospital of Hebei Medical University were included in this study. Rats were housed at constant temperature and humidity under a 24-h light/dark cycle (lights on 07:00 h), and given food and water ad libitum. A week later,

the rats were selected with Y-maze method (Chinese Pharmacological University, China). The test was performed in a quiet and dark room. There was a lamp at the end of each of three pathways in Y-maze and the base of maze had electric net. In the training phase, running from unsafe area to safe area at one time was justified as successful. Such test was repeated for 10 times for each rat and the successful frequency was recorded. The qualified standard of maze test was that the rat arrived in the safe area 9 times during 10 experiments. Then in the testing phase each rat was tested 30 times and the error number was recorded. The qualified standard of maze test was that the error number less than 20 times during 30 experiments in the first day and 15 times during 30 experiments in the second day. Rats with bad scores were excluded from the study.

Drug treatment

Pentylenetetrazol (PTZ, Sigma, USA.), a GABA_A-receptor antagonist, was dissolved in saline to prepare concentrations of 10 mg/ml. A dose of 35 mg/kg was injected intraperitoneal (i.p.) for making chronic epileptic model. Memantine (H.Lundbeck A/S) at dose of 10 mg/kg was administered by intraperitoneal injection to drug intervention group.

Group of experiment

In addition to the low scores rats, other rats were randomly divided into four groups, including normal control group (NC group, n=20), epileptic group (PTZ group, n=35), epileptic + memantine group (PTZ+MMT, n=35), and memantine control group (MMT group, n=20).

Induction of seizures

The 20 rats in the normal control group (NC) were injected saline intraperitoneally (i.p) by 3.5 ml/kg/d for consecutive 44 days. The 35 rats in PTZ group received intraperitoneal injection of 1% pentylenetetrazol (PTZ) by 35 mg/kg/d for consecutive 44 days. The 35 rats in PTZ+MMT group received intraperitoneal injection of 1% pentylenetetrazol (PTZ) by 35 mg/kg/d for consecutive 30 days. From the 31st day on, the rats in PTZ+MMT group were injected memantine intraperitoneally by 10 mg/kg/d 15 minutes before PTZ in the rest 14 days. Rats in MMT group (20 rats) were injected intraperitoneally saline by 3.5 ml/kg/d for consecu-

Memantine improves learning and memory of pentylenetetrazole-kindled rats

tive 30 days. From the 31st day on, the rat in MMT group were injected MMT intraperitoneally by 10 mg/kg/d 15 minutes before saline in the rest 14 days.

Motor assessment

Motor ability was assessed at 24 hours after the models completed [16-18]. In this test, rats were trained to grasp a horizontal copper bar (0.5 cm diameter, 50 cm above the ground) with both front limbs. The suspending time that each rat kept on hanging was recorded in seconds.

Morris water maze test

After motor assessment, rats (n=18 per group) were evaluated for spatial learning and memory with MWM according to the classic Morris protocol [19]. The MWM (Shanghai Mobiledatum Information Technology Co. Ltd., P. R. China) consisted of a circular tank (150 cm diameter × 60 cm height) was filled with water (26±1°C) to a depth of 30 cm. Four points on the perimeter of the pool were designed north east (NE), south east (SE), south west (SW), and north west (NW). A hidden platform (12 cm diameters) made of roughened Plexiglas, was submerged 1 cm under water in one of the four designated positions within the tank. Each rat was placed in the pool without the platform for 120 s to habituate to the environment on the day before trials. On the first day to the fourth day, rats were trained for 32 trials (eight trials a day) to find the platform within 120 s. The time which rats spent in finding the platform was called escape latency. For each rat, the quadrant in which the platform was located remains constant, but the point of immersion in the pool varied between NE, SE, SW, and NW in a random order for the 32 trials. On mounting the platform, the rats were given a 30-second rest period, after which the next trial was started. Rats failing to find the platform within 120 s were guided to the platform and placed on it for a 30-second rest, and the escape latency would be 120 s. After trial, rats were dried and returned to their home cages. All sessions were recorded by a video camera located above the tank. On day 5, the platform was removed. The rat was allowed 120 s of free swimming (probe trial). The times that the rats cross the quadrant where the platform was previously located (crossing times) and the time that rats spent in the goal quadrant (swimming time) were recorded. The testing procedure used during the 4

days of locating the hidden platform is considered a measurement of spatial reference memory, whereas the probe trial is considered to measure the strength of spatial learning. After the Morris water maze test the rats would be sacrificed within 24 hours.

Reverse transcription-polymerase chain reaction

RT-PCR was used to analyse the levels of Arc and GluR1 mRNA. Total mRNA was extracted from hippocampi of the rats in each group according to the instructions of TRIzol reagent (Invitrogen, U.S.A.). The yield of total mRNA was determined by measuring the absorption at 260 nm and 280 nm separately, 2 µg of which was in reverse transcribed to first strand cDNA in a 20 ml reaction volume. The primers of Arc, GluR1 and β-actin designed by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China) according to the serial number from GenBank were as follows, the forward primer for Arc [20] was 5'-CCAGGAAGCTGATGG CTACGAC-3' (bases 693-714, NM0193) and the reverse primer was 5'-GTGTCAGCCCCAGCTCAATCAAG-3' (bases 1471-1494), the forward primer for GluR²¹ was 5'-GGAATATGCCGTACATCTTTG-3' (bases 193-203, NM031608) and the reverse primer was 5'-AAGTCATCTCAAAGCTGTCGC-3' (bases 391-411), the forward primer for β-actin was 5'-GCCATGTACGTAGCCATCCA-3' (bases 472-491, NM031144) and the reverse primer was 5'-GAACCGCTCATTGCCGATAG-3' (bases 827-846). The lengths of amplified fragments were 801 bp, 223 bp and 375 bp, respectively. The reaction was started at 95°C for 2 min and amplified for Arc of 35 cycles of 30 s at 95°C, 30 s at 62°C, 40 s at 72°C and ended with 5 min extension at 72°C; It was started at 95°C for 2 min and amplified for GluR1 of 33 cycles of 30 s at 95°C, 30 s at 57°C, 40 s at 72°C and ended with 5 min extension at 72°C. 5 µl of each PCR product was observed after electrophoresis on 2% agarose gel, and the density of each band was analyzed on the gel image analysis system (Syngene, U.K.). The levels of Arc mRNA and GluR1 mRNA were determined by calculating the density ratio of Arc mRNA/β-actin mRNA and GluR1 mRNA/β-actin mRNA.

Western blotting

Protein samples of hippocampi for Arc were homogenized in 100 mM Tris containing 200

Memantine improves learning and memory of pentylenetetrazole-kindled rats

mM NaCl, 10% glycerol, 2 mM NaF, 2 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM DTT, 1 mM EDTA, 0.1 mM Na_3VO_4 , 1 mM pepstatin, 10 mg/ml trypsin inhibitor, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mM PMSF at pH 7.4 (all chemicals were from Amresco Inc., USA). After 15 min lysis in ice, samples were centrifuged at 20,500 g for 15 min. For GluR1 and phospho-Thr286-CaMKII α the hippocampi protein samples were homogenized by using a sonicate (4-6 bursts for 1 s each) on ice in approximately 7 volumes of 50 mM HEPES (Ph7.5) containing 2 mM EDTA, 100 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ aprotinin, and then centrifuged at 12,000 g for 30 min at 4°C. The supernatants were used as total extracts.

The protein concentration of the supernatant was determined to use a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). Extracts (50 μg protein each lane) were diluted in an equal volume of electrophoresis buffer and boiled for 5 min. The extracts were separated by 10% SDS-PAGE and then electrophoretically transferred on to polyvinylidene difluoride (PVDF) membranes (Promega, U.S.A.), and the membranes were blocked with 5% (w/v) nonfat milk in PBS for 1 h at room temperature. After 3~5 min washing with PBS/Tween-20, the membranes were probed overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-Arc antibody (1:400, sc-15325, Santa Cruz), rabbit polyclonal GluR1 antibody (1:200, BA0896, Boster), and rabbit polyclonal anti-phospho-CaMKII α antibody (1:500, sc-12886-R, Santa Cruz). For all antibody dilutions and for blocking nonspecific reactions, PBS containing 5% nonfat dry milk, and 0.05% Tween-20 was used. After washing 5 times in PBS/Tween-20, the membranes were incubated with goat anti-rabbit fluorescent antibody (1:5000, Rockland, U.S.A.) for 60 min at room temperature. Following four washes in PBS/Tween-20 and one in PBS alone, immunolabeled marker protein bands on the membranes were scanned and analyzed by a far infrared fluorescent image scanner and analyzer (Odyssey, U.S.A.). Since multiple gels were analyzed for quantification of marker proteins, immunopositive bands of the control protein GAPDH (1:1000, Santa Cruz, U.S.A.) were used for normalization of optical densities of marker protein bands of each probe. Data were expressed as normalized optical density. Negative controls were

performed by omitting the first antibody and did not show any signals.

Immunohistochemistry

Brains were removed quickly, immersed with 4% paraformaldehyde in PBS for 24 h at 4°C. Brain sections (5- μm thick) were blocked in 3% H_2O_2 , 3% normal goat serum, and incubated with phospho-CaMKII α rabbit polyclonal antibody (1:200, Santa Cruz) in 0.01 mol/L phosphate-buffered saline overnight. The secondary antibodies, secondary biotinylated conjugates and diaminobezidine were from the Vect ABC kit (Zhongshan Biology Technology Company, China). Five visual fields of hippocampi were selected and the immunoreactive cells were counted under a 400 \times light microscope.

Statistical analysis

Quantitative data were expressed as mean \pm SD. Statistical comparisons were conducted using one-way ANOVA followed by SNK and LSD tests for intergroup comparisons. Differences with $P < 0.05$ were considered statistically significant. We used SPSS22.0 (Chicago, Illinois) for all Statistical analysis.

Results

Behavioral features of PTZ-induced seizures

Rats in PTZ group had seizures induced by PTZ since 6th day. The seizures were characterized by head shaking, squealing and crawling. Wild running, loss of righting reflex and generalized tonic-clonic convulsions were seen from the 16th day to the 18th day.

Rats in PTZ+MMT group developed the seizures of stages 4~5, while the lasting time and recovering time were shorter than those in epileptic group. No seizures were found in rats of NC group and MMT group during the experiment period.

Results of motor assessment

In overhanging test, the suspending times (mean \pm standard deviation, s) of rats were 34.57 ± 4.88 s, 32.67 ± 6.54 s, 34.89 ± 6.41 s, 35.24 ± 8.71 s in normal control, epileptic, epileptic + memantine and memantine groups, respectively. No significant difference was observed in over hanging test among the four groups ($P > 0.05$).

Memantine improves learning and memory of pentylenetetrazole-kindled rats

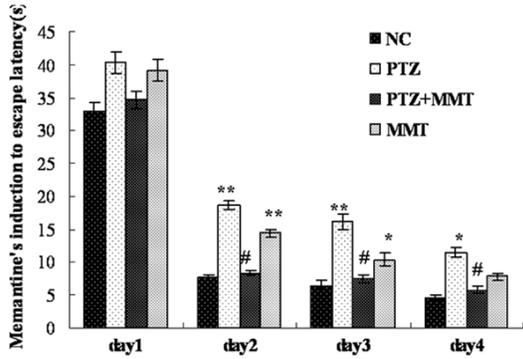


Figure 1. Escape latencies on day 1 to day 4. MWM was used to test the ability of the rats' learning and memory. On day 1 to day 4, each rat tried eight times each day. The mean escape latency each day was used to measure the ability of learning and memory. Each bar represents the mean \pm S.E.M. Bar graph illustrating the induction of memantine, * $P < 0.05$, ** $P < 0.01$ vs. NC group; # $P < 0.05$, ## $P < 0.01$ vs. PTZ group.

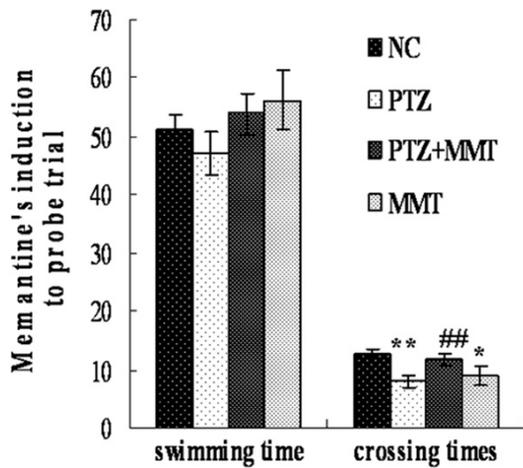


Figure 2. Swimming time and crossing times in the goal quadrant within 120 s in the probe trial on day 5. Bar graph illustrating the induction of memantine, * $P < 0.05$, ** $P < 0.01$ vs. normal control group, # $P < 0.05$, ## $P < 0.01$ vs. PTZ group.

Results of MWM

Spatial learning and memory was assessed through the MWM task. As shown in **Figure 1**, there was significant difference on the second day to the fourth day. On the first day, no significant differences were found among the four groups ($P > 0.05$). However, on the following three days, the mean escape latencies of the rats in PTZ+MMT group were strikingly shortened, compared with those of the rats in PTZ group ($P = 0.011$) (**Figure 1**).

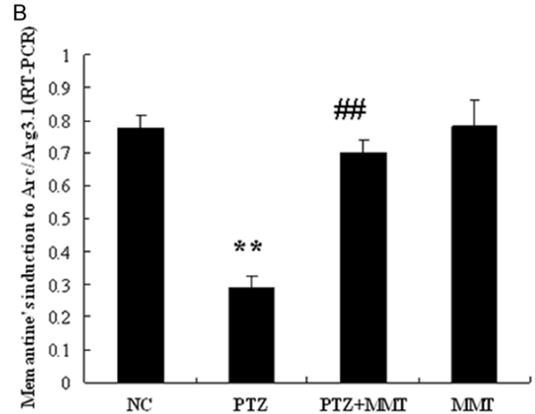
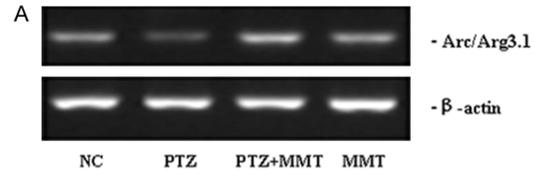


Figure 3. RT-PCR of Arc/Arg3.1 genes in the rat hippocampus after seizures. Representative photographs RT-PCR of dynamic expression (A) of Arc/Arg3.1 mRNA, and β -actin. (B) Bar graph illustrating the mRNA levels for Arc/Arg3.1 in normal control (NC), epileptic (PTZ), epileptic + memantine (PTZ+MMT) and memantine (MMT) group, * $P < 0.05$, ** $P < 0.01$ vs. NC group; # $P < 0.05$, ## $P < 0.01$ vs. PTZ group.

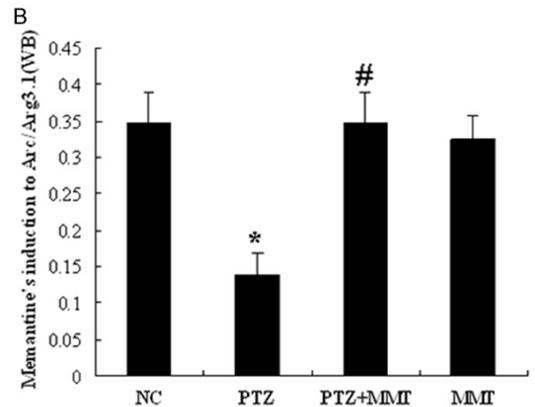
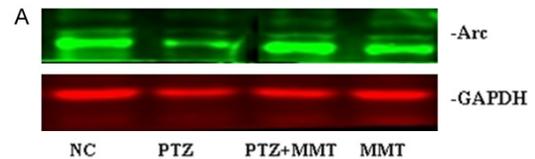


Figure 4. Representative photographs Western blot of dynamic expression (A) of Arc protein and GAPDH control in the rat brain after seizures. (B) Bar graph illustrating the Memantine induction to protein of Arc. * $P < 0.05$ vs. NC group; # $P < 0.05$ vs. PTZ group.

In probe trial, no significant difference was found in swimming time spending in the goal

Memantine improves learning and memory of pentylenetetrazole-kindled rats

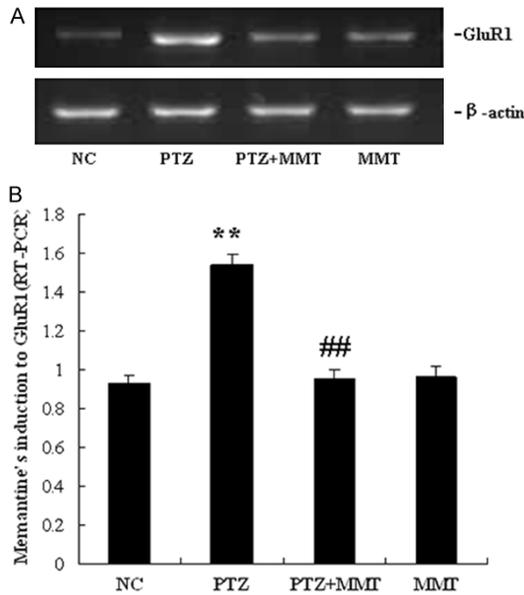


Figure 5. RT-PCR of GluR1 genes in the rat hippocampus after seizures. Representative photographs RT-PCR of dynamic expression (A) induction (10 mg/kg, ip) of GluR1 mRNA, and β -actin. (B) Bar graph illustrating the mRNA levels for GluR1 in normal control, epileptic, epileptic + memantine and memantine group, * $P < 0.05$, ** $P < 0.01$ vs. normal control group; # $P < 0.05$, ## $P < 0.01$ vs. epileptic group. Each bar represents the mean \pm S.E.M. Equal amount of the reverse transcription products was used for the PCR reactions. The PCR products were separated on 2% agarose gels. The intensity of each band was calculated as an integrated density value. β -actin was used as an internal control; the level of GluR1 mRNA was determined by GluR1 mRNA/ β -actin mRNA. The lengths of amplified fragments of GluR1 and β -actin were 223 bp and 375 bp, respectively.

quadrant among the groups ($P > 0.05$). But there were significant differences regarding crossing times among the groups. The number of crossing times through the platform quadrant within 120 s in PTZ group decreased obviously compared with that in NC group ($P < 0.01$). The number of crossing times in PTZ+MMT group increased obviously compared with that in PTZ group ($P < 0.01$). In addition, the number of crossing times in MMT group decreased compared with that in NC group ($P < 0.05$) (Figure 2).

Memantine attenuated the aberrant expression of Arc, GluR1 and phospho-CaMKII α induced by epilepsy

Memantine elevated the mRNA ($P < 0.01$) and protein ($P < 0.05$) levels of Arc in PTZ+MMT group compared with the PTZ group (Figures 3A, 3B, 4A, 4B). Correspondingly, memantine

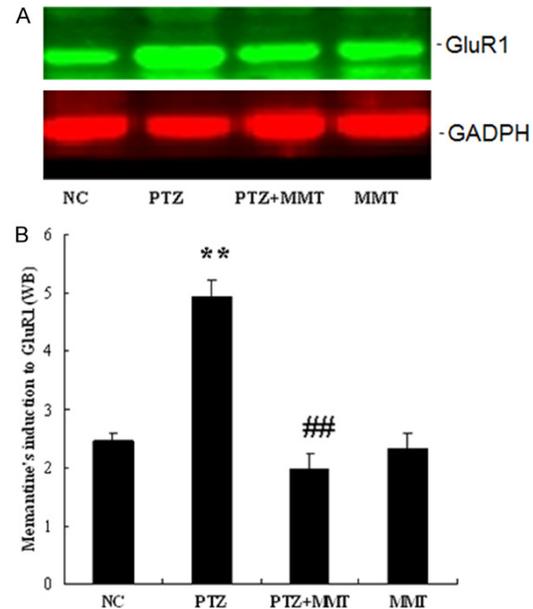


Figure 6. Representative photographs Western blot of dynamic expression (A) of GluR1 protein and GAPDH control in the rat brain after seizures. (B) Bar graph illustrating the Memantine induction to protein of GluR1. * $P < 0.05$, ** $P < 0.01$ vs. NC group; # $P < 0.05$, ## $P < 0.01$ vs. PTZ group.

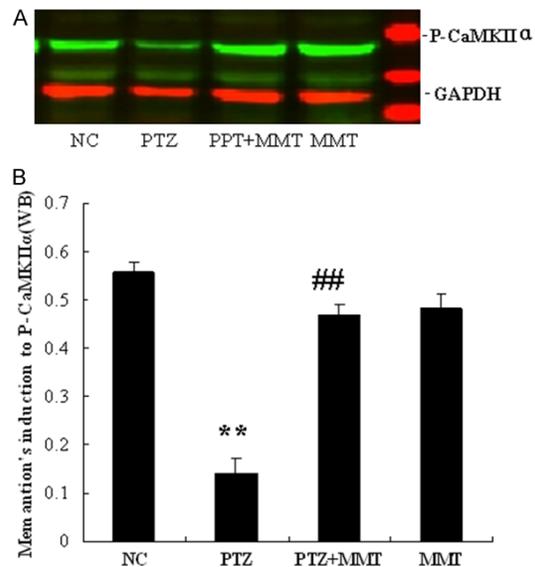


Figure 7. Representative photographs Western blot of dynamic expression (A) of p-CaMKII α protein and GAPDH control in the rat brain after seizures. (B) Bar graph illustrating the Memantine induction to protein of p-CaMKII α . * $P < 0.05$, ** $P < 0.01$ vs. NC group; # $P < 0.05$, ## $P < 0.01$ vs. PTZ group.

could counteract the decreased mRNA and protein levels of GluR1 induced by epilepsy ($P < 0.05$) (Figures 5A, 5B, 6A, 6B). The p-CaMKII α

Memantine improves learning and memory of pentylenetetrazole-kindled rats

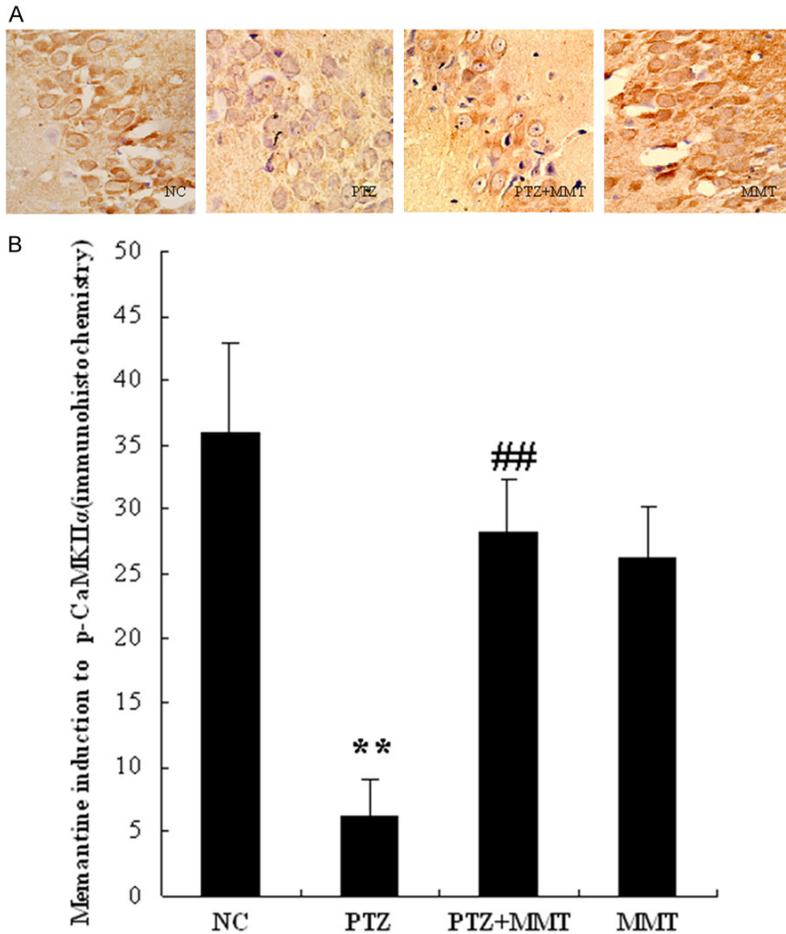


Figure 8. The expression of phospho-CaMKII α in immunohistochemistry. A and B. Bar graph showed memantine induction of p-CaMKII α expression, * $P < 0.05$, ** $P < 0.01$ vs. NC group; # $P < 0.05$, ### $P < 0.01$ vs. PTZ group.

protein levels in PTZ+MMT group were increased significantly compared with those in PTZ group ($P < 0.05$) (Figure 7A, 7B). Consistently, immunohistochemistry results showed that the stained p-CaMKII α were significantly increased ($P < 0.01$) in the hippocampus of rats of PTZ+MMT group compared with the PTZ group (Figure 8A, 8B).

Discussion

The exact neurophysiological basis for the learning deficits in epileptic patients has not yet been established. Alterations in the ability of hippocampal synapses to sustain long-term potentiation (LTP) could be a contributing factor [22]. Memantine, as a unique NMDA antagonist has been approved for treatment of AD by FDA and it has been proved that memantine produces symptomatic improvement of cognition

in animal models, though the specific mechanism is not fully understood [23]. Furthermore, memantine is a neuroprotective agent in various animal models based on both neurodegenerative and vascular processes, as it ameliorates cognitive and memory deficits [24]. Importantly, memantine treatment is able to partially normalize information processing in the hippocampus, suggesting that given memantine in the early development of the pathology, could provide neuronal and cognitive protection to indirectly prevent pathological microglial activation [25]. But little study concerned to the effects of memantine on the cognitive dysfunction of epilepsy. Under conditions of tonic activation of NMDA receptors, uncompetitive NMDA receptor antagonists can paradoxically reverse deficits in learning and synaptic plasticity [26]. Therefore this study aimed to evaluate the effects of memantine

on PTZ-kindled rats and its underlying mechanism.

In our study, the application of memantine could not prevent the seizures of the rats kindled by PTZ, but might play an alleviative effect. Furthermore, we found that memantine could attenuate the impairment of learning and memory of rats in epileptic group and also reversed the expression of GluR1, Arc/Arg3.1 and phospho-CaMKII α in hippocampus of rats in epileptic group. It suggested that the effect of memantine on the spatial learning and memory might be associated with the Arc/Arg3.1, CaMKII α and GluR1-containing AMPARs, which had been indicated playing an important role in synaptic plasticity in numerous studies. Interestingly, Water maze test showed that memantine had played a negative role to the normal rats. We speculated that memantine had inter-

Memantine improves learning and memory of pentylenetetrazole-kindled rats

ferred NMDA receptors existing on the normal postsynaptic membrane due to the low or moderate affinity to NMDA receptors, which further to affect neuronal transmission. However, no significant difference were found between the control group and the memantine group in the next testing of Arc, CaMKII α and GluR1 ($P > 0.05$). These results suggested that memantine improve the cognitive impairment in pathological state but to physiological state, the effects of memantine on cognitive function were not so optimistical as such. Therefore, the bidirectional effects of memantine in the different conditions is needed to be further investigated.

But Dhir. et al. reported that memantine have protective role in the management of new epilepsy seizures [27], we supposed that's may cause by the difference of conditions.

In summary, our data validated memantine exerted beneficial effects on improving cognitive impairment in the epilepsy, and this effect might be associated with the aberrant expression of GluR1, Arc/Arg3.1 and pospho-CaMKII α which would open new perspectives for therapeutic targets of memantine in patients with epilepsy.

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Disclosure of conflict of interest

None.

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Memantine improves learning and memory of pentylenetetrazole-kindled rats

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