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Abstract:

Ischemic stroke, the second leading cause of death worldwide, leads to excessive glutamate release, over-activation of N-methyl-D-aspartate receptor (NMDAR), and massive influx of calcium (Ca^{2+}), which may activate calpain and caspase-3, resulting in cellular damage and death. Memantine is an uncompetitive NMDAR antagonist with low-affinity/fast off-rate. We investigated the potential mechanisms through which memantine protects against ischemic stroke *in vitro* and *in vivo*. Middle cerebral artery occlusion-reperfusion (MCAO) was performed to establish an experimental model of ischemic stroke. The neuroprotective effects of memantine on ischemic rats were evaluated by neurological deficit scores and infarct volumes. The activities of calpain and caspase-3, and expression levels of microtubule-associated protein-2 (MAP2) and postsynaptic density-95 (PSD95) were determined by Western blotting. Additionally, Nissl staining and immunostaining were performed to examine brain damage, cell apoptosis, and neuronal loss induced by ischemia. Our results show that memantine could significantly prevent ischemic stroke-induced neurological deficits and brain infarct, and reduce ATP depletion-induced neuronal death. Moreover, memantine markedly suppressed the activation of the calpain-caspase-3 pathway and cell apoptosis, and consequently, attenuated brain damage and neuronal loss in MCAO rats. These results provide a molecular basis for the role of memantine in reducing neuronal apoptosis and preventing neuronal damage, suggesting that memantine may be a promising therapy for stroke patients.

Abbreviations: NMDAR, N-methyl-D-aspartate receptor; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion-reperfusion; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; ATP-D, ATP depletion; MEM, memantine; CBF, cerebral blood flow; MAP-2, microtubule-associated protein-2; PSD95, postsynaptic density-95; TTC, 2,3,5-triphenyl tetra-zolium chloride; TUNEL, TdT-mediated dUTP Nick-End Labeling;

1. Introduction

Ischemic stroke is one of the most common causes of mortality and morbidity in both pediatric and adult populations [1, 2]. The reduction of cerebral blood flow that occurs during acute ischemic stroke leads to oxygen and glucose deprivation and to subsequent hyperactivation of postsynaptic glutamate/glycine-gated ion channels, specifically, the N-methyl-D-aspartate receptor (NMDAR) [1, 3]. Overstimulation of NMDAR induces an excessive influx of Ca^{2+}, which may activate calpain and caspase-3, eventually resulting in neuronal dysfunction and cell death by necrosis or apoptosis [3-7]. Therefore, NMDARs have been considered drug targets in treating stroke.

Many previous NMDA receptor antagonists tested in clinical trials have been unsuccessful because of their unacceptable neurotoxic side effects, which are partially caused by their blockade of physiological synaptic transmission [8-10]. However, memantine is an uncompetitive NMDA receptor antagonist with a low-affinity and a fast off-rate, which preferentially blocks pathological NMDA receptor activity, particularly at the extrasynaptic position, without disrupting physiological synaptic transmission [10-13]. Previous studies have revealed that memantine exerts a neuroprotective in different *in vitro* and *in vivo* models of excitotoxicity [14-16]. Moreover, memantine reduces ischemic brain injury and enhances recovery in rats and mice when administered in the acute or chronic stroke phase [17-21]. Additionally, clinical trials of memantine for the treatment of ischemic stroke are currently under way (Clinical Trials.gov identifier: NCT02144584). These findings suggest that memantine may be a viable pharmacological agent for treating stroke patients.

Memantine has received considerable attention and has been examined as a potential therapeutic drug for several neurological disorders, including Alzheimer's disease, vascular dementia, neuropathic pain, and stroke. However, the molecular mechanisms of memantine in treating ischemic stroke remain largely unknown. In the

present study, we investigated whether memantine could exert neuroprotective effects against ischemic stroke and attenuate neuronal apoptosis by suppressing calpain-caspase-3 pathways in the penumbra of MCAO rats.

2. Experimental procedures

2.1. Animals

Sprague-Dawley (SD) rats were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). They were individually housed in plastic cages at a controlled temperature (22 ± 1°C), relative humidity (55 ± 10%), and photoperiod (light/dark conditions 12/12 h lights on 7:00 a.m.). All experiments were performed strictly in accordance with the International Ethical Guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Primary hippocampal neuronal culture

Primary hippocampal neurons were prepared and cultured similarly to our previous reports [22]. Briefly, hippocampi were dissected out from 18-day rat embryos of either sex in cold HBSS. Then, the tissue was digested with 0.05% trypsin–EDTA for about 20 min at 37°C, followed by trituration with pipettes in the plating media (DMEM with 10% FBS, 10% F12 and 25 u/ml penicillin/streptomycin). After rinsing twice, cells were counted and plated onto glass coverslips precoated with 0.1 mg/ml poly-D-lysine (Sigma). After culturing for 1 day, half of the media were changed into neuronal culture media (neurobasal media (GIBCO) containing 2 mM GlutaMAX™-I Supplement, 2% B27 and 25 u/ml penicillin/streptomycin). 2 μM Ara-C (Sigma) was added 6–8 d after plating during the culture medium change, and cells were fed twice weekly thereafter. All cells were grown at 37°C and in 5% CO2. Unless stated otherwise, all tissue culture reagents were obtained from Invitrogen (San Diego, CA).

2.3. ATP depletion and assessment of neuronal death

Primary cultured neurons (11–12 DIV) were washed twice with PBS and then were incubated at 37°C for 20 min in either normal growth medium (control cells) or glucose-free DMEM containing 3 mM 2-deoxyglucose and 5 mM NaN3 to yield ATP-depleted cells. Following ATP depletion, hippocampal neurons were exposed with or without memantine (1, 10 and 50 μM). Neuronal death was determined either by visualizing survival neurons stained with NeuN or measuring lactate dehydrogenase (LDH) activity released from damaged cells into culture medium at 24 h after memantine treatment. Measurement of LDH release was performed by using a Cytotox 96 Kit (Promega) with the instructions of the manufacturer. Data are presented as the difference in LDH levels as a percentage of control.

2.4. Transient focal cerebral ischemia

Transient focal ischemia was induced by intraluminal suturing of the middle cerebral artery occlusion and reperfusion (MCAO), which was originally described by Longa et al [23]. Briefly, SD rats were anesthetized with isoflurane (3% initial, 2% maintenance) and nitrous oxide (60%) in oxygen with spontaneous respiration via a mask. A Laser Doppler Flowmetry device (LDF100C, Biopac Systems, Goleta, CA), attached to the skull (5 mm lateral and 1 mm posterior to bregma) with dental cement, was used for measuring ipsilateral cerebral blood flow. Under the operating microscope, proximal portions of the left common carotid artery (CCA) and external carotid artery (ECA) were ligated, and a 4–0 silicon-coated nylon suture was introduced into the CCA and advanced about 18 mm beyond the carotid bifurcation for transient occlusion of the middle carotid artery (MCA). Ischemic rats that showed a stable drop of 80% in Blood Perfusion Units compared with baseline levels (before MCAO) were used for further experimentation. Reperfusion was achieved by removing the intraluminal occlusive embolus 1 h after MCAO. Animals subjected to the sham operation were treated similarly, except without ligations and occlusions.

2.5. Experimental design and drug treatment

Experimental animals were divided into sham, saline, and memantine (MEM) groups. Immediately after MCAO, drugs were injected intraperitoneally. (1) The MEM group received memantine at a dose of 20 mg/kg (Sigma, 5 mg/ml in 0.9 % sterile saline), followed by a maintenance dose of 1 mg/kg at 12 h intervals to sustain levels of approximately 1–10μM in brain tissue because the half-life of the drug is approximately 12 h [24]. (2) The saline group animals were subjected to MCAO and received an equivalent volume of vehicle. (3) The sham group served as sham-operated control and received 0.9% sterile saline.

2.6. Neurological assessment

The neurological deficit score of each rat was measured 24 h after MCAO induction in a blinded fashion according to a well established five-point neurological scale [23]: score 0 = no apparent deficits; score 1 = failure to fully extend the right forepaw; score 2 = circling to the right; score 3 = falling or leaning over to the right; score 4 = no spontaneous walking and a depressed level of consciousness; score 5 = dead.

2.7. Measurement of cerebral infarct volume

For determination of the infarct volume, rats were deeply anesthetized with 3.5% isoflurane and sacrificed by decapitation. Brains were quickly removed, and six 2-mm-thick coronal sections were prepared with a brain matrix device (ASI Instruments, Inc.). Brain sections were stained with 2% TTC (w/v) at 37°C for 15 min and fixed in 4% paraformaldehyde (PFA). Images were analyzed using Image J software (NIH, Bethesda, MD, USA). The infarct volume data was expressed as a percentage of the contralateral hemisphere and calculated as described previously [25].

2.8. Western blotting

At 24 h after MCAO, rats (n = 4 per group) were deeply anesthetized, and the ischemic penumbras were microdissected according to established protocols in rodent models of unilateral proximal MCAO [26]. Briefly, a 4-mm thick coronal brain slice was cut, beginning 5 mm from the anterior tip of the frontal lobe. Then a longitudinal cut (from top to bottom) was made approximately 2 mm from midline through the ischemic hemisphere to remove medial portions. Finally, a transverse diagonal cut was made at approximately the "2 o'clock" position to separate the wedge-shaped penumbra.

The harvested brain tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktails (Roche Diagnostics) and PMSF. After centrifugation, the protein concentration was quantified by the BCA assay (Pierce Biotechnology). Brain homogenates (30 μg) were separated by SDS-PAGE and transfered onto PVDF membranes, which were blocked for 2 h with 5% non-fat dry milk at room temperature. The blots were incubated overnight at 4°C with the appropriate primary antibodies: calpain 1 (1:1000; Abcam), calpain 2 (1:1000; Abcam), GAPDH (1:3000; CST), MAP2 (1:1000; Sigma), PSD95 (1:500; Sigma), Cleaved caspase-3 (1:300; CST) and NeuN (1:2000; Millipore), followed by the appropriate HRP-conjugated secondary antibody for 2h. The bands were visualized with enhanced chemiluminescence (ECL) development (Pierce Biotechnology), and images were taken using a Bio-Image Analysis System (Bio-Rad). Normalization of the results was acomplished by running parallel western blots and probing for GAPDH. The value of the sham-operated group was designated as 100%.

2.9. Tissue section preparation

Deeply anesthetized animals were transcardially perfused with saline, 24 h after MCAO, followed by 4% PFA in 0.1 M phosphate buffer (pH 7.4). Rats' brains were post-fixed overnight at 4°C and cryoprotected with successive 15 and 30% sucrose sinks in PBS at 4°C for 3 d. The brains were then immersed with JUNG tissue

freezing medium (Leica). Coronal cryostat sections (15-µm thick) were made on a Leica CM1950 cryostat (Leica), air dried, and stored at -20°C until immunostaining.

2.10. Nissl staining

Coronal brain sections from four equidistant brain levels, 1 mm apart, were stained with cresyl violet according to a standard protocol [27]. For Nissl staining, air-dried sections were fixed again in 4% PFA solution for 15 min. Next brain sections were immersed in 100, 95, 85, 70 % ethanol and double distilled water for 3 min each, stained for 15 min in filtered cresyl violet solution (Sigma, 0.2% (w/v)), and briefly rinsed in doubly distilled water. Finally, they were dehydrated again in 70, 95, 100% ethanol for 1 min each, placed in xylenes for another 10 min and then coversliped.

Nissl staining were attempted to examine neuronal injury. Necrotic neurons (Red triangles) showed the disappearance of Nissl's bodies in the cytoplasm, shrunken intercellular space, and deep staining. The following four scores were used to evaluate necrotic neurons in the infarct area: 0, normal; 1, damaged neurons were <25%; 2, damaged neurons were 25–50%; 3, damaged neurons were 50–75%; and 4, damaged neurons were >75% [27].

2.11. TUNEL staining

Cerebral ischemia-induced DNA fragmentation was quantified in frozen sections using the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Promega) according to the manufacturer’s instructions. Briefly, air-dried sections were fixed with methanol-free 4 % PFA at room temperature for 15 min and washed twice with PBS. The sections were immersed in equilibration buffer for 10 min, which was then replaced by a mixture of 1 µl of TdT enzyme, 5 µl of nucleotide mix, and 45 µl of equilibration buffer. The sections were then kept at 37°C for 90 min. SSC (2×) was added for 15 min at room temperature to terminate the TdT enzyme reaction.

2.12. Immunohistochemistry

Immunohistochemical analyses were performed in a humidified chamber. Sections were dried at room temperature for at least 30 min, washed in PBS (10 min), and permeabilized with 98% methanol for 2 min. Permeabilization solution was removed and the sample washed three times in PBS for 10 min each. After blocking with 10% donkey serum, sections were incubated overnight at 4 °C with the following primary antibodies: guinea pig anti-NeuN (1:500; Millipore) or mouse anti-MAP2 (1:300; Sigma). After washing three times in PBS, sections were incubated for 2 h at room temperature with the corresponding secondary antibodies: anti-Guinea Pig Alexa 647-conjugated or anti-mouse Alexa 594-conjugated secondary antibodies (1:300; Invitrogen). Sections were again washed three times in PBS and then mounted onto glass slides with ProLong Gold antifade reagent (Molecular Probe). Images were visualized using an Olympus BX51 microscope with DP controller 3.2 software (Olympus) or Nikon A1R-A1 Confocal System under ×10/0.45 or ×20/0.75 objective with NIS Elements 4.2 Software (Nikon Instruments).

2.13. Statistical analysis

All data are expressed as mean ± standard error mean (SEM), and were analyzed using the SPSS package for Windows (Version 18.0). Statistical analysis of the data was performed with Student's t test or a one-way ANOVA followed by the LSD post hoc test. Differences with $p<0.05$ were considered statistically significant.

Results

3.1. Memantine protects neurons against ATP depletion-induced neuronal death in primary hippocampal cultures

The neuroprotective efficacy and dose response profile of the memantine against ATP depletion exposure in primary hippocampal neurons was evaluated either by visualizing survival neurons or measuring LDH release, which is an accepted

indicator of neuronal death. The data in Fig. 1A-C show that ATP depletion exposure caused a large increase in neuronal death (Ctrl: 71.0 ± 7.5, n=3; ATP: 21.7 ± 0.9, n=3; $p < 0.01$) and LDH release (Ctrl: 100 ± 5.3 %, n=4; ATP: 295.3 ± 6.4 %, n=4; $p < 0.001$). At the lowest dose, memantine (1 μM) failed to protect cultured neurons, but at the high doses, memantine (10 or 50 μM) significantly increased survival neurons to 37.6 ± 3.8 (n=4, $p < 0.05$) and 59.3 ± 2.4 (n=4, $p < 0.001$). Simultaneously, memantine treatment displayed the same profile of inhibition of ATP depletion-induced LDH release in hippocampal neurons. The lowest level of LDH release was observed for 50 μM memantine (145 ± 10.5 %, n=4) compared to the control (100 ± 5.3 %, n=4; $p < 0.001$). Based on the in vitro data, it is suggested that memantine has the ability to protect against ATP depletion-induced neuronal death in primary hippocampal cultures.

3.2. Memantine alleviates neurological deficits and reduces infarct volume after ischemic stroke

To further identify the neuroprotective effects of memantine on ischemic stroke *in vivo*, neurological deficit scores and cerebral infarct volumes were measured at 24 h after ischemic stroke. As shown in Fig. 2, the cerebral infarct volume of MCAO rats treated with memantine (MEM, 20 mg/kg, i.p.) was significantly reduced from 33.2 ± 3.7 % (n=5) in control saline-treated rats to 21.9 ± 2.0 % (n=5) ($p < 0.05$; Fig. 2E). The reduction of infarct volume was associated with a neurological improvement at 24 h after MCAO. The neurological deficit scores in the saline and MEM groups were 2.2 ± 0.2 (n=14) and 1.7 ± 0.1 (n=17), respectively, indicating that MEM significantly improved neurological symptoms in the MCAO model ($p < 0.05$; Fig. 2F). Consistent with previous reports [18, 20, 21], these data demonstrated the therapeutic efficacy of memantine against ischemic stroke *in vivo*.

3.3. Memantine inhibits calpain 1/2 over-activation and reduces MAP2 and PSD95

cleavage in the penumbra area of MCAO rats

During acute ischemic stroke, excessive glutamate induces NMDA receptor over-stimulation, which permits Ca^{2+} entry into neuronal cells. Increased intracellular Ca^{2+} leads to over-activation of calpain, which subsequently induces PSD95 and/or MAP2 cleavage. To further determine the underlying neuroprotective mechanisms of memantine in ischemic stroke, we attempted to measure the activities of calpain 1 and calpain 2, and the expression levels of MAP2 and PSD95 in ischemic penumbra by western blotting. As shown in Fig. 3a, focal cerebral ischemia significantly over-activated the expression of both calpain 1 and calpain 2 and decreased the levels of PSD95 and MAP2 at 24 h after MCAO as compared with the sham control group (n=4, $p < 0.05$, and $p < 0.001$ respectively). However, treatment with memantine significantly suppressed MCAO induced activation of both calpain 1 and calpain 2 and the degradation of PSD95 and MAP2 (n=4, $p < 0.05$, Fig. 3a(B-D)).

Among calpain substrates, MAP2 has been identified as one of the neuronal proteins most vulnerable to calpain [7]. The Ca^{2+} overload usually triggers extensive pathological calpain activation and then MAP2 degradation, which likely results in destabilization of microtubules in the cell body and dendrites [28, 29]. Histochemical studies of brain sections stained for MAP2 showed the disappearance of immunostaining signal not only in the striatum, but also in the ischemic cortex (Fig. 3b(A)). This finding was in accordance with earlier reports of MAP2 alterations [28-30]. In Fig. 3b(B-C), the representative micrographs and analytical results clearly illustrate that memantine treatment could partially, but significantly, prevent the disruption of MAP2 in penumbral regions (Area 2-6) at 24 h after MCAO, compared with the comparable MCAO area in saline group. Taken together, these results suggested that calpain-mediated cleavage are critically involved in the treatment of ischemic stroke with memantine.

3.4. Memantine attenuates cleaved caspase-3 release and cell apoptosis in MCAO

rats

Well-documented experimental evidence from both *in vitro* and *in vivo* models of stroke has clearly demonstrated that the calpain family of cysteine proteases has a critical role in apoptosis[3, 6, 7]. In addition, calpain is known to activate caspase-3, and can thereby cause caspase-3-dependent neuronal cell death [3, 7]. Herein, immunoblotting for cleaved caspase-3 showed that the levels of active caspase-3 in the penumbra were significantly elevated (n=4, $p < 0.05$) at 24 h after MCAO. In contrast, memantine treatment significantly prevented in the elevation of caspase-3 levels as compared with saline injection (saline: 134.2 ± 11.6%, n=4; MEM: 87.4 ± 1.5%, n=4; $p < 0.05$) (Fig. 4A-B). In order to examine whether memantine could suppress neuronal apoptosis in MCAO brain, TUNEL staining was also used to quantify apoptotic cells after ischemic injury and memantine treatment. The data in Fig. 4C-D show that there were massive TUNEL positive staining in both the penumbral and core regions 24 h after MCAO. Memantine treatment significantly decreased the density of TUNEL+ cells in the ischemic penumbra (Area 2: saline: 221.6 ± 35.5 /mm^2, MEM: 15.5 ± 2.6 /mm^2, $p < 0.01$; Area 3: saline: 444.3 ± 129.0 /mm^2, MEM: 85.7 ± 35 /mm^2, $p < 0.05$; Area 4: saline: 557.4 ± 110.7 /mm^2, MEM: 239.2 ± 126.2 /mm^2, $p < 0.05$) as compared with the saline control MCAO group, demonstrating the anti-apoptotic activity of memantine.

3.5. Memantine prevents brain damage after ischemic stroke

In addition, Nissl staining was also performed to identify neuronal injury in ischemic rats with and without memantine treatment. Nissl staining images in Fig. 5A show that the infarcts were clearly demarcated from the surrounding region, indicating brain tissue damage. In the present study, we have compared equivalent regions in the ischemic cortex in MEM-treated (Fig. 5B, box a-c) and saline-treated (Fig. 5B, box d-f) rats. The images demonstrated that, in non-injury area, normal neurons were arranged in an orderly fashion and had normal morphology with intact structure,

abundant cytoplasm, and a clear nucleolus. Additionally, most neurons in the damaged area appeared shrunken and deep stained, which are generally indicative of injury that can progressively lead to neuronal loss in the saline control MCAO group. These characteristic changes in the penumbra and the numbers of necrotic neurons (saline: 2.6 ± 0.2, n=5; MEM: 1.9 ± 0.1, n=8; $p < 0.05$) in coronal brain sections were markedly alleviated by MEM treatment 24 h after MCAO, indicating that neuronal injury resulting from stroke could be attenuated by memantine treatment.

3.5. Memantine suppresses ischemia-induced neuronal death in MCAO rats

Neuroprotectants capable of reducing neuronal loss in the ischemic penumbra are urgently needed for the treatment of stroke patients. Thus, we decided to evaluate the effect of memantine treatment on neuronal death during ischemic stroke. As shown in Fig. 6, there was a robust loss of neurons, measured by a specific neuronal marker, NeuN, immunostaining, in the cortex after ischemic stroke. Neuronal damage was maximally decreased in the immediate vicinity of the infarct region in MCAO rats injected with saline. The immunohistochemical images (Fig. 6A) and densitometric analysis (Fig. 6B) clearly demonstrated that memantine treatment could markedly suppress neuronal loss in the penumbral regions (Area 2: saline: 730 ± 55.6 /mm^2, MEM: 924.9 ± 22.1 /mm^2, $p < 0.001$; Area 3: saline: 625.8 ± 64.6 /mm^2, MEM: 777.2 ± 30.3 /mm^2, $p < 0.01$; Area 5: saline: 634.5 ±22.0 /mm^2, MEM: 745.4 ± 42.1 /mm^2, $p < 0.05$) 24 h after MCAO. In accordance with the immunohistochemical analysis, western blot analysis also revealed that expression of NeuN in the ischemic penumbra was significantly decreased after MCAO to 25.5 ± 7.2% (n=4, $p < 0.05$) of the sham control, but was significantly rescued by MEM treatment (65.7 ± 13.3%; n=4, $p < 0.05$) (Fig. 6C-D). Together, these results suggested that the loss of neurons caused by ischemic stroke could be attenuated by memantine treatment.

4. Discussion

In this current study, we showed that memantine, an uncompetitive NMDA receptor antagonist, protects against brain damage in ischemic stroke, and reduces cell apoptosis and neuronal death by suppressing the activation of the calpain-caspase-3 pathway in the ischemic penumbra.

NMDA receptors are composed of NR1 (constitutively present), NR2, and/or NR3 subunits. Additionally, the NR2A subunit is found mostly in synaptic NMDARs, whereas NR2B is present predominantly in extrasynaptic receptors. It is thought that over-activation of extrasynaptic NR2B might contribute to neurotoxicity, whereas synaptic NR2A might be neuroprotective. Neurotoxicity caused by over-activation of the NMDA receptor is one of the major causes of neuronal cell apoptosis after ischemic stroke. Therefore, NMDA receptors have been suggested as potential therapeutic targets [3, 15, 31]. Although several NMDAR antagonists have been found to be neuroprotective in animal models of stroke [10, 32, 33], previous clinical trials have met with limited success because of the narrow therapeutic window, clinically intolerable side effects, poor clinical trial design, and even the lack of efficacy [1, 34, 35]. Recently, clinical and preclinical data have indicated that memantine, an FDA approved drug for AD treatment, may be a viable pharmacological agent for therapeutic intervention in reducing brain damage in stroke [17, 19, 36-38].

NMDA receptors are ligand- and voltage-gated ion channels activated by glutamate, co-agonist glycine, and cell membrane depolarization to remove the magnesium (Mg^{2+}) block. It has been well established that NMDARs are essential for both physiological and pathological activity in the nervous system. In order to be acceptable in clinical, anti-excitotoxic drugs must block excessive activation of NMDARs while preserving normal synaptic activity to avoid side effects. Memantine, an uncompetitive NMDA receptor antagonist with low-affinity/fast off-rate, has the potential to preferentially block higher, pathological levels of glutamate over normal, physiological levels. Furthermore, memantine selectively binds to a site overlapping

the Mg^{2+}-blocking site of the NMDA receptor only when channel is open, and it rapidly dissociates from the receptor as soon as the ion channel is closed [39-41]. Owing to these properties, memantine can preferentially block excessive NMDA receptor activity without accumulating in the ion channels to disrupt normal physiological activity, which may explain the limited side effects in patients treated with memantine [10, 38, 42]. In the present study, our results showed that memantine has the ability to promote the survival of primary hippocampal neurons, and markedly reduced neurological deficits and cerebral infarct volume at 24 h after MCAO when delivered immediately after ischemia. The results are in line with other work, in which memantine was administered before, and sometimes within the first 2 hours after ischemia [18, 20, 21, 37].

During ischemic stroke, excessive Ca^{2+} influx activates a cellular cascade that includes calpain activation and caspase release, which may contribute to cell necrosis or apoptosis [4, 5]. Calpain is a calcium-dependent cysteine protease that plays important roles in both physiological and pathological conditions [43, 44]. Numerous studies [3, 7] over the years have shown that activated calcium-bound calpain cleaves postsynaptic cytoskeletal and scaffolding proteins, thereby promoting reorganization of the PSD and synaptic remodeling. Both MAP-2 and PSD-95 are two major substrate proteins that undergo limited proteolysis by over-activated calpain [7]. In the present study, we observed that over-activation of calpain 1/2 was inhibited, and degradation of PSD95 and MAP2 was significantly reduced by treatment with memantine, when compared with saline-treated group. This demonstrates a critical role for calpain-mediated cleavage in response to memantine treatment after ischemic stroke.

Additionally, previous studies identified that calpain may be responsible for cleaving of caspase-3, which is believed to be a final step in apoptosis. During acute cerebral ischemia, calpain and caspase-3 are over-activated and contribute to neuronal death, which have been revealed through investigating the neuroprotective

mechanisms of calpain and caspase-3 inhibitors [43-46]. It's well known that cell survival in the core of the cerebral infarction in the penumbra are key prognostic factors [47]. Herein we found that treatment with NMDA antagonist memantine could markedly attenuate the amount of active caspase-3 and ischemic cell apoptosis in the penumbra (Area 2-4) after stroke. No significant change in the core area (Area 5-8) was observed between the vehicle and treatment groups. Furthermore, memantine significantly suppressed neuronal loss in the same penumbral area of MCAO rats. These data indicated that memantine may attenuate neuronal apoptosis and death by suppressing the activation of the calpain-caspase-3 pathway.

From the foregoing discussion, it is clear that prevention of over-activation of the NMDA receptors mediated excitotoxicity is an important step to protect against apoptosis after stroke. Calpain-caspase-3 pathway, activated by calcium influx through the NMDA receptors to induce neuronal death, is one of the most important apoptotic signaling pathways. However, ischemic stroke could trigger a complex sequence of biochemical and molecular mechanisms that work synergistically to induce neuronal death. There are two general pathways involved in apoptosis: the intrinsic and the extrinsic pathways. Although many of the key apoptotic regulatory molecules have been identified, our understanding of the underlying mechanisms of memantine in treating ischemic stroke remains a daunting task.

5. Conclusions

In summary, the results of the present study demonstrated that memantine could significantly block over-stimulated NMDARs to antagonize calpain-caspase3 pathway, reduce MAP2 and PSD95 cleavage, and attenuate neuronal apoptosis and death, reducing neurological deficits and infarct volume in MCAO rats. All together, these results provide a molecular basis for the role of memantine in reducing neuronal apoptosis and protecting brain damage after cerebral ischemia, suggesting that memantine may be a potential pharmacological agent in the treatment of ischemic

stroke.

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Author Contributions

Bin Chen, Yun Wang and Lidian Chen conceived the idea and designed the experiments. Bin Chen, Guoxiang Wang and Ruhui Lin performed the experiments and interpreted the experimental results. Weiwei Li, Min Jiang and Jing Tao participated in the study design and data analysis. Bin Chen, Weilin Liu, Yun Wang and Lidian Chen wrote the manuscript. All authors read and approved the current version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Memantine protects neurons against ATP depletion-induced neuronal death in primary hippocampal cultures

(**A-B**) Representative micrographs and quantification of hippocampal neurons stained with a NeuN immunostaining. After exposed to ATP depletion, the hippocampal neurons were treated with or without memantine (1, 10 and 50 μM) for 24 h. Enlarged versions of the areas indicated by white squares. **(C)** Quantification of neuronal death measured by LDH release. All data are expressed as mean ± SEM. n = 3~4 for each group. $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. Ctrl group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ATP-D group. Ctrl, control; ATP-D: ATP depletion; MEM, memantine.

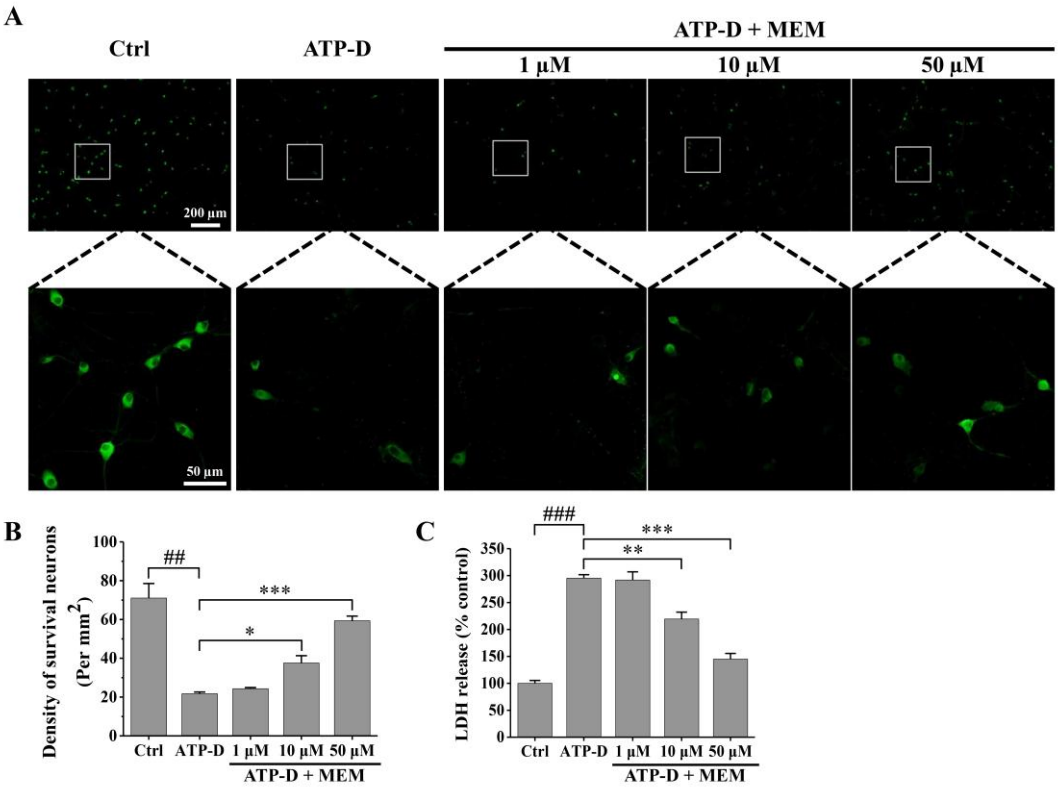


Fig. 2. Memantine alleviates neurological deficits and reduces infarct volume after ischemic stroke

(**A**) Cerebral blood flow in the ischemic brains as measured using a laser Doppler perfusion monitor. MCAO that immediately decreased the cerebral blood flow to 20% of the base value were regarded as a successful. (**B**) Standard lines for rat brain slices in TTC staining. (**C**) Calculation formula of TTC-defined infarct volume from rat brains. In the formula, *A*: infarct lesion; *B*: Contralateral region; *C*: ipsilateral intact region. (**D**, **E**) Representative photographs of TTC-stained brain slices (2 mm), and quantitative results of infarct volume (n = 5) at 24 h after MCAO. TTC-stained red color indicates normal region, and the white color indicates an infarct lesion. (**F**) Neurological deficit scores of the saline group (n=14) and MEM group (n=17) 24 h after MCAO. All data are represented as mean ± SEM. * $p < 0.05$, vs. saline group. CBF, cerebral blood flow; MCAO, middle cerebral artery occlusion.

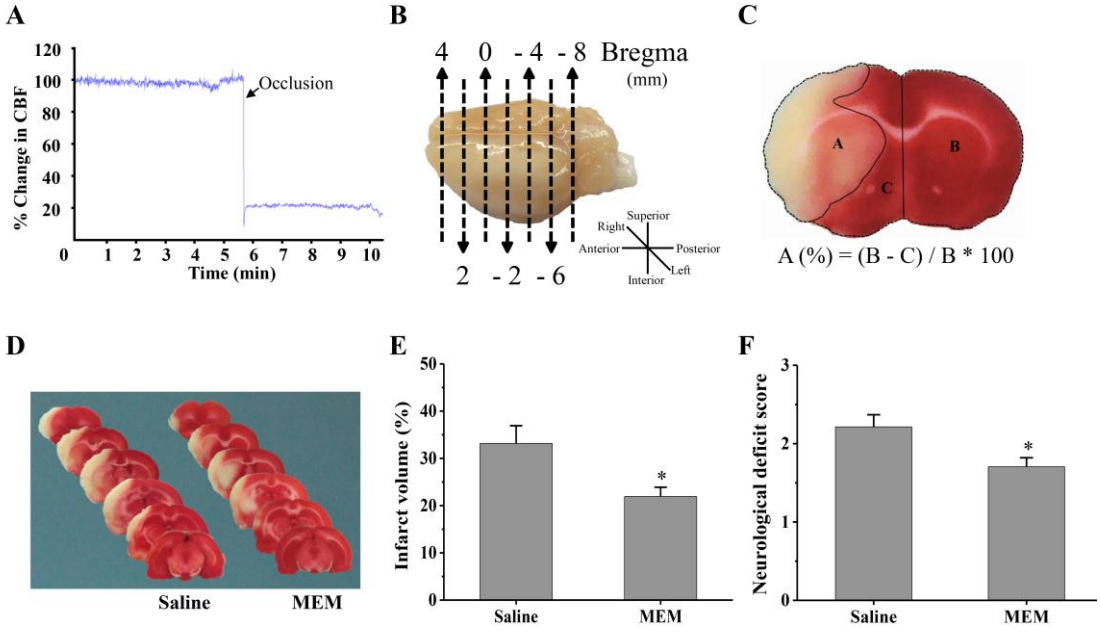


Fig. 3. Memantine inhibits calpain 1/2 over-activation, and reduces MAP2 and PSD95 cleavage in the penumbra area of MCAO rats

Fig. 3a. (**A**) A schematic diagram of the ischemic penumbra and core in MCAO rats. (**B**, **C**) Western blot micrographs of calpain 1/2, PSD95, and MAP2 in the penumbra after ischemic stroke. (**D**, **E**) Quantitative analysis of the western blots. GAPDH was used as the internal control. The results are shown as a percentage of the levels in sham rats. Data are expressed as mean ± SEM. n = 4 for each group. $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ vs. sham group; * $p < 0.05$ vs. saline group.

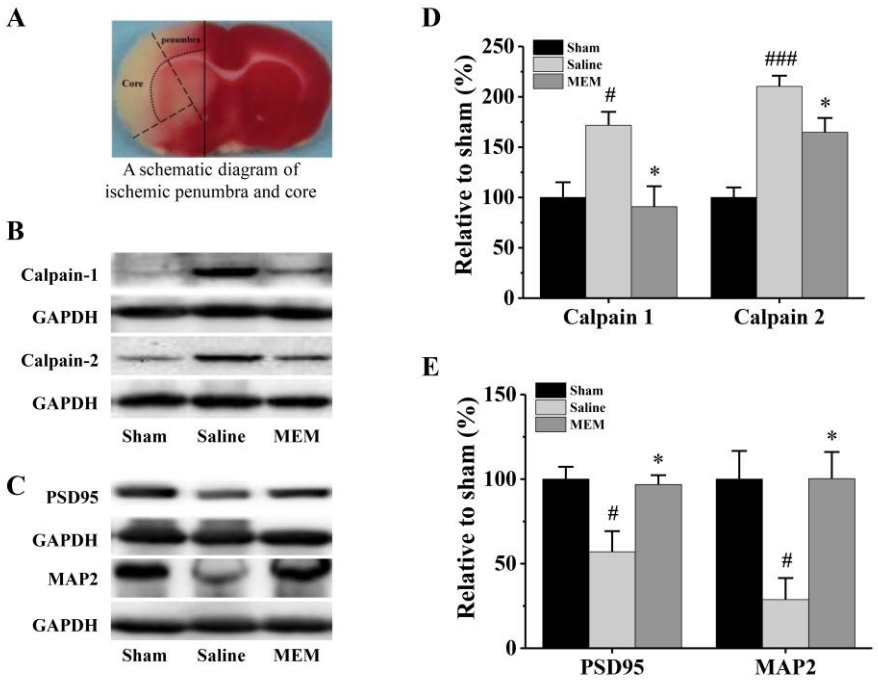


Fig. 3b. (**A**) A schematic drawing of the rat ischemic cortex, divided into 8 regions from the midline for immunohistochemical analysis. (**B**) Representative images of MAP2 immunostaining from saline and MEM-treated groups in different regions (Area 1-8). Scale bar = 50 μm. (**C**) Quantitative analysis of MAP2 intensity in Area 1 to Area 8. Data expressed as the mean ± SEM from 5 (saline group) and 8 (MEM group) individual rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline group

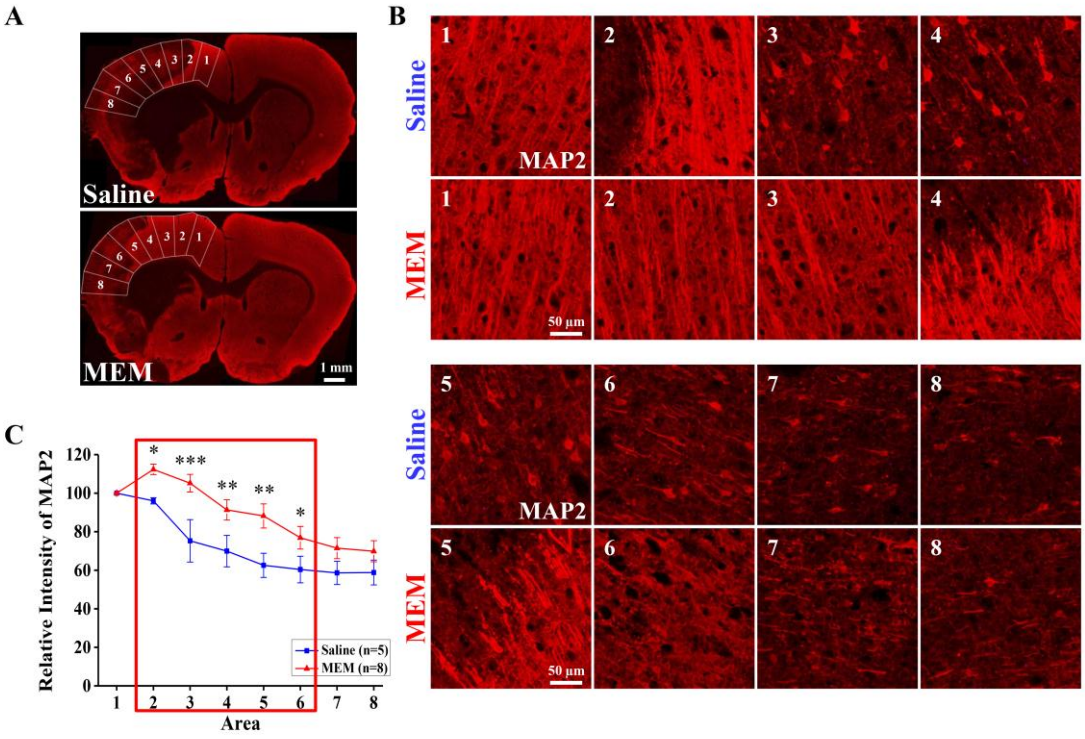


Fig. 4. Memantine attenuates cleaved caspase-3 release and cell apoptosis in MCAO rats

(**A, B**) Representative blot and quantification of cleaved caspase-3 in the ischemic penumbra 24 h after MCAO. n = 4 for each group. (**C**) Micrographs of TUNEL staining in different ischemic regions (Area 1-8) in MCAO rats injected with saline and memantine. Scale bar = 50 μm. (**D**) Summary of the density of TUNEL+ cells. Data shown as the mean ± SEM from 5 (saline group) and 8 (MEM group) individual rats. $^{\#}$ $p < 0.05$ vs sham group; * $p < 0.05$, ** $p < 0.01$ vs. saline group.

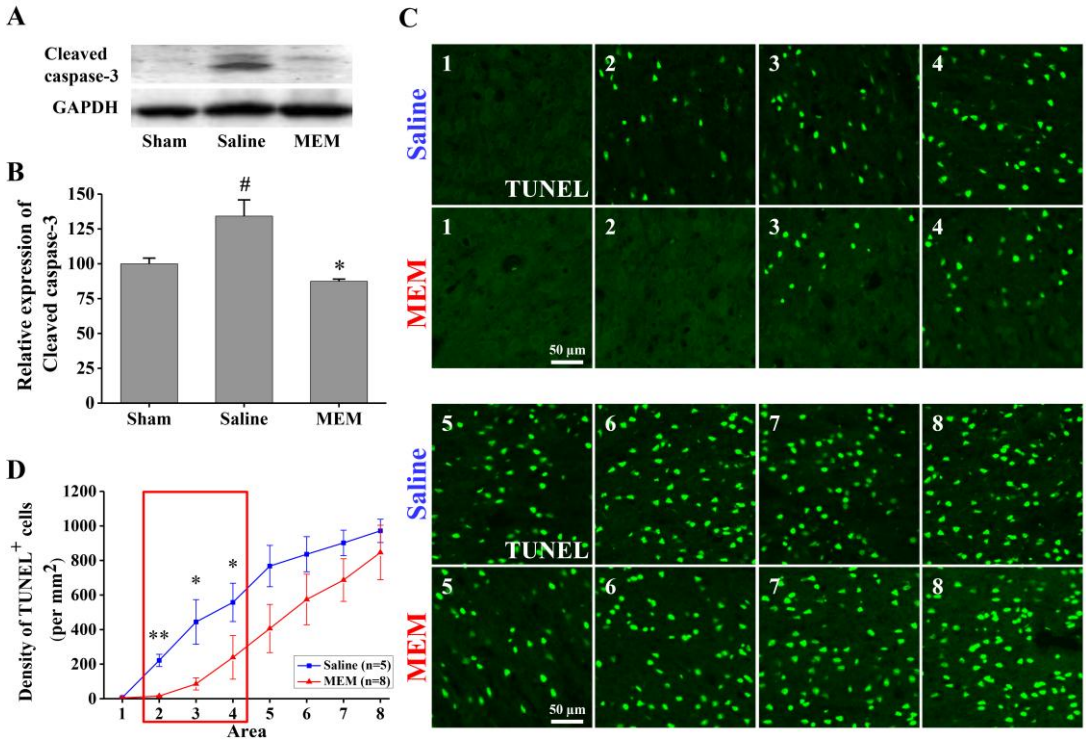


Fig. 5. Memantine prevents brain damage after ischemic stroke

(**A**) Representative equidistant series of Nissl-stained coronal sections, 1 mm apart, showing the brain damage of rats with injections of saline or memantine after ischemic stroke. The white lines outline the infarct regions. (**B**) Higher magnification of insets is indicated by white squares in A (Ⅰ , Ⅱ , Ⅲ, Ⅳ, Ⅴ and Ⅵ). Red triangles indicate necrotic neurons that appeared shrunken and deeply stained, whereas black arrows are normal neurons. Scale bars indicate 200 μm or 50 μm as labeled. (**C**) Quantitative results of the histological deficit score from rats with saline or memantine treatment at 24 h after MCAO. Data shown as the mean ± SEM from 5 (saline group) and 8 (MEM group) individual rats. * $p < 0.05$ vs. saline group.

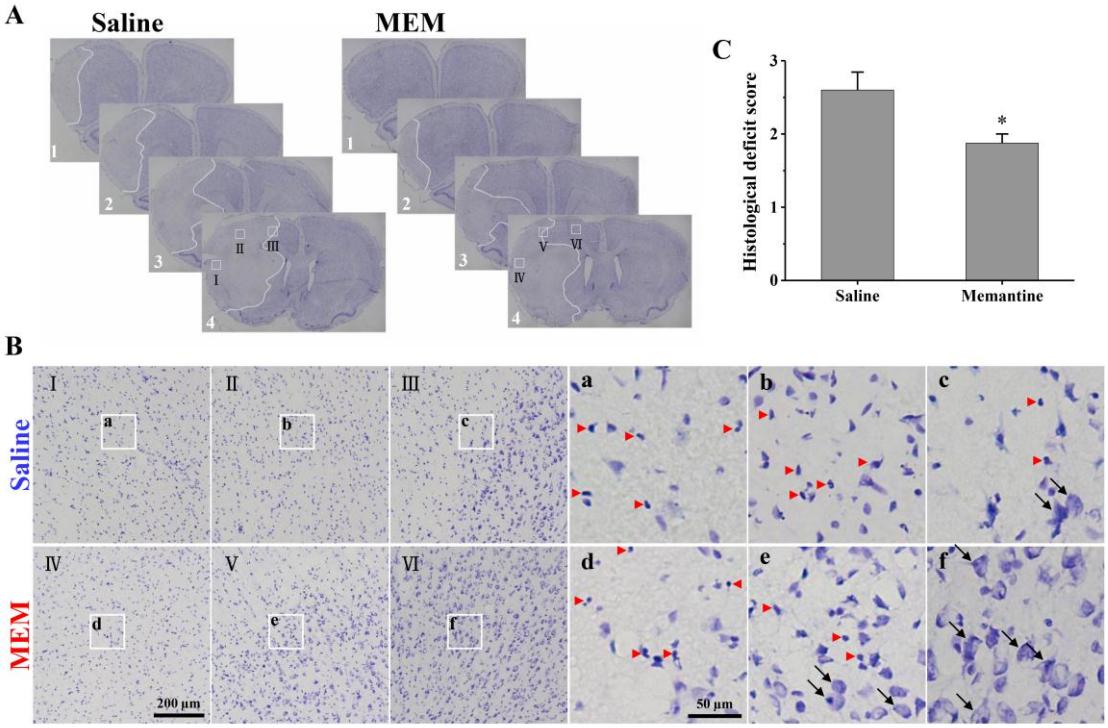
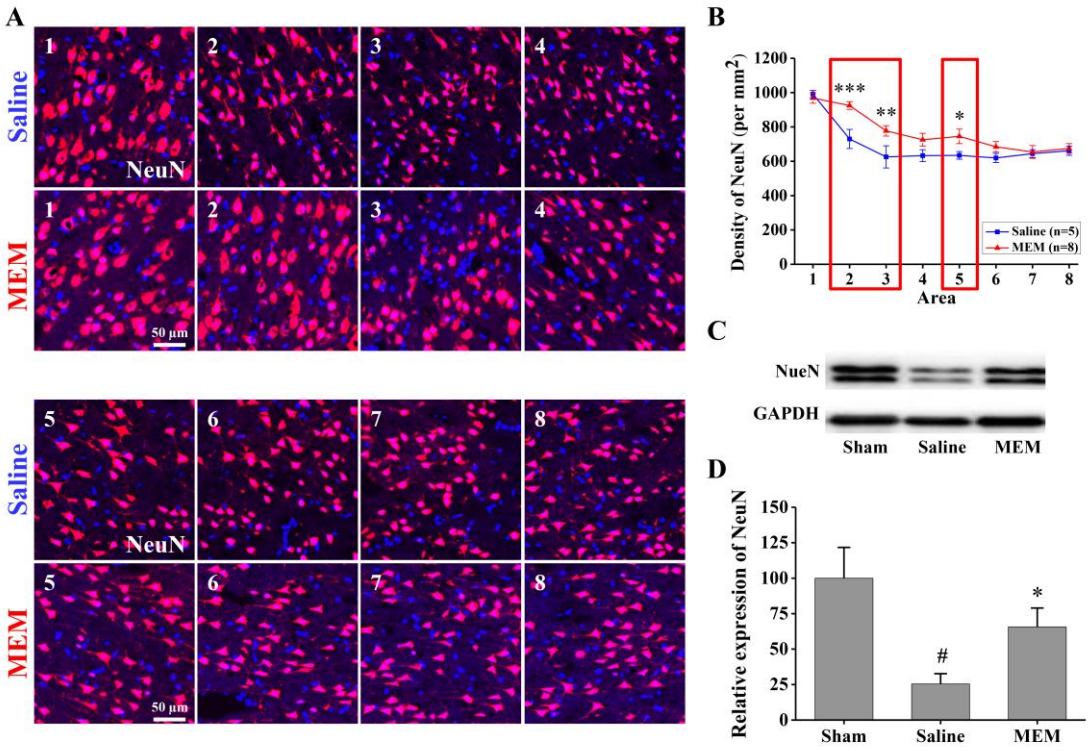
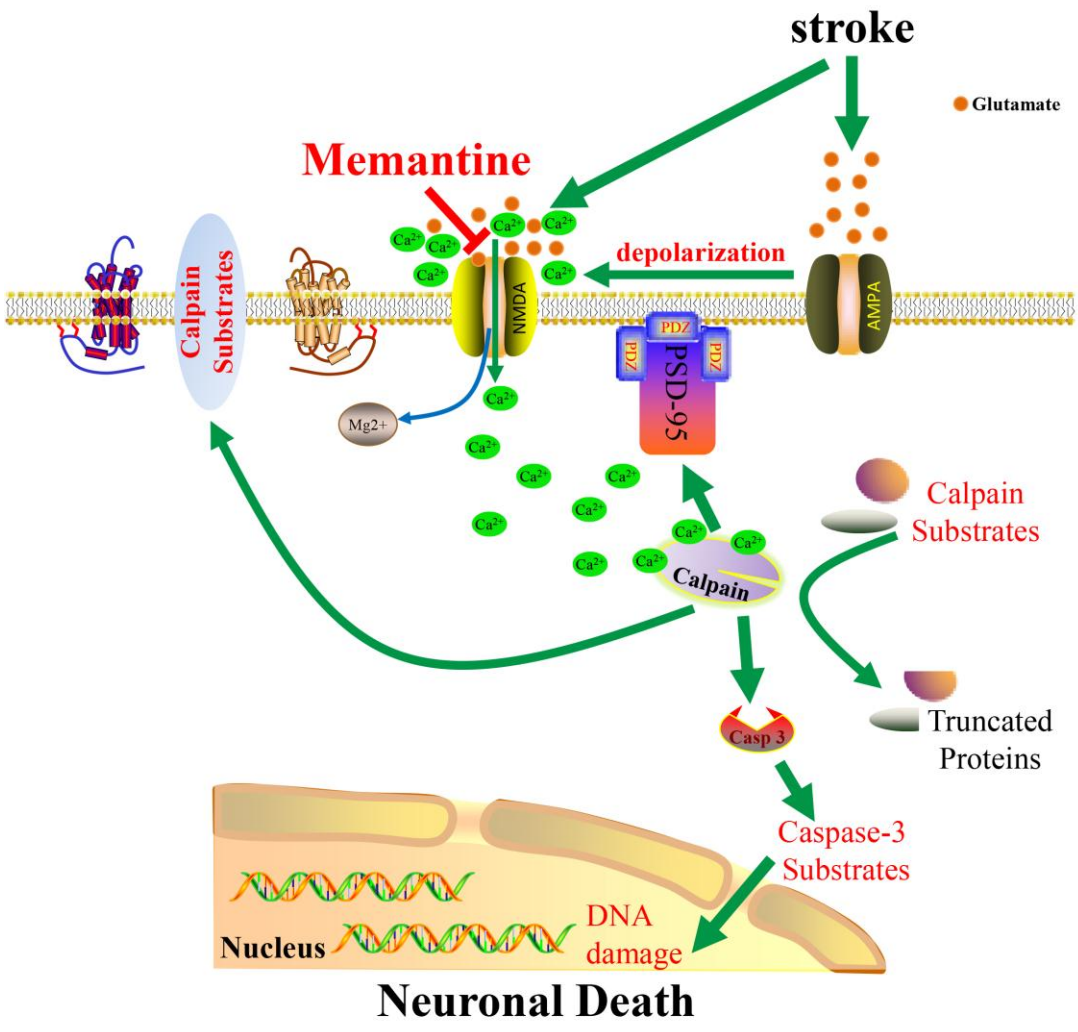


Fig. 6. Memantine suppresses ischemia-induced loss of neurons in MCAO rats (**A**) Representative images of immunohistochemical staining of a specific neuronal marker (NeuN) in saline and MEM-treated groups in different regions (Area 1-8). Nuclei are counterstained with DAPI (blue). Scale bar = 50 µm. (**B**) Quantitative analysis of NeuN+ density in Area 1 to Area 8. (**C, D**) The protein expression levels of NeuN measured by western blot 24 h after MACO. n = 4 for each group. GAPDH was used as an internal control. The results are presented as percentage of the levels in sham-operated rats. All data are expressed as mean ± SEM. $^{\#}$ $p < 0.05$ vs sham group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline group.



Graphical Abstract. Schematic illustration of memantine-mediated regulation of neuronal apoptosis through modulating the calpain-caspase-3 pathway after ischemic stroke

During acute ischemic stroke, excessive release of glutamate causes AMPA receptor over-stimulation to remove the Mg^{2+} block on NMDA receptor, which permits large amounts of Ca^{2+} to enter the cell. The increased intracellular Ca^{2+} leads to over-activation of calpain, subsequent PSD95 and MAP2 cleavage, and caspase-3 release, eventually contributing to cellular damage and death by necrosis or apoptosis. We have demonstrated that memantine, an uncompetitive NMDA receptor antagonist, blocks excessive NMDA receptor activity and Ca^{2+} influx which, in turn, prevents further cellular damage.



Highlights

Memantine protects against ATP Depletion-induced neuronal death in cultured neurons

Memantine also alleviates neurological deficits and infarct volume in MCAO rats

Memantine suppresses the activation of calpain-caspase-3 pathway in MCAO rats

Memantine reduces calpain-mediated cleavage of PSD95 and MAP2 in the ischemic penumbra

Memantine attenuates cell apoptosis in the penumbral area of MCAO rats