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Ginsenoside Rg_1 protects neurons from hypoxic–ischemic injury possibly by inhibiting Ca^{2+} influx through NMDA receptors and L-type voltage-dependent Ca^{2+} channels

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Abstract

The purpose of this study is to assess the neuroprotective effect of Rg_1, a ginsenoside. We measured cell viability and lactate dehydrogenase (LDH) release from primary culture of rat hippocampal neurons and electrical activities in hippocampal slices of rats, before and after the neurons were deprived of oxygen and glucose. In addition, cerebral damage was evaluated with magnetic resonance imaging after middle cerebral artery was occluded transiently. Nissl staining was used for histological observation and immunohistochemistry analysis for activated caspase-3 expression of the brain. Furthermore, calcium influx was measured with laser confocal microscopy in neurons perfused with KCl (50 mM) or *N*-methyl-D-aspartate (NMDA, 1 mM), or deprived of oxygen and glucose. The influences of ginsenoside Rg_1 on these parameters were determined simultaneously. We found that treatment of Rg_1: 1) increased the neuronal viability; 2) promoted the recovery of electrical activity in hippocampal slices; 3) reduced the release of LDH, cerebral damage area, neuronal loss and expression of caspase-3; and 4) inhibited calcium influx induced by NMDA, KCl or oxygen/glucose deprivation. However, the protective effect of Rg_1 was blocked by mifepristone, an antagonist of glucocorticoid receptors. Taken together, these results suggest that ginsenoside Rg_1 can reduce neuronal death, including apoptotic cell death, induced by hypoxic–ischemic insults. This neuroprotective effect is probably mediated by the activation of glucocorticoid receptors, and by the inhibition of calcium influx through NMDA receptors and L-type voltage-dependent Ca^{2+} channels and the resultant reduction of intracellular free Ca^{2+}.

Keywords: Ginsenoside Rg_1; Neuroprotection; Focal cerebral ischemia/reperfusion; NMDA receptors; L-type voltage-dependent calcium channels; Calcium overload

1. Introduction

Although ischemic cerebral stroke has become one of the leading causes of human death and disability in the world, the treatment of stroke remains much unsatisfactory. Almost all neuroprotective agents proved to be promising in animal stroke experiments were disappointing in clinical human trials (Cheng et al., 2004; Curry, 2003; Gladstone et al., 2002; Jonas et al., 2001), but the recent success of "Fast-Mg^{2+}" therapy (Saver et al., 2004) has stimulated our interests in searching for new neuroprotective agents for cerebral stroke management.

Panax ginseng C.A. Meyer (Araliaceae), a well-known herb in Traditional Chinese Medicine has been used widely for thousands of years, and ginsenosides or ginseng saponins are popularly considered as one of the principal bioactive ingredients (Kiefer and Pantuso, 2003). Up to now, over thirty different ginsenosides have been identified from ginseng. They are classified into three subclasses: panaxadiol, panaxatriol and oleanolic acid type ginsenosides (Attele et al., 1999). Ginsenoside Rg_1 belongs to the panaxatriol ginsenosides family. Growing evidence indicates a neuroprotective effect of total saponins or a part of single ginsenosides in several experimental

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models such as excitotoxicity insults, traumatic brain injury and cerebral ischemia (Ji et al., 2005; Van Kampen et al., 2003; Kim et al., 1998, 2007; Lee et al., 2002; Leung et al., 2007; Liao et al., 2002; Lim et al., 1997; Tian et al., 2005; Wen et al., 1996; Zhang and Liu, 1996). To search for effective neuroprotective agents, we have compared the neuroprotective effects of 9 single ginsenosides (Rb_1, Rb_3, Rd, Re, Rg_1, Rg_2, Rh_1, Rh_2, F_{11}) in hippocampal slices, and found that Rb_1, Rb_3, Rg_1, Rh_2, and F_{11} could protect hippocampal neurons and alleviate neuronal injury induced by oxygen/glucose deprivation (Jiang et al., 2000, 2001; Jiang and Jiang, 2003). The neuroprotective effect of ginsenoside Rg_1 has been investigated recently by other researchers in several cell-damaging models (Chen et al., 2003; Leung et al., 2007; Li et al., 1997; Li and Zhang, 1997; Liao et al., 2002; Liu and Zhang, 1995; Rudakewich et al., 2001), but less in hypoxic–ischemic conditions. Moreover, the mechanisms underlying the neuroprotective effect of Rg_1 are still unclear.

A number of reports have shown that ginsenosides could modulate the functions of many receptors and ion channels on neurons, including *N*-methyl-D-aspartate (NMDA) receptors (Kim et al., 2002, 2004; Kim and Rhim, 2004; Lee et al., 2006a,b) and Ca^{2+} channels (Rhim et al., 2002). Due to the central involvement of the activation of NMDA receptors and calcium overload in ischemic brain injury (Berliocchi et al., 2005), the current investigation was designed mainly to assess the inhibitory influence of ginsenoside Rg_1 on Ca^{2+} influx evoked by oxygen/glucose deprivation and potential involvements of NMDA receptors and L-type voltage-dependent Ca^{2+} channels.

2. Materials and methods

2.1. Animals and chemicals

Sprague–Dawley rats were obtained from the Experimental Animal Center of Nantong University, Nantong, China. All procedures used in this study were in accordance with our institutional guidelines, which comply with international rules and policies. Ginsenoside Rg_1, extracted from the stem and leaf of Jilin *P. ginseng*, was purchased from the Department of Organic Chemistry, Medical School of Jilin University, Changchun, China, and the purity (HPLC analysis) was 98.2% (Lot # 20060208). Common inorganic salts were purchased in China, culture mediums were purchased from Invitrogen Corporation (Carlsbad, USA), Fluo 3-AM from Dojindo Laboratories (Kumamoto, Japan), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) from BDH Chemicals Ltd. (Poole, England). Mifepristone (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one, RU486), cytosine β-D-arabinofuranoside, sodium dodecylsulphate, and other chemicals except those indicated elsewhere were purchased from Sigma-Aldrich Corporation (Saint Louis, USA).

2.2. Cell culture

Hippocampi were removed from embryonic rats (E17-18). Briefly, hippocampal cells were isolated and plated immediately unto 96- and 24-well plates. These plates were coated with poly-L-lysine overnight prior to the experiment. Cells were grown in the plating medium with 85% high glucose Dulbecco's Minimum Essential Medium (DMEM, 12100-046), 15% fetal bovine serum (Hyclone, Logan, USA), and 10^6 U/l penicillin–streptomycin. Six hours later, the plating medium was changed with the feeding medium with 98% neurobasal medium (21103-049), 2% B27 (17504-044), 2 mM glutamine, and 10^6 U/l penicillin–streptomycin. The day of plating was counted as day-in-vitro 0. On day-in-vitro 3, cytosine β-D-arabinofuranoside (5 μM) was added to suppress the proliferation of glial cells. The feeding medium was changed every 3 days. Cultures were maintained in a 5% CO_2 incubator at 37 °C.

2.3. Hippocampal slice preparation and recording of orthodromic population spikes

Male rats (120–150 g body weight) were anesthetized with ether and then sacrificed by decapitation. Each brain was rapidly removed and dissected in cold (0–4 °C) artificial cerebrospinal fluid (aCSF) containing (in mM) NaCl 124, $NaHCO_3$ 25.7, KCl 3.3, $MgSO_4$ 2.4, $CaCl_2$ 2.4, NaH_2PO_4 1.24, Glucose 10.0, pH 7.35–7.45, and saturated with 95% O_2–5% CO_2 (oxygenated). Transverse hippocampal slice, 400 μm thick, was made with a vibroslicer (MA 752, Campden Instruments Ltd., Loughborough, England) and placed into a recording well with temperature maintained at 32±0.5 °C, and the upper surface of the slice was submerged 2 mm under the oxygenated aCSF. The slice was continuously perfused with oxygenated aCSF at a flow rate of 1.5–2 ml/min. One hour later, the orthodromic response in area CA_1 of each slice was elicited by stimulating CA_3 Schaffer collaterals at a frequency of 0.1 Hz through a bipolar electrode with square wave pulses of 0.1 ms duration and 0.2–0.7 mA intensity. Orthodromic population spikes were recorded in the CA_1 pyramidal layer of the hippocampal slice at a depth of about 0.1 mm under the cut surface with a glass microelectrode. The glass microelectrode had a resistance of 3–5 MΩ when filled with 2 mM NaCl solution. Orthodromic population spikes were recorded by an X–Y recorder, each trace was the average of 4 responses. Only the slice with the orthodromic population spike amplitude of 3 mV or greater was utilized for further testing.

2.4. Oxygen/glucose deprivation

Oxygen/glucose deprivation was carried out in three experiments: two measurements of cell damage and Ca^{2+} influx in cultured hippocampal neurons and the recording of electrical activity in hippocampal slices. For the measurement of cell damage, primarily cultured hippocampal neurons on days-in-vitro 12–13 were rinsed twice with extracellular solution containing (in mM): NaCl 145, KCl 3, HEPES 10, $CaCl_2{\cdot}2H_2O$ 3, $MgCl_2{\cdot}6H_2O$ 2, glucose 8, incubated in glucose-free DMEM (11966-025) at 37 °C and placed in an incubator containing 94% N_2, 5% CO_2 and 1% O_2 for 5 h. The neurons were then fed with high glucose DMEM, and returned to the normal incubator for a 24-h recovery. Those neurons for control underwent the same procedure except the oxygen/glucose

deprivation treatment. In addition, oxygen and glucose were deprived for 14 min and 10 min respectively, during the recording of electrical activity in hippocampal slices and the measurement of Ca^{2+} influx in cultured hippocampal neurons, by perfusion with aCSF and extracellular solution saturated with 95% N_2–5% CO_2. Glucose in these two solutions was substituted with sucrose.

2.5. Measurements of cell damage

Cell viability was assessed with the MTT method. Briefly, 24 h after the oxygen/glucose deprivation treatment, the culture medium was removed from the 96-well plate, the neurons were washed twice with phosphate-buffered saline, and the MTT solution of 25 μl was then added into each well (final concentration 1 g/l). Following incubation at 37 °C for 4 h, 100 μl of 20% sodium dodecylsulphate solution (dissolved in dimethylformamide) was mixed into each well to dissolve the resultant dark blue crystal for 20 h. Absorbance of each well was measured at a wavelength of 570 nm (OD 570) with the Universal Microplate Reader (ELx 800, BioTek instruments, Inc., Winooski, USA).

Neuronal damage was also evaluated by measurement of lactate dehydrogenase (LDH) released into the culture medium. LDH activity was determined according to the protocol of an LDH kit (Jiancheng Bioengineering Institute, Nanjing, China). At the end of various treatments, 0.4 ml of medium was mixed with 1.3 ml nicotinamide adenine dinucleotide (NADH) solution and 1.3 ml sodium pyruvate solution. Both agents were dissolved in potassium phosphate buffer (100 mM K_2HPO_4, adjusted to pH 7.5 with KH_2PO_4). The mixed solution was immediately assayed with a UV-2450 visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by monitoring the conversion of NADH to NAD at 340 nm at 37 °C, coupled with the reduction of pyruvate to lactate. LDH activity is expressed as units/ml, with one unit of activity representing the amount of LDH that causes a decrease of 0.001 absorbance unit of NADH per minute in the presence of sodium pyruvate.

2.6. Surgery of middle cerebral artery occlusion

Male rats (230–270 g body weight) were anesthetized using 2 ml enflurane in an ether jar, and maintained with 10% chloral hydrate (400 mg/kg, i.p.). The middle cerebral artery was occluded with a 4-0 silicone-coated nylon suture by surgical operation as described by Gerriets et al. (2003). Reperfusion was induced 2 h after middle cerebral artery occlusion by filament withdrawal. Sham-operated animals were subjected to the same surgical procedure, but the suture was not advanced beyond the internal carotid bifurcation. Rectal temperature of rats was maintained at 37 °C throughout the anesthetic period including the surgical procedures using a temperature-regulated heating pad. After revival from anesthesia, animals were housed back at room temperature (24±1 °C), with free access to food and tap water.

Neurological evaluation was performed at both 2 h and 24 h after reperfusion of the middle cerebral artery and scored on a 6-point scale: 0, no neurological deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, inability to bear weight on the left; 4, no spontaneous walking with depressed level of consciousness; and 5, death.

2.7. Magnetic resonance imaging (MRI)

A subgroup of rats which were subjected to middle cerebral artery occlusion was examined with MRI in a 3.0 T scanner (Signa Excite HD 3.0T, GE Healthcare, Connecticut, USA). An actively shielded gradient coil with a 120-cm inner diameter driven by the standard gradient power supply was applied. In this configuration, 180 mT/m could be reached in 180 ms. As a rapid frequency coil, we adopted a home-made birdcage resonator with a 125-mm inner diameter. MRI examination was performed at 4 h and on days 1, 2 and 5 after middle cerebral artery occlusion, respectively. For each animal, a T_2-weighted MRI with a FRFSE sequence (repetition time=5200 ms, echo time=85 ms, number of averages=2, field of view=4.0 cm×4.0 cm, matrix=320×288, 6 slices, slice thickness=2 mm) was performed. Image data of T_2-weighted MRI were analyzed with Image-Pro Plus 5.1. We calculated the damage size as described by Heiland et al. (1997). The ischemic damage on T_2-weighted MRI was defined as hyperintense areas on the image with the highest T_2 weighting (echo time=96 ms). The lesion area was subdivided into cortical and subcortical parts according to neuroanatomic landmarks.

2.8. Nissl staining and immunohistochemistry

After 2-h ischemia and 22-h reperfusion, rats were anesthetized with 10% chloral hydrate (400 mg/kg, i.p.) and perfused with 200 ml saline and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer saline (pH 7.4). Rat brains were then removed and post-fixed for 24 h in the same fixative. The post-fixed brains were dry-protected in 25% sucrose in phosphate-buffered saline.

For Nissl staining, the brain tissue was then sectioned coronally 20 μm in thickness from 2.3 mm posterior to the bregma with a cryostat slicer (CM1900, LAICA, Bensheim, Germany). Six sections were collected by selecting one slice from every 6 slices. These sections were mounted with neutral balata and blotted onto slides before being processed through different baths in the following order (and time): chloroform (30 min), acetone (15 min), 100% ethanol (30 s), 95% ethanol (30 s), 70% ethanol (30 s), distilled water (30 s, twice), cresyl violet (20 min), distilled water (30 s, three times), 70% ethanol (1 min), 95% ethanol (1 min), 100% ethanol (1 min), chloroform (5 min), differentiator (95% ethanol, added glacial acetic acid till pH was 4.1) (6 min), 95% ethanol (2 min), 100% ethanol (3 min, twice), xylene (2 min), xylene (3 min, twice), and then covered with a coverslip.

For immunohistochemical assay of neuronal expression of the activated caspase-3, the rat brain was immersed in paraffin and then embedded. The paraffin-embedded brain was coronally sectioned 5 μm in thickness from 2.3 mm posterior to the bregma. Six sections were collected by selecting one from every 10 slices. As the primary antibody, the rabbit polyclonal antibody against cleaved caspase-3 (Asp175, Cell Signaling Technology Inc.,

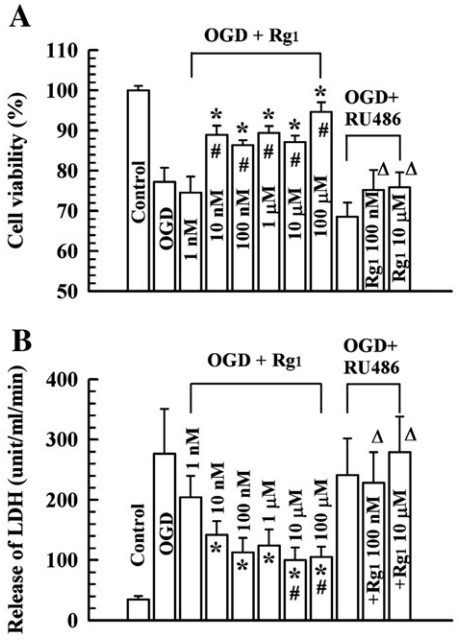

Fig. 1. Ginsenoside Rg_1 elevates cell viability and reduces LDH release of cultured hippocampal neurons subjected to oxygen/glucose deprivation (OGD). A, cell viability ($n=12$). B, release of LDH ($n=6$). RU486, an antagonist of glucocorticoid receptors significantly attenuated the effect of Rg_1. *, $P<0.05$, OGD+Rg_1 *vs* OGD; #, $P<0.05$, *vs* that of Rg_1 at 1 nM; Δ, $P<0.05$, *vs* that of Rg_1 without RU486. Note that RU486 (10 μM) itself did not induce significant changes in cell viability and LDH release in OGD-treated neurons.

Boston, USA) was used at 1:100 dilution. The secondary antibody was biotin-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA, USA). An ABC kit (Zhongshan Biotechnology Company, Beijing, China) was adopted to localize the secondary antibody. Finally, a diaminobenzidine kit (Zhongshan Biotechnology Company) was applied to visualize the catalyzed peroxidase-reaction product. Simultaneously, negative control was performed without the primary antibody.

2.9. Intracellular Ca^{2+} imaging

For intracellular free Ca^{2+} imaging, the acetoxymethyl-ester form of Fluo 3 (Fluo 3-AM) was used as a fluorescent Ca^{2+} indicator. The cultured hippocampal neurons on day-in-vitro 13 were loaded with 5 μM Fluo 3-AM, which was added in extracellular solution for 45 min at 37 °C. After being washed three times with normal extracellular solution, the neurons were incubated at 37 °C for another 30 min to complete the deesterification of Fluo 3-AM. The intensity of fluorescence with the excitation wavelength at 485 nm and emission wavelength at 525 nm was recorded every 10 s using a laser scanning confocal microscope (TCS SP2, Leica Microsystems, Heidelberg, Germany). All image data were collected and analyzed with the Leica Control software of the microscope. The increase of intracellular free Ca^{2+} was determined according to the following equation: Ca^{2+} influx (%)$=(F_{525}-F_{base,\ 525})/F_{base,\ 525}\times 100$, where F_{525} is the fluorescence intensity we measured after each treatment, and $F_{base,\ 525}$ the basal fluorescence intensity. High extracellular K^+ solution containing (in mM): 98 NaCl, 50 KCl, 10 HEPES, 3 $CaCl_2{\cdot}2H_2O$, 2 $MgCl_2{\cdot}6H_2O$, 8 glucose was used for the induction of Ca^{2+} influx through the L-type Ca^{2+} channels.

2.10. Application of Rg_1 and RU486

Ginsenoside Rg_1 was dissolved in dimethyl sulphoxide as a concentrated stock, diluted with normal saline, extracellular solution or culture medium as needed just before use. In *in vivo* experiments, Rg_1 was given intraperitoneally (20 mg/kg) 1 h before middle cerebral artery occlusion and repeated every 12 h until each experiment was completed. In *in vitro* experiments, Rg_1 was applied to cultured hippocampal neurons simultaneously with high KCl or NMDA-containing extracellular solution, or immediately before oxygen/glucose deprivation. For hippocampal slices, Rg_1 was administered to bath solution 10 min before oxygen/glucose deprivation and maintained until 15 min after the recovery of oxygen/glucose supply. RU486, an antagonist of glucocorticoid receptors, was also dissolved in dimethyl sulphoxide as a stock, diluted with cell culture medium before application. RU486 (final concentration of 10 μM) was administered to neurons 30 min before oxygen/glucose deprivation. The final concentration of dimethyl sulphoxide was 0.1%.

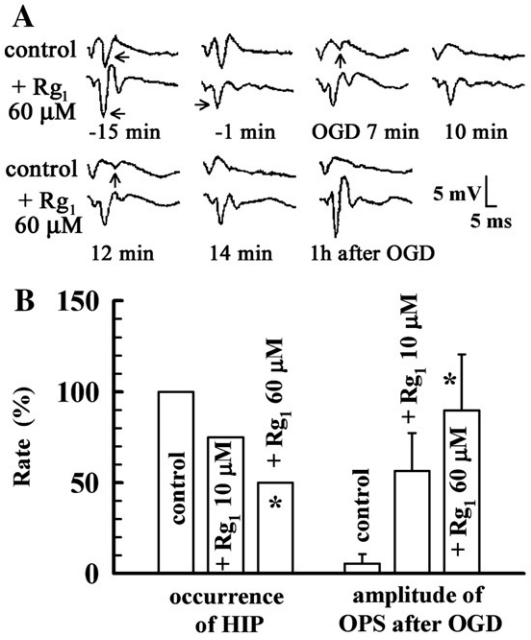

Fig. 2. Ginsenoside Rg_1 facilitates the recovery of orthodromic population spike (OPS, the large and downward wave (←), Fig. 2A) in CA_1 area of rat hippocampal slices after oxygen/glucose deprivation (OGD). A, examples of OPS recorded in Rg_1-treated slice and paired control slice at the time indicated. During OGD, OPS decreased (at 7 min, ↑), disappeared (at 10 min), and then reappeared with reduced amplitude (at 12 min, ↑) in the control slice. This reappeared OPS is termed as hypoxic injury potential (HIP). HIP disappeared (at 14 min) rapidly after its occurrence in the control slice. OPS did not recover in the control slice (1 h after OGD). With application of Rg_1, OPS was reduced (1 min before OGD, →), did not disappear during OGD, and recovered completely (1 h after OGD). Scale bar was shown at the right lower side. B, mean values of control group ($n=7$) and Rg_1-treated groups ($n=6$). *, $P<0.05$ *vs* control.

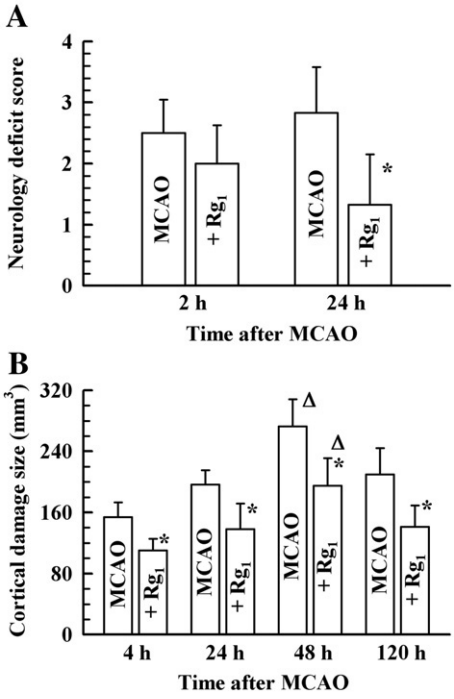

Fig. 3. Ginsenoside Rg_1 improves the neurological behavior and decreases the cerebral damage size on MRI at different time after middle cerebral artery occlusion (MCAO). A, Rg_1 treatment significantly decreased the neurology deficit score of rats ($n=16$) 24 h after MCAO. B, Rg_1 treatment significantly reduced cortical damage size of rat brain at all time points ($n=4$). *, $P<0.05$, *vs* MCAO; $^{\Delta}$, $P<0.05$, *vs* that at 4 h.

2.11. Statistical analysis

All values are presented as mean±S.D. Data were analyzed with Student's *t*-test or analysis of variance for two or more than two groups, respectively. Differences were considered statistically significant at a level of $P< 0.05$.

3. Results

3.1. Protective effect of Rg_1 on cultured hippocampal neurons subjected to oxygen/glucose deprivation

We firstly evaluated the protective effect of Rg_1 by comparing the cell viability and LDH release. After oxygen/glucose deprivation insult, the cell viability of the cultured hippocampal neurons was decreased significantly to 77.2±3.6% of control ($n=12$, $P<0.05$, Fig. 1A), and LDH release was increased from 34.9±5.6 in control to 276.7±74.4 U/ml/min ($n=6$, $P<0.05$, Fig. 1B). These changes were attenuated prominently by the treatment of ginsenoside Rg_1 (at the concentration range from 10 nM to 100 μM, $P<0.05$). An application of Rg_1 (100 μM) significantly improved the cell viability to 94.7±2.4% ($n=12$, $P<0.05$, Fig. 1A), and reduced LDH release to 105.5±17.0 U/ml/min ($n=6$, $P<0.05$, Fig. 1B). However, the effects of Rg_1 were significantly blunted by the pretreatment of RU486, an antagonist of glucocorticoid receptors (right columns in Fig. 1A and B). Specifically, addition of RU486 reduced the cell viability to 75.9±3.7% (from 87.1±1.7% in 10 μM Rg_1 group, $P<0.05$, $n=12$, Fig. 1A) and increased LDH release to 279.5±58.7 U/ml/min (from 100.5±20.4 U/ml/min in 10 μM Rg_1 group, $n=6$, $P<0.05$, Fig. 1B).

3.2. Protective effect of Rg1 on hippocampal slice subjected to oxygen/glucose deprivation

To further assess the protective effect of Rg_1, we measured electrical activities in hippocampal slices. As illustrated in the upper panel in Fig. 2A, orthodromic population spikes in the control slices were depressed progressively and disappeared during the exposure of oxygen/glucose deprivation. In addition, in all control slices we observed a hypoxic injury potential (Fairchild et al., 1988), which occurred at about 8–12 min after the beginning of oxygen/glucose deprivation and subsided within 1–3 min (Fig. 2A). After 1-h reoxygenation, the orthodromic population spikes recovered with mean amplitude of only 5.4±5.4% of that before oxygen/glucose deprivation. When Rg_1 (60 μM) was added to the bath solution, however, the orthodromic population spikes were inhibited initially before oxygen/glucose deprivation in 5 of 6 slices tested (Fig. 2A). Rg_1 induced-inhibition was reversible as the orthodromic population spikes recovered after the removal of Rg_1 (data not shown). In the presence of Rg_1, at 60 μM in particular, the occurring rate of hypoxic injury potential was reduced from 100% in control ($n=7$) to 50% ($P<0.05$, $n=6$, Fig. 2B), and the orthodromic population spikes, after 1-h reoxygenation, recovered significantly better (89.8±31.0% in Rg_1 at 60 μM, $n=6$) than that of the control group (5.4±5.4%, $n=7$, $P<0.05$, Fig. 2B).

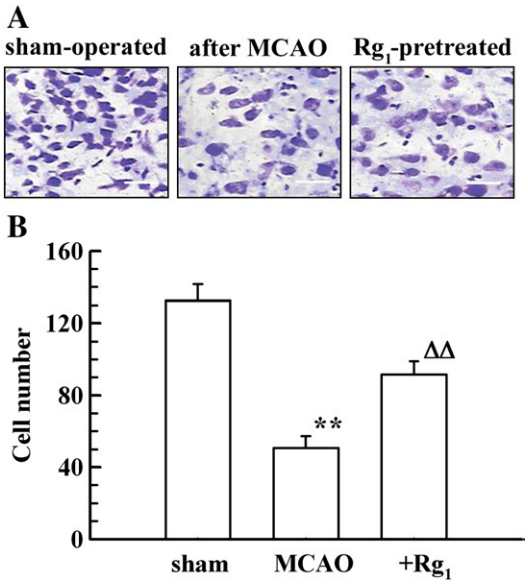

Fig. 4. Nissl staining indicates reduced neuronal loss by ginsenoside Rg_1 in the cerebral cortex of rat after middle cerebral artery occlusion (MCAO). A, examples of Nissl stained neurons show that MCAO significantly reduced the cell number (center), comparing to the sham group (left panel). MCAO-induced cell loss was significantly blunted by Rg_1 pretreatment (right panel). Scale bar=20 μm. B, mean values (±S.D.) of cell counting (from 6 rats). Cell number was counted in one vision under microscope (× 400). **, $P<0.01$, *vs* sham (sham-operated); $^{\Delta\Delta}$, $P<0.01$, *vs* MCAO and sham.

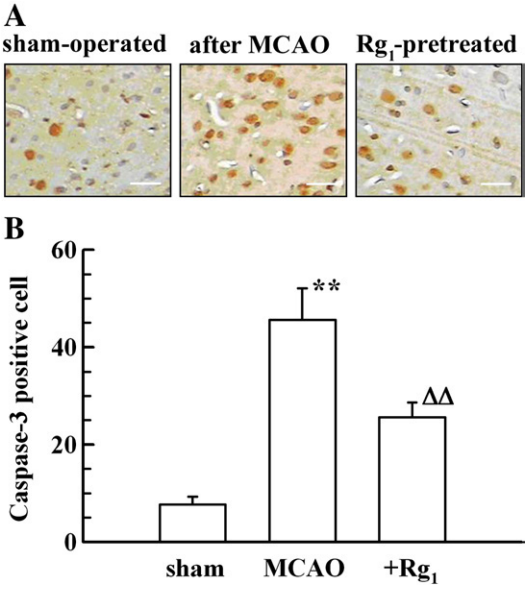

Fig. 5. Ginsenoside Rg_1 inhibits positive expression of activated caspase-3 in the cerebral cortex of rats after middle cerebral artery occlusion (MCAO). A, examples show that MCAO significantly increased the number of cells with positive expression of activated caspase-3 (center), comparing to the sham group (left panel). MCAO-induced increase in the expression of activated caspase-3 was significantly inhibited by Rg_1 pretreatment (right panel). Positive cells were stained deeply as brown. Scale bar=20 μm. B, mean values of positive expression cell counting (n=6). Cell number was counted in one vision under microscope (×400). **, $P<0.01$, *vs* sham (sham-operated); $^{\Delta\Delta}$, $P<0.01$, *vs* MCAO and sham.

3.3. Protective effect of Rg_1 on rat brain subjected to middle cerebral artery occlusion

For another index of the protective effect of Rg_1, we examined the influence of Rg_1 on ischemic brain tissue. Rats displayed some functional deficits in neurology after middle cerebral artery occlusion. The neurology deficit score was respectively 2.5±0.55 at 2 h and 2.83±0.75 at 24 h (Fig. 3A). However, the neurology deficit score was reduced significantly at 24 h after middle cerebral artery occlusion in the group of rats treated with Rg_1 (1.33±0.82, n=16, $P<0.05$, Fig. 3A). Cerebral damage area was measured according to images of MRI at 4 h to 120 h after middle cerebral artery occlusion. With the damage area of the rat brain divided into the cortical and subcortical parts, we found that the cortical damage area extended with time and reached the peak at 48 h after middle cerebral artery occlusion (272.8±35.8 mm^3 *vs* 153.8±19.5 mm^3 at 4 h, n=4, $P<0.05$, Fig. 3B). By contraries, the subcortical damage size did not change significantly with time (data not shown). The cerebral damage size was reduced markedly after Rg_1 treatment. This reduction was detected primarily in the cortical part and at all the time points examined, with the mean value of 141.3±28.0 mm^3 at 120 h in Rg_1 group (*vs* 209.5±34.9 mm^3 of control, n=4, $P<0.05$, Fig. 3B).

We next compared the number of neuronal cells in parietal cortex in the border of the infarct area with Nissl staining. As illustrated in Fig. 4A and B, in the sham-operated group, the number of neuronal cells was 132.5±9.0, which was significantly reduced to 50.7±6.6 after middle cerebral artery occlusion (n=6, $P<0.01$, Fig. 4B). When the rats were treated with Rg_1, the number of neurons in the same brain area was elevated to 91.5±7.5 (n=6, $P<0.01$, Fig. 4B).

3.4. Inhibition of Rg_1 on activated caspase-3 expression of the brain after middle cerebral artery occlusion

In order to evaluate the effect of Rg_1 on neuronal apoptosis after middle cerebral artery occlusion, the expression of activated caspase-3 was examined with immunohistochemistry in parietal cortex in the border of the infarct area (Fig. 5A). The number of caspase-3 positive neurons was increased from 7.7±1.6 in sham-operated group to 45.7±6.4 after middle cerebral artery occlusion ($P<0.01$, Fig. 5B). In contrast, the number of caspase-3 positive neurons in the same brain area was decreased to 25.7±3.0 by Rg_1 treatment ($P<0.01$, Fig. 5B).

3.5. Inhibition of Rg_1 on oxygen/glucose deprivation-induced Ca^{2+} influx

We next determined calcium influx with laser confocal microscopy. As illustrated in Fig. 6A, Ca^{2+} influx of cultured hippocampal neurons was increased greatly after oxygen/glucose deprivation. Fig. 6B shows that a 10-min oxygen/glucose deprivation increased Ca^{2+} influx by 73.5±5.3%. This increase

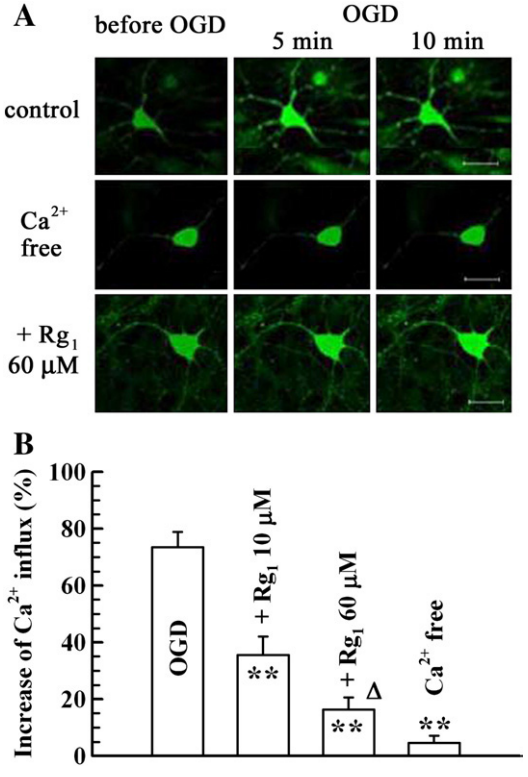

Fig. 6. Ginsenoside Rg_1 inhibits Ca^{2+} influx in cultured hippocampal neurons deprived of oxygen/glucose (OGD). A, examples show that Ca^{2+} fluorescence intensity was significantly increased after OGD for 5 (central panel) and 10 min (right panel). These changes were not observed in neurons perfused with Ca^{2+} free solution, or treated with Rg_1 (60 μM). Scale bar=15 μm. B, mean values of four different treatments (n=7). **, $P<0.01$, *vs* OGD; $^{\Delta}$, $P<0.05$, *vs* Rg_1 at 10 μM.

was inhibited remarkably (Fig. 6B) in the presence of Rg_1 (10 μM, to 35.5±6.5%, $n=7$, $P<0.01$ and 60 μM to, 16.4±4.3%, $n=7$, $P<0.01$) or in the absence of extracellular Ca^{2+} (Ca^{2+} free, to 4.5±2.6%, $n=7$, $P<0.01$).

3.6. Inhibition of Rg_1 on high K^+-elicited Ca^{2+} influx

The above result indicates that Rg_1 reduces Ca^{2+} influx induced by oxygen/glucose deprivation. In an attempt to determine the mechanism underlying this phenomenon, we conducted the following experiments. Ca^{2+} influx was enhanced prominently when KCl (50 mM) was infused to the cultured hippocampal neurons (Fig. 7A and B). This enhancement was inhibited dose-dependently by nifedipine (at 0.2 μM and 2 μM, $P<0.01$, Fig. 7A and B), indicating that the Ca^{2+} influx was mediated by the L-type voltage-dependent Ca^{2+} channels. When Rg_1 was administered (1 nM–100 μM), high K^+-elicited Ca^{2+} influx was diminished as a function of Rg_1 concentrations, with the maximal inhibition by Rg_1 at 100 μM (Fig. 7A and B). The mean (±S.D.) values of Ca^{2+} influx elevation in KCl, Rg_1 (10 μM) and nifedipine (2 μM) groups were 115.6±9.7% ($n=8$), 39.2±8.2% ($n=8$) and 31.1±4.3% ($n=8$), respectively (Fig. 7B).

3.7. Inhibition of Rg_1 on NMDA-elicited Ca^{2+} influx

An addition of NMDA (1 mM) to the cultured hippocampal neurons remarkably elicited Ca^{2+} influx (Fig. 8A and B). This effect of NMDA was blocked dose-dependently by AP-4 or MK-801, the NMDA receptor antagonists, or by removing Ca^{2+} from the extracellular solution ($P<0.01$, Fig. 8A and B). The NMDA-elicited Ca^{2+} influx was reduced dose-dependently by Rg_1 (from 100 nM to 1 mM, Fig. 8A and B). The mean values of Ca^{2+} influx elevation in NMDA, Rg_1 (10 μM), MK-801 (10 μM), AP-4 (1 mM) and Ca^{2+} free groups ($n=8$) were 141.0±5.9%, 88.8±7.3%, 7.2±4.4%, 38.4±11.7% and 15.6±12.1%, respectively (Fig. 8B).

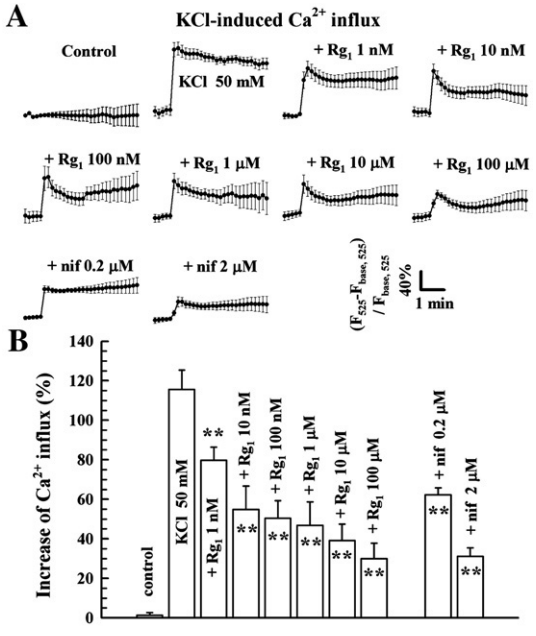

Fig. 7. Ginsenoside Rg_1 dose-dependently inhibits KCl-induced Ca^{2+} influx in cultured hippocampal neurons ($n=8$). A, KCl (50 mM)-induced Ca^{2+} influx or co-applied with Rg_1 (from 1 nM to 100 μM, as indicated), as well as nifedipine (0.2 and 2 μM, respectively, as indicated), an antagonist of L-type voltage-dependent Ca^{2+} channels. Scale bar was shown at the right lower side. B, mean values of all points after use of KCl of each curve in Fig. 7A. **, $P<0.01$, *vs* KCl; $^{\Delta}$, $P<0.05$, $^{\Delta\Delta}$, $P<0.01$, *vs* Rg_1 at 1 nM.

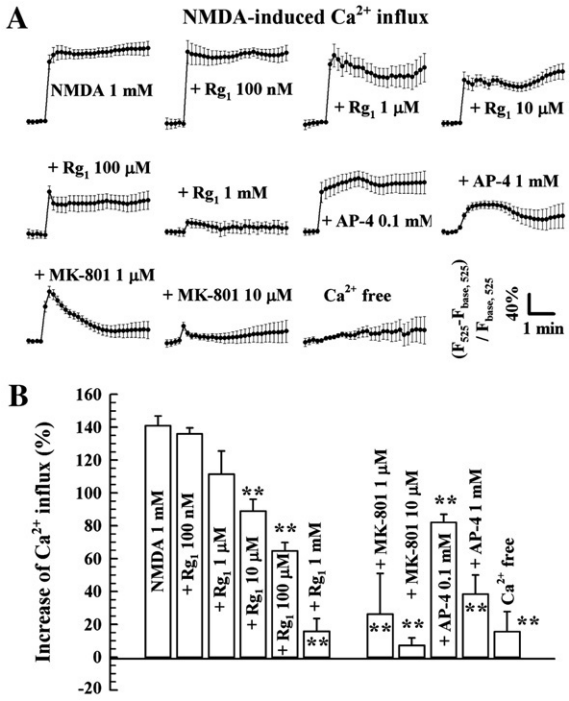

Fig. 8. Ginsenoside Rg_1 dose-dependently inhibits NMDA-induced Ca^{2+} influx in cultured hippocampal neurons ($n=8$). A, NMDA (1 mM)-induced Ca^{2+} influx without and with the co-application of Rg_1 (at the indicated concentrations) and MK-801 and AP-4, the two antagonists of NMDA receptors. Rg_1 or each antagonist of NMDA receptors was applied simultaneously with NMDA to neurons. Scale bar was shown at the right lower side. B, mean values of all points after use of NMDA of each curve in Fig. 8A. **, $P<0.01$, *vs* NMDA; $^{\Delta}$, $P<0.05$, $^{\Delta\Delta}$, $P<0.01$, *vs* Rg_1 at 100 nM; $^{\#}$, $P<0.01$, *vs* Rg_1 at other concentrations. Note that NMDA-induced Ca^{2+}influx was inhibited almost completely in the absence of Ca^{2+} in the medium (Ca^{2+} free).

4. Discussion

The present study in rats provided the first evidence that ginsenoside Rg_1 could protect neurons from damage induced by hypoxic–ischemic insults. We found that Rg_1 improved neuronal viability and reduced LDH release in cultured hippocampal neurons after oxygen/glucose deprivation. With Rg_1 treatment, hypoxic injury potential appeared less frequently, and the orthodromic population spike recovered better than that in the control hippocampal slices after oxygen/glucose deprivation. In addition, in *in vivo* experiments, Rg_1 decreased the neurology deficit score of rats 24 h after middle cerebral artery occlusion, indicating a promoting effect of Rg_1 on the recovery of the neurological function. Moreover, Rg_1 reduced the cerebral damage area on MRI, neuronal loss and expression of activated caspase-3 in the cerebral cortex. These results suggest a potential

application of Rg_1 as a neuroprotective agent for the treatment of ischemic cerebral stroke.

The protective effect of Rg_1 on neurons subjected to hypoxic–ischemic insults had been less studied before. This is likely because of the earlier negative results reported by Zhang and Liu (1996) in investigating the protective effect of Rg_1 (40 mg/kg, i.v.) on the rat brain subjected to both permanent and reversible (2-h) middle cerebral artery occlusion. Nevertheless, many researchers have found Rg_1 to be protective in other cell-damaging models in the recent decade. In a glutamate excitotoxic model, Liu and Zhang (1995) have observed that Rg_1 elevates the cell viability and reduces the LDH release of cortical neurons. Moreover, Liao et al. (2002) have documented that Rg_1 could protect the cultured spinal neurons from excitotoxicity induced by glutamate and kainic acid, as well as oxidative stress induced by H_2O_2. Li et al. (1997) and Li and Zhang (1997) have reported that Rg_1 inhibits the apoptosis of the cultured cortical neurons with serum withdrawal. Rudakewich et al. (2001) have reported that Rg_1 exerts a protective effect on the SN-K-SH cell line in a MPTP-induced cell toxicity model, but not on the beta-amyloid-induced cell death. In another report, Chen et al. (2003) have displayed that Rg_1 reduces the apoptosis of PC12 cells induced by exogenous dopamine. Recently, Leung et al. (2007) have shown that Rg_1 could protect nigral neurons from rotenone toxicity. The results of our present study are consistent with most of these reports.

In order to investigate the mechanisms underlying the protective effect of Rg_1 on the ischemic brain or oxygen/glucose-deprived neurons, we carried out additional *in vitro* experiments. We found that Rg_1 inhibited the increase of Ca^{2+} influx in hippocampal neurons subjected to oxygen/glucose deprivation. Furthermore, both high K^+- and NMDA-induced Ca^{2+} influx was reduced dose-dependently by Rg_1. It is suggested that the neuroprotective effect of Rg_1 is probably mediated by the inhibition of Ca^{2+} influx through both NMDA receptor channels and L-type voltage-dependent Ca^{2+} channels, and the resultant reduction of intracellular free Ca^{2+}. Similar inhibition of Ca^{2+} influx by Rg_1 has been observed by Liu and Zhang (1995) in a glutamate (0.5 mM) toxicity model of cultured hippocampal neurons of rats. In addition, other researchers have identified the inhibition of Ca^{2+}influx by other ginsenosides: inhibition of glutamate- or NMDA-gated Ca^{2+} influx in rat hippocampal neurons by Rg_3 (Kim et al., 2002, 2004; Lee et al., 2006a,b), Rh_1 (Lee et al., 2006a,b), Rh_2 (Lee et al., 2006a,b) and by total saponins (Kim et al., 2002); inhibition of voltage-dependent Ca^{2+} channels-mediated Ca^{2+} influx in neurons by total saponins (Kim et al., 2002; Lee et al., 2006a,b) and by some single ginsenosides (Lee et al., 2006a,b; Nah et al., 1995; Rhim et al., 2002). However, in contrast to our observation, Zhang and Liu (1996) have reported that Rg_1 at 40 mg/kg i.v. has neither any effect on Ca^{2+} accumulation in the infarct area and nor protective effect in rats subjected to permanent or 2-h middle cerebral artery occlusion. This inconsistency is possibly attributed to the differences in experimental conditions, including the induction of middle cerebral artery occlusion, sex and body weight of rats, dose, interval and route of use of Rg_1, as well as whether it is an *in vitro* or *in vivo* experiment.

It is generally believed that caspases are a family of intracellular proteins involved in the initiation and execution of cell apoptosis. The induction of apoptosis through extrinsic or intrinsic death mechanisms results in the activation of initiator caspases, where caspase-3 is a common and key executor caspase (Graham and Chen, 2001; Ashe and Berry, 2003). There are many evidence indicating that cerebral ischemia could induce the activation of caspases including caspase-3, up-regulation and activation of which have been proved to precede neuronal death. Caspase-mediated neuronal death after transient focal cerebral ischemia is more extensive than that after the permanent ischemia and may contribute to the delayed loss of neurons from the penumbral region of infarct (Graham and Chen, 2001; Love, 2003). In the present study, we documented neuronal loss and increased positive cells of activated caspase-3 expression in the border of the infarct area of the parietal cortex in rat after transient focal brain ischemia. These results are in line with those previously reviewed by Love (2003). After administration of Rg_1, the expression of activated caspase-3 was significantly inhibited. This anti-apoptotic effect of Rg_1 is consistent with the reports of several laboratories (Li et al., 1997; Li and Zhang, 1997). Generally, it is accepted that calcium overload is centrally involved in ischemic brain injury (Berliocchi et al., 2005), and activation of caspases is one of the downstream pathways of calcium signaling during ischemic cell death (White et al., 2000). Therefore, the inhibition of the caspase pathway could at least in part account for the neuroprotective effect of Rg_1 in the present study.

Ginsenosides have been identified to modulate the functions of many receptors and channels on neurons, and the present study extends these findings. However, the underlying mechanisms are still unclear. Given the structural similarity of ginsenosides with steroid hormones, Lee et al. (1997) and Chung et al. (1998) have investigated the interaction of Rg_1 with glucocorticoid receptor, and proved that Rg_1 is possibly a functional ligand of glucocorticoid receptor. On the basis of this, we applied RU486, an antagonist of glucocorticoid receptor, and observed its influence on the neuroprotective effect of Rg_1 in cultured rat hippocampal neurons deprived of oxygen and glucose. We found that RU486 blocked the effects of Rg_1 on neuronal viability and LDH release. This result suggests that the neuroprotective effect of Rg_1 is potentially mediated by the activation of glucocorticoid receptors. This is consistent with a recent report (Leung et al., 2007). It has been identified that the activation of glucocorticoid receptors could acutely inhibit NMDA receptor activity in the CA_1 area of the mouse hippocampal slices (Sato et al., 2004). Nevertheless, the same research group has reported an opposite result in rats (Takahashi et al., 2002). The differences in the experimental conditions of these two studies may contribute to the controversial results. Moreover, there are many controversies about the effect of glucocorticoid or its receptors in neuroprotection (Abraham et al., 2001; Anderson and Cranford, 1979). Therefore, more precise experiments are needed to clarify these issues.

In the present study, we found an interesting phenomenon, i.e., pretreatment with ginsenoside Rg_1 reversibly reduced the amplitude of orthodromic population spikes in hippocampal slices

before any other treatments. This inhibition has also been observed previously in our experiments upon the neuroprotective effect of total saponins of ginseng (Jiang et al., 2000, 2001) and ginsenoside Rb_1 (unpublished data). The orthodromic population spike is a field potential recorded extracellularly, a synchronous response of action potentials of many post-synaptic hippocampal neurons in CA_1 area. It is known that the synaptic transmission is herein mediated by the neurotransmitter glutamate (Amaral and Witter, 1989). Hence, it is suggested that ginsenoside Rg_1 could inhibit glutamatergic transmission. At present, the exact mechanisms of this inhibition are unclear. An inhibition of Rg_1 on NMDA receptors and L-type voltage-dependent Ca^{2+} channels found in the present study is potentially involved. Whether other pre-synaptic or post-synaptic mechanisms are attributable to this phenomenon warrants further study.

In conclusion, ginsenoside Rg_1 exerted a neuroprotective role on the oxygen/glucose-deprived hippocampal neurons and slices, as well as on the ischemic/reperfused brain in rats. This effect is probably mediated by the activation of glucocorticoid receptors, the inhibition of Ca^{2+} influx through both NMDA receptor channels and L-type voltage-dependent Ca^{2+} channels, and the resultant reduction of intracellular free Ca^{2+}. As a result, neuronal death including apoptotic cell death is diminished.

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Further reading

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