Research Report

Activation of c-Jun NH$_2$-terminal kinase 3 is mediated by the GluR6·PSD-95·MLK3 signaling module following cerebral ischemia in rat hippocampus

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Abstract

Kainate receptor glutamate receptor 6 (GluR6) binds to the postsynaptic density protein 95 (PSD-95), which in turn anchors mixed lineage kinase 3 (MLK3) via SH3 domain in rat brain tissue. MLK3 subsequently activates c-Jun NH$_2$-terminal kinase (JNK) via MAP kinase kinases (MKKs). We investigated the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, the interaction of MLK3 with JNK3, and JNK3 phosphorylation following cerebral ischemia in rat hippocampus. Our results indicate that the GluR6·PSD-95·MLK3 complex peaked at 6 h of reperfusion. Furthermore, MLK3 autophosphorylation and the interaction of MLK3 with JNK3 occurred with the alteration of GluR6·PSD-95·MLK3 signaling module. To further prove whether JNK3 activation in ischemic hippocampus is mediated by GluR6·PSD-95·MLK3 signaling pathway, the AMPA/KA receptor antagonist 6,7-dinitroquinoxaline-2, (1H, 4H)-dione (DNQX), the GluR6 antagonist 6,7,8,9-Tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione-3-oxime (NS102), the AMPA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzo diazepine (GYKI52466), and the NMDA receptor antagonist ketamine were given to the rats 20 min prior to ischemia. Our findings indicate that both DNQX and NS102 significantly attenuated the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, interaction of MLK3 with JNK3, and JNK3 phosphorylation, while GYKI52466 and ketamine had no effect. Moreover, administration of NS102 before cerebral ischemia significantly increased the number of the surviving hippocampal CA1 pyramidal cells at 5 days of reperfusion. Consequently, GluR6, one subunit of kainate receptor, plays a critical role in inducing JNK3 activation after ischemic injury.
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1. Introduction

Glutamate receptors (GluRs) are classified into two subgroups: ionotropic receptors and metabotropic receptors. Ionotropic glutamate receptors can be divided into two classes, N-methyl-D-aspartate (NMDA) receptors (NR1, NR2A–NR2D, NR3A–B) and non-NMDA receptors. Non-NMDA receptors can be further subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (GluR1–4) and kainate acid (KA) receptors (GluR5–GluR7, KA1 and KA2) [1,12,32]. Ionotropic glutamate receptors mediate excitatory synapse transmission in the mammalian central nervous system and play a central role in learning and memory. Furthermore, calcium

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influx through ionotropic glutamate receptors modulates many physiological functions in the central nervous system. Also note that considerable evidence suggests that glutamate receptors are also involved in neuronal death after ischemic insults [25,33]. Excessive activation of glutamate receptors can cause excitotoxicity and cell death by increasing transcellular calcium concentration. Thus, neuroprotective action has mainly focused on blocking excitotoxicity induced by the excitatory transmitter glutamate and Ca^{2+} overload in neurons. The NMDA receptors are highly Ca^{2+}-permeable, and cerebral ischemia induces enhancement of the expression of Ca^{2+}-permeable AMPA receptors [25]. However, this view that excitotoxicity with attendant neuronal Ca^{2+} overload is the predominant mechanism underlying ischemic brain injury faces challenge due to negative results from several recent trials with antagonists of NMDA receptor [16]. Previous studies report GluR6 knockout mice are more resistant to kainate-induced seizures and excitotoxic neuronal death in the hippocampus [22]. Consequently, we are particularly interested in establishing whether and how ischemic brain damage is mediated by KA receptor GluR6 subunit.

The postsynaptic density protein 95 (PSD-95) is a scaffold protein characterized by the presence of several protein binding domains including three N-terminal PDZ, a signal Src homology region 3 domain, and a C-terminal guanylate kinase-like domain [3,15,23]. The PDZ domains are known to bind to the C-terminus of NMDA receptor NR2 and KA receptor GluR6 subunit, and these interactions are very crucial for the clustering of NMDA receptors and KA receptors in the postsynaptic membrane [8,21,40]. In addition, PSD-95 also interacts with some cytoplasmic signaling proteins. For example, PSD-95 can connect NR2 with neuronal nitric oxide synthase (nNOS) [30]. Some studies indicate that PSD-95, GluR6, and MLK3 form an association, and GluR6-deficient mice show strong resistance to kainate-induced seizures [22,31]. Therefore, PSD-95 plays a critical role for GluR6-mediated MLK3 activation and neuronal excitotoxicity induced by kainate in cell lines.

MLK3, a member of mixed lineage kinase family, is composed of an N-terminal SH3 domain, a middle kinase domain, and a C-terminal proline-rich region [7]. It is proven that C-terminal proline-rich region in MLK3 binds to SH3 domain of PSD-95 [31]. MLKs are the direct activators of MKK4/7 which subsequently phosphorylate and activate JNKs. The c-Jun NH2-terminal kinase (JNKs), or stress-activated protein kinase (SAPK), belongs to the mitogen-activated protein kinase (MAPK) family of proline-directed serine/threonine kinase that participates in intracellular signaling pathway by transmission of signals from the plasma membrane to the nucleus. Additionally, JNKs are critically involved in the apoptosis of NGF-deprived PC12 cells [2,6]. It is important to note that current studies demonstrate that the knockout mice of JNK3 gene resist the kainate-induced seizure [41], just as GluR6-deficient mice. Although JNK1/2 are widely expressed in a variety

of tissues, JNK3 is found predominately in neurons [11]. On the other hand, studies from both our group and others show that JNKs are activated and implicated in neuronal degeneration in response to ischemic insult [9,14]. However, JNKs' specific upstream activator and signaling pathway in cerebral ischemia remain complicated.

Although studies from Savinainen et al. [31] show that PSD-95 plays a critical role in GluR6-mediated JNK activation and excitotoxicity by anchoring MLKs to the GluR6 complex, the results were mainly obtained from cell lines experiment and kainate stimulation. Meanwhile, our preliminary studies show that the MLK3 and JNK3 activation induced by cerebral ischemia–reperfusion in animal models is related to the activation of non-NMDA receptors, but not NMDA receptors [35,36]. Therefore, the present study was undertaken to investigate whether JNK3 activation in response to cerebral ischemia was mediated by GluR6 activation. We hypothesize that ischemic injury induced the formation and alteration of GluR6·PSD-95·MLK3 signaling module, subsequently activated MLK3 and JNK3, and finally mediated the neuronal death in hippocampus.

2. Experimental procedures

2.1. Animal surgical procedures

Adult male Sprague–Dawley (SD) rats weighing 250–300 g were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Science. The experiment procedures were approved by the local legislation for ethics of experiments on animals. Transient brain ischemia was induced by four-vessel occlusion [9,29]. Briefly, the rats were anesthetized by chloral hydrate (350 mg/kg, i.p.), their vertebral arteries were then electrocauterized, and their common carotid arteries were exposed. The rats were allowed to recover for 24 h. Ischemia was induced by occluding the common arteries with aneurysm clips. The rats which lost their righting reflex within 30 s, and their pupils were dilated and unresponsive to light, were selected for the experiments. The rats with seizures were discarded. An EEG was monitored to ensure isoelectricity after carotid artery occlusion. Carotid artery blood flow was restored by releasing the clips. Rectal temperature was maintained at about 37 °C during and 2 h after ischemia. Sham animals received the same surgical procedures except bilateral carotid arteries were not occluded.

2.2. Brain tissues and drug treatment

In order to study time courses of the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, interaction of MLK3 with JNK3, and JNK3 phosphorylation, rats were decapitated immediately (0 min) and at different times of reperfusion (30 min, 3 h, 6 h, 1 day, 3

days, and 5 days) after 15 min of global cerebral ischemia. To evaluate the effects of AMPA/KA receptor antagonist DNQX [13,34], GluR6 receptor antagonist NS102 [10,15,17,37], specific AMPA receptor antagonist GYKI52466 [19,26,39], and NMDA receptor antagonist ketamine [38] on the association of the signal model and phosphorylation of JNK3 in response to cerebral ischemia, the rats were given ketamine (50 mg/kg) by abdominal injection 20 min before ischemia. Ketamine was dissolved in physiological saline (0.9% NaCl). Vehicle control rats received an equal volume of 0.9% NaCl by abdominal injection. DNQX (D-0540, Sigma), NS102 (N-179 Sigma), and GYKI52466 (No. 1454, Tocris) were dissolved in DMSO at a concentration of 10 mM. The drug infusion was performed using a micro-injector through both cerebral ventricles (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) 20 min before ischemia. A volume of 5 μl each was infused over 5 min. An equal volume of DMSO infusion in the rats served as vehicle control. Whole brains were removed by dissection, and then the hippocampi were immediately frozen in liquid nitrogen. All samples were stored at −80 °C until use.

2.3. Immunoprecipitation and immunoblotting

Briefly, tissue samples were homogenized in 1.5 ml of 50 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 10 mM β-phosphoglycerol, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 1 mM benzamidine, and enzyme inhibitors: 5 μg/ml each of phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, aprotinin (Sigma), and then centrifuged for 15 min at $1000 \times g$. The supernatant was removed and stored at −80 °C until use. Immunoprecipitation was performed as in Pei et al. [27]. Tissue homogenizations (400 μg of protein) were diluted 4-fold with 50 mM HEPES buffer, pH 7.4, containing 10% glycerol, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and 1 mM each of EDTA, EGTA, PMSF, and Na_3VO_4. The supernatants were incubated with 2 μg antibody A for 4 h at 4 °C. Protein A–Agarose (Sigma) 20 μl was added, and the incubation continued for 2 h. Samples were centrifuged at $10,000 \times g$, and the pellets were washed three times with HEPES buffer. Bound proteins were eluted by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (10 μl) and boiled for 5 min. Samples were centrifuged, and supernatants were separated by 10% SDS-PAGE gel and were electrotransferred onto nitro-cellulose membrane by semidry blotting system. After blocked for 2 h in phosphate-buffered saline with 0.1% Tween 20 (PBST) and 3% bovine serum albumin (BSA), membranes were incubated overnight at 4 °C with antibody B in PBST containing 3% BSA. Membranes were then washed and incubated with alkaline-phosphatase-conjugated secondary antibodies in PBST for 2 h and developed using NBT/BCIP color substrate (Promega, Madison,

USA). After immunoblot, the bands on the membrane were scanned and analyzed with an image analyzer (LabWorks Software, UVP-Upland, CA, USA). (Above-mentioned antibody A was used to immunoprecipitate, while antibody B was used to immunoblot.)

2.4. Histology

The rats were perfusion-fixed with 10% formalin under anesthesia after 5 days of reperfusion. Paraffin sections (5 μm) were prepared and stained with cresyl violet. The sections were examined with a light microscopy (400×), and the numbers of the surviving hippocampal CA1 pyramidal cells per 1 mm length were counted as neuronal density.

2.5. Antibodies

The following primary antibodies were used and were purchased from Santa Cruz Biotechnology: goat polyclonal anti-GluR6 (sc-7618), rabbit polyclonal anti-MLK3 (sc-13072), and mouse monoclonal anti-p-JNKs (sc-6254). Rabbit polyclonal anti-JNK3 (06-749) was obtained from Upstate Biotechnology. Mouse monoclonal anti-PSD-95 (CP35–100 UL) was bought from Oncogene. Rabbit polyclonal anti-p-ser antibody was purified by our laboratory. The secondary antibodies used in our experiment which were purchased from Sigma were goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-goat IgG.

2.6. Statistical analysis

Values were expressed as mean ± SD. Statistical analysis of the results was carried out by one-way analysis of the variance (ANOVA) followed by alternatives of the Duncan's new multiple range method and Newman–Keuls test. P values < 0.05 were considered significant.

3. Results

3.1. The alteration of GluR6·PSD-95·MLK3 signaling module during reperfusion after cerebral ischemia

To test our hypothesis, we first examined whether PSD-95 was associated with GluR6 during reperfusion in rat hippocampus. Because neuronal death induced by ischemia is identified as delayed neuronal injury, that is, cell death appears during reperfusion after ischemia, we explored alteration of GluR6·PSD-95·MLK3 signaling module at different times of reperfusion after 15 min of ischemia. Thus, anti-GluR6 antibody and anti-PSD-95 antibody were incubated with hippocampal preparation, respectively, and then immunoprecipitates were blotted by anti-PSD-95 antibody and anti-GluR6 antibody, respectively. As shown in Figs. 1A and B, PSD-95 and GluR6 were detected in anti-
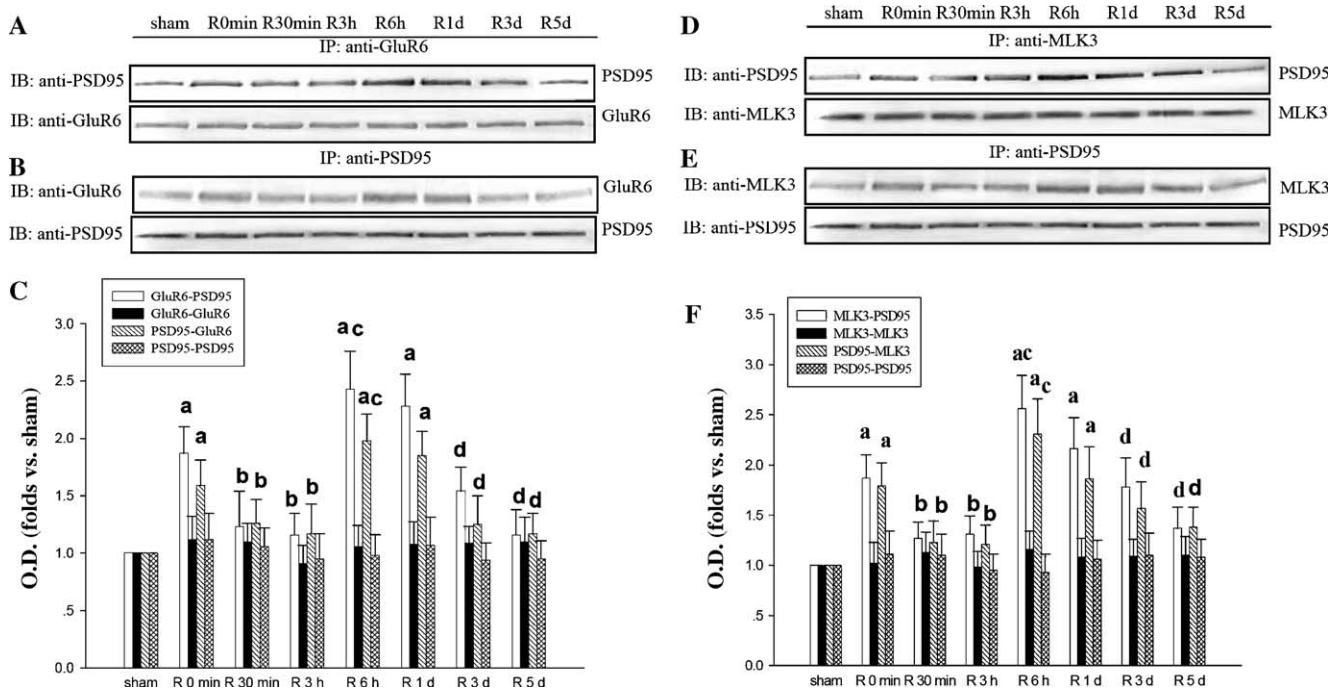
Fig. 1. Time courses of reperfusion induced the alteration of GluR6·PSD-95·MLK3 signaling module from sham and ischemic animals that had 0, 30 min, 3 h, 6 h, 1 day, 3 days, and 5 days of reperfusion. (A) Homogenates of rat hippocampus from animals in various reperfusion time points after 15 min of ischemia were immunoprecipitated (IP) with anti-GluR6 specific antibody and then immunoblotted (IB) with anti-PSD-95 antibody. Similar amounts of GluR6 were detected by immunoblotting with anti-GluR6 antibody. (B) Homogenates of rat hippocampus from animals in above reperfusion time points were immunoprecipitated (IP) with anti-PSD-95 antibody and immunoblotted (IB) with anti-GluR6 antibody. Similar amounts of PSD-95 were detected by immunoblotting with anti-PSD-95 antibody. (D) Homogenates of rat hippocampus from animals in various reperfusion time points after 15 min ischemia were immunoprecipitated (IP) with anti-MLK3 antibody and immunoblotted (IB) with anti-PSD-95 antibody. Similar amounts of MLK3 were detected by immunoblotting with anti-MLK3 antibody. (E) Homogenates of rat hippocampus from animals in different reperfusion time points were immunoprecipitated with anti-PSD-95 antibody and immunoblotted (IB) with anti-MLK3 antibody. Similar amounts of PSD-95 were detected by immunoblotting with anti-PSD-95 antibody. (C, F) Bands were scanned, and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD from four independent animals ($n = 4$). [a]$P < 0.05$ vs. sham control, [b]$P < 0.05$ vs. R 0 min, [c]$P < 0.05$ vs. R 3 h, [d]$P < 0.05$ vs. R 6 h.

GluR6 antibody immunoprecipitates and anti-PSD-95 antibody immunoprecipitates, respectively. Next, we further examined whether PSD-95 was associated with MLK3 during reperfusion after 15 min of ischemia. As shown in Figs. 1D–E, the immunoprecipitates of anti-MLK3 antibody contained PSD-95, and those of anti-PSD-95 antibody contained MLK3.

These results indicate that cerebral ischemia without reperfusion induces the formation of GluR6·PSD-95·MLK3 signaling module, as shown in $t = 0$ time point. During reperfusion, the association of PSD-95 with GluR6 and MLK3 first decreased and then peaked at 6 h of reperfusion, finally reduced again after 1 day of reperfusion. However, the protein levels of GluR6, PSD-95, and MLK3 had no noticeable alteration (Figs. 1C and F), indicating that the reperfusion-induced elevation of the assembly does not result from the change of protein level.

3.2. The further confirmation of the alteration of GluR6·PSD-95·MLK3 signaling module during reperfusion

To further clarify the enhancement of GluR6·PSD-95·MLK3 signaling module during reperfusion, we

examined the interaction among GluR6, PSD-95, and MLK3 at 6 h of reperfusion after 15 min of ischemia. Endogenous GluR6, PSD-95, and MLK3 were immunoprecipitated from the hippocampal samples with three specific antibodies, respectively. The proteins in precipitates were analyzed by blotting with the same three antibodies, respectively. As shown in Fig. 2, the antibodies of PSD-95 and MLK3 can co-precipitate the other two proteins in the reperfusion groups. Additionally, the association of PSD95 with GluR6 and MLK3 enhanced up to about 2-fold compared with sham groups, while nonspecific IgG was used in immunoprecipitation as negative control. No significant corresponding band was detected. These data further confirm the results we described above which indicate the definite existence and alteration of GluR6·PSD-95·MLK3 signaling module during reperfusion.

3.3. The activation of MLK3 and JNK3 during reperfusion after 15 min of ischemia

Because GluR6 can mediate MLK3 and JNK activation via PSD-95 and evoke cell death in HN33 cell line induced
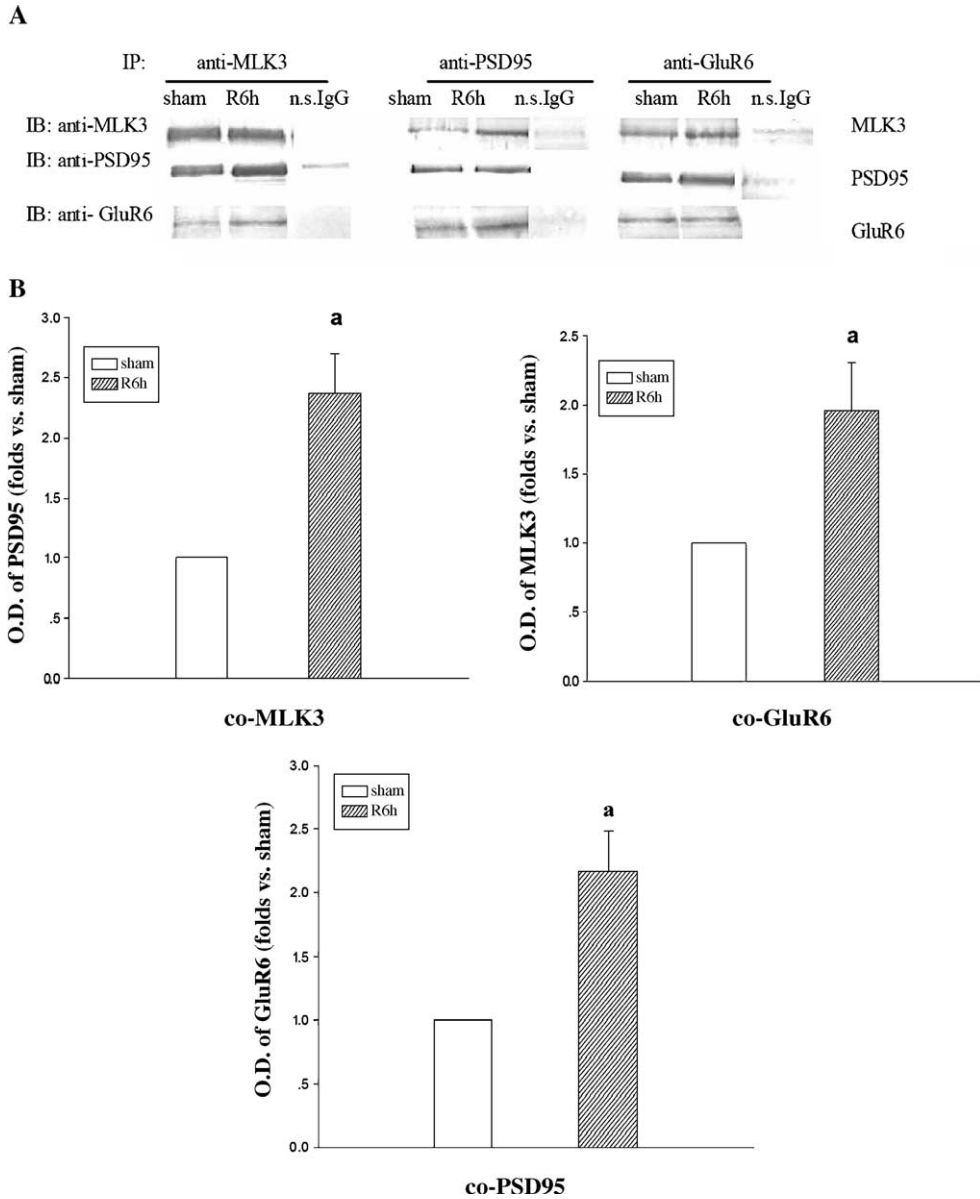
Fig. 2. GluR6, PSD-95 and MLK3 coimmunoprecipitated with each other. (A) Homogenates from sham-operated controls and ischemic animals at 6 h of reperfusion were immunoprecipitated (IP) with anti-MLK3, anti-PSD-95, anti-GluR6 antibodies, and nonspecific IgG (n.s.IgG), and the precipitates were analyzed by immunoblotting (IB) with anti-MLK3, anti-PSD95, and anti-GluR6 antibodies. Nonspecific IgG was used in immunoprecipitation as negative control. (B) Bands corresponding to PSD-95, MLK3, and GluR6 were scanned, and the intensities were expressed as folds vs. sham control. 'co-MLK3', 'co-GluR6', and 'co-PSD-95' represented the MLK3, GluR6, and PSD-95 coimmunoprecipitated with PSD-95, MLK3, and GluR6, respectively. Data were expressed as mean ± SD from four independent animals ($n = 4$). [a] $P < 0.05$ vs. sham control.

by kainate [31]. Furthermore, as mentioned above, the GluR6·PSD-95·MLK3 signaling module can be altered by cerebral ischemia in rat hippocampus. Because of this, we were particularly interested in establishing whether MLK3 and JNK3 can be activated following with the alteration of the signaling module after cerebral ischemia. And, because the autophosphorylation of MLK3 and the diphosphorylation of JNK3 are crucial for their activation, we examined MLK3 autophosphorylation and JNK3 diphosphorylation, respectively. The alteration of MLK3 autophosphorylation was detected by immunoprecipitating with anti-MLK3 specific antibody then blotting with anti-p-ser antibody.

As shown in Figs. 3A and C, the MLK3 autophosphorylation and the interaction between MLK3 and JNK3 coincided with the change of GluR6·PSD-95·MLK3 signaling module and also reached their maximums at 6 h of reperfusion. As shown in Fig. 3B, however, two peaks were observed in JNK3 diphosphorylation. The first peak occurred at 30 min, while the second peak lasted from 6 h to 3 days. These data indicate that MLK3 and JNK3 can be activated by reperfusion after ischemia and also that the activation of MLK3 and JNK3 approximately coincided with the alteration of GluR6·PSD-95·MLK3 signaling module.
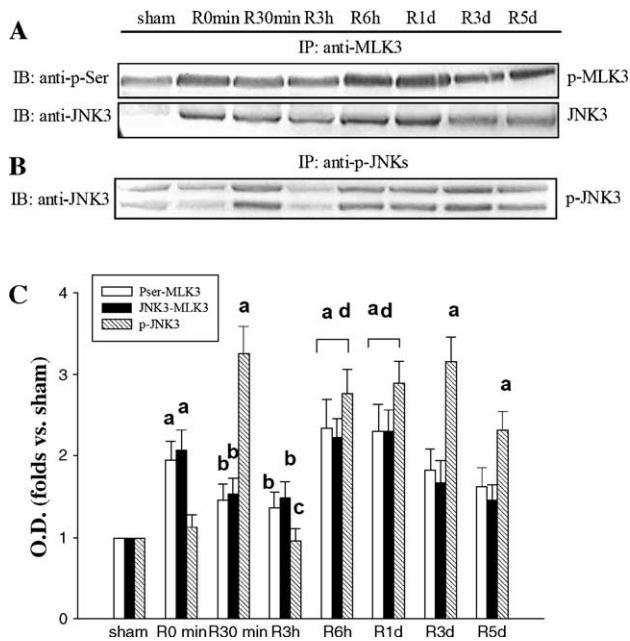
Fig. 3. Time courses of reperfusion-induced MLK3 autophosphorylation, interaction of MLK3 with JNK3, and JNK3 phosphorylation from sham and ischemic animals that had 0, 30 min, 3 h, 6 h, 1 day, 3 days, and 5 days of reperfusion. (A) Immunoprecipitation (IP) followed by immunoblotting (IB) analysis of the MLK3 autophosphorylation and interaction of MLK3 with JNK3 at various times of reperfusion (0, 30 min, 3 h, 6 h, 1 day, 3 days, and 5 days). (B) Immunoprecipitation (IP) with anti-p-JNKs antibody followed by immunoblotting (IB) with anti-JNK3 antibody analysis at above times of reperfusion. (C) Bands were scanned, and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD from four independent animals ($n = 4$). [a]$P < 0.05$ vs. sham control, [b]$P < 0.05$ vs. R 0 min, [c]$P < 0.05$ vs. R 30 min, [d]$P < 0.05$ vs. R 3 h.

3.4. The effects of some non-NMDA receptor antagonists and NMDA receptor antagonist on the assembly of GluR6·PSD-95·MLK3 signaling module and on the activation of MLK3 and JNK3 at 6 h of reperfusion after 15 min of ischemia

To clarify whether the activation of MLK3 and JNK3 in response to ischemia was mediated by GluR6 activation, we first selected an AMPA/KA receptor antagonist DNQX to give rats 20 min before 15 min of ischemia. Because 6 h of reperfusion is the common peak time point of the formation of GluR6·PSD-95·MLK3 signaling module and the activation of MLK3 and JNK3, the effect of DNQX on rats was observed at this time point. As shown in Figs. 4A–F, not only was the association of PSD-95 with GluR6 and MLK3 attenuated approximately 50% by DNQX, but the activation of MLK3 and JNK3 was also diminished. Because DNQX is an AMPA/KA receptor antagonist, not a specific KA or GluR6 receptor antagonist, we further examined whether NS102, which effectively diminished GluR6 activation, has the same inhibiting effect as DNQX on the formation of GluR6·PSD-95·MLK3 signaling module and the activation of MLK3 and JNK3. As shown in Figs. 4A–F, the activation of MLK3 and JNK3 was also limited by NS102. In addition, we further excluded the action of AMPA receptor and NMDA

receptor to observe the change of signaling module and the activation of MLK3 and JNK3 by using GYKI52466 (a specific AMPA receptor antagonist) and ketamine (a specific NMDA receptor antagonist). As shown in Figs. 5 and 6, GYKI52466 and ketamine had no effect on either of them. Consequently, our results show that NS102, but not GYKI52466 or ketamine, significantly inhibits the formation of the GluR6·PSD-95·MLK3 signaling module as well as the activation of MLK3 and JNK3 at 6 h of reperfusion.

Taken together, these data reveal that the activation of MLK3 and JNK3 via the GluR6·PSD-95·MLK3 signaling module is related to GluR6 activation in response to ischemia, but not AMPA receptor or NMDA receptor activation.

3.5. The neuroprotective effects of NS102 against ischemia–reperfusion-induced neuronal loss in hippocampus

NS102 did inhibit the assembly of GluR6·PSD-95·MLK3 signaling module and the activation of MLK3 and JNK3. Thus, we further explore whether NS102 has a neuro-protective effect. Because CA1 region is more sensitive to
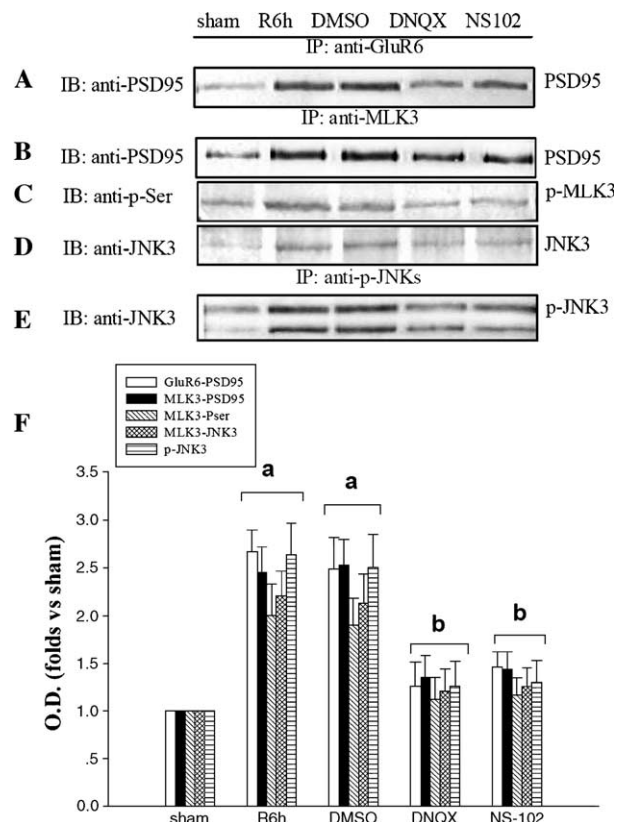

Fig. 4. Effects of DNQX and NS102 on the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, interaction of MLK3 and JNK3, and JNK3 phosphorylation during reperfusion. (A–E) DNQX and NS102 obviously attenuated the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, the interaction of MLK3 and JNK3, and JNK3 phosphorylation at 6 h of reperfusion. (F) Bands were scanned, and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD from four independent animals ($n = 4$). [a]$P < 0.05$ vs. sham control, [b]$P < 0.05$ vs. R 6 h + DMSO.
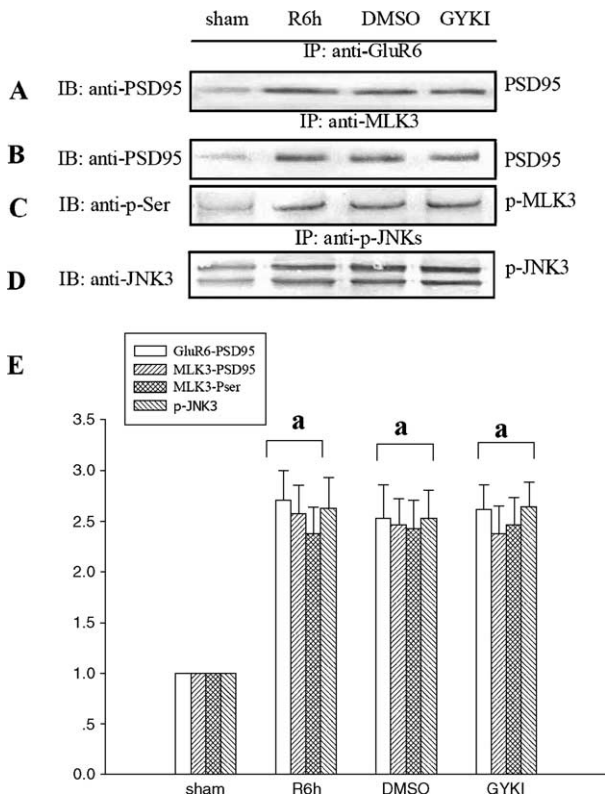
Fig. 5. Effects of GYKI52466 on the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, and JNK3 phosphorylation during reperfusion. (A–D) GYKI52466 had no effect on the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, and JNK3 phosphorylation at 6 h of reperfusion. (E) Bands were scanned, and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD from four independent animals ($n = 4$). $^aP < 0.05$ vs. sham control.
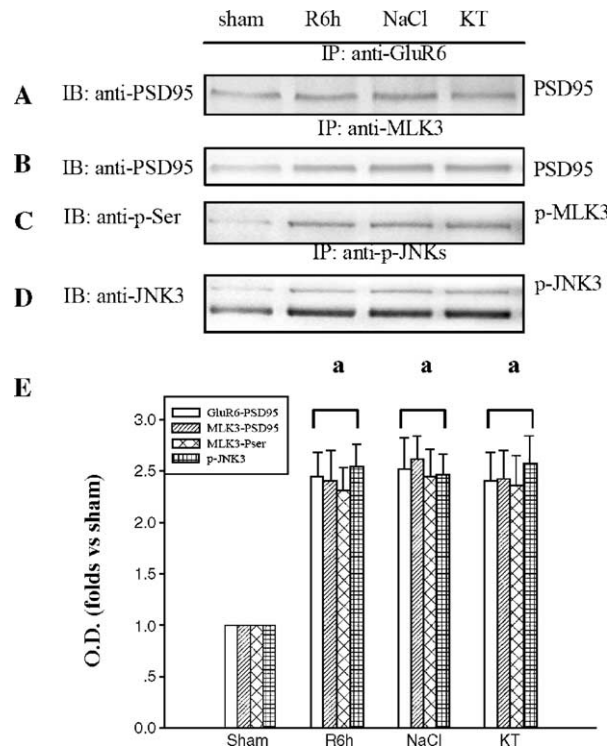

Fig. 6. Effects of ketamine on the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, and JNK3 phosphorylation during reperfusion. (A–D) Ketamine had no effect on the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, and JNK3 phosphorylation at 6 h of reperfusion. (E) Bands were scanned, and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD from four independent animals ($n = 4$). $^aP < 0.05$ vs. sham control.

global cerebral ischemia, we examined the role of NS102 on the neuronal survival of CA1 pyramidal neurons in rat hippocampus after 5 days of reperfusion. Cresyl violet staining was used to examine survival in the CA1 pyramidal neurons. Normal cells showed round and pale stained nuclei, while shrunken cells with pyknotic nuclei after ischemia were counted as dead cells. As shown in Table 1 and Fig. 7, transient cerebral ischemia followed by 5 days reperfusion induced severe cell death. However, administration of NS102 (10 mM in 5 μl DMSO) 20 min before cerebral ischemia limited the neuronal degeneration. These results indicate that NS102 is capable of protecting against neuronal injury induced by reperfusion after ischemia. Taken together, these data suggest that neuronal degeneration after ischemia was at least partially mediated by GluR6 activation and, furthermore, that the decrease of GluR6 activation inhibited the formation of GluR6·PSD-95·MLK3 signaling module, the activation of JNK3 and MLK3, and further neuronal degeneration.

4. Discussion

In this study, we first provided evidence for the existence and alteration of GluR6·PSD-95·MLK3 signaling

module in response to cerebral ischemia and, furthermore, that the alteration of GluR6·PSD-95·MLK3 signaling module induced the activation of MLK3-JNK pathway and resultant neuronal cell death in hippocampal CA1 region after cerebral ischemia. It is known that JNK activation is closely connected with ischemic injury and glutamate excitotoxicity [4,24,43]. However, the exact type of ionotropic glutamate receptors involved in JNK activation and its molecular mechanism remains unclear. Our current study addressed this question. In this in vivo global ischemic model, we reveal that the JNK activation and the neuronal injury are especially induced by GluR6 activation.

Table 1
Quantitative analysis of the protective effects of NS102 against transient ischemia followed by reperfusion

Groups	Neuronal density (mean ± SD)
Sham	210.0 ± 25.1
I/R5d	38.6 ± 7.6
DMSO+I/R5d	30.3 ± 5.4
NS102+I/R5d	145.7 ± 10.6*

Neuronal density was expressed as the number of surviving neurons per 1 mm length of CA1 pyramidal cells counted under light microscopy. Data were mean ± SD ($n = 7$).

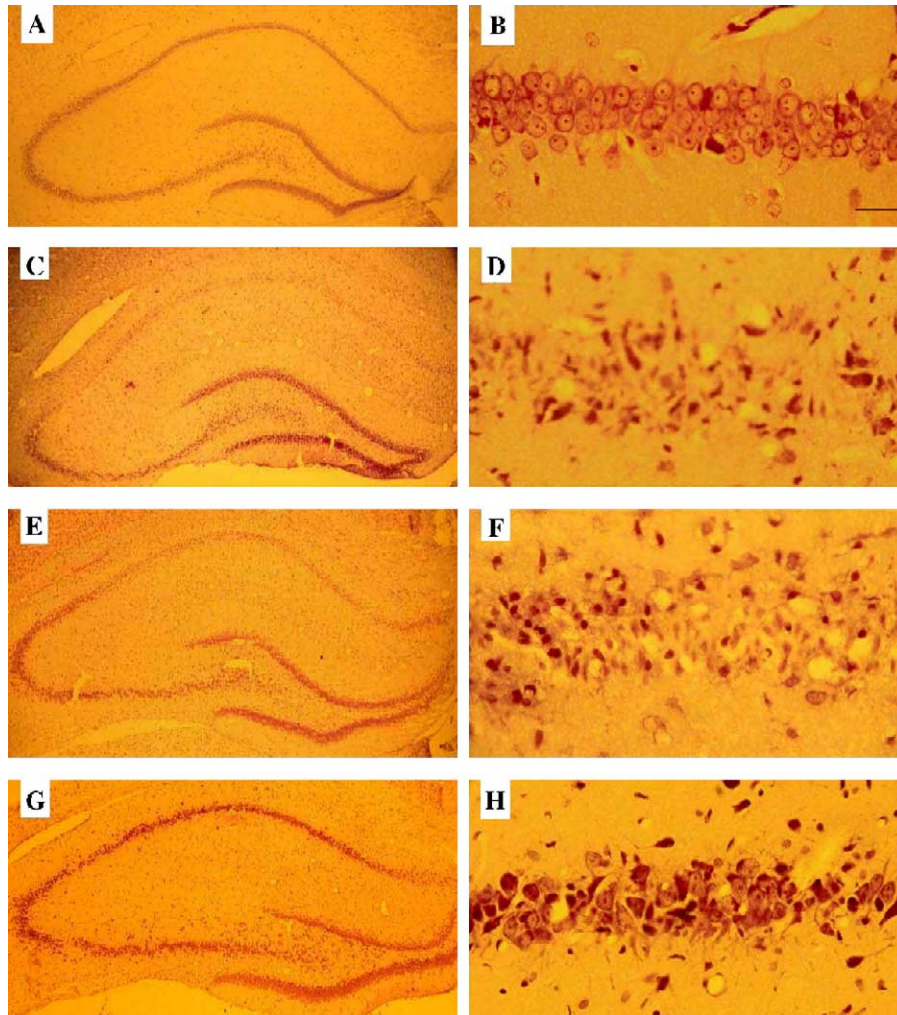* $P < 0.05$ vs. vehicle-treated group.

Fig. 7. Example of cresyl-violet-stained sections of the hippocampi of sham-operated rats (A, B), and rats subjected to 5 days of reperfusion after 15 min of ischemia (C, D), administration of the vehicle (E, F) and NS102 (10 mM in 5 μl DMSO) 20 min before ischemia (G, H). Data were obtained from 7 independent animals, and the results of a typical experiment are presented ($n = 7$). Scale bars: (A, C, E, G) = 200 μm; (B, D, F, H) = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This is different from previous studies which mainly focused on exploring the molecular basis for the association of PSD-95 with GluR6 and MLK3 in culture cell line and fusion proteins [8,31]. In our present study, the coimmunoprecipitation of GluR6, PSD-95, and MLK3 with one another further confirms the existence of GluR6·PSD-95·MLK3 signaling module, particularly in the in vivo ischemic rat hippocampus.

Previous in vitro studies show that the deletion of JNK3 gene in mice can inhibit kainate-induced epileptic seizures and neuronal toxicity [41], which is similar to GluR6-null mice showing resistance to neuronal degeneration and seizure induced by kainic acid [22]. This suggests that GluR6 subunit may be implicated in JNK3-mediated neuronal degeneration in hippocampus. In our current in vivo study, we provide evidence that GluR6 activates JNK3 via the GluR6·PSD-95·MLK3 signaling module and also that ischemic insult enhanced the association of PSD-95 with GluR6 and MLK3 in hippocampal preparations.

Coimmunoprecipitation experiments showed that ischemia–reperfusion significantly elevated the interaction among PSD-95, MLK3, and GluR6. Note that our laboratory showed that PSD-95 antisense oligodeoxynucleotides can inhibit the activation of MLK3 and JNK3 via the GluR6·PSD-95·MLK3 signaling module after transient cerebral ischemia in rat hippocampus [28], which is a more direct evidence that JNK is activated via this signaling module. In order to observe the JNK3 activation mechanism responding to brain ischemia, we selected the AMPA/KA receptor antagonist DNQX and the GluR6 antagonist NS102 to reveal whether the antagonists can attenuate the formation of GluR6·PSD-95·MLK3 signaling module, autophosphorylation of MLK3, and phosphorylations of JNK3 during reperfusion. Our data reveals that both DNQX and NS102 significantly inhibit the formation of GluR6·PSD-95·MLK3 signaling module, the interaction of MLK3 with JNK3, as well as the activation of MLK3 and JNK3. Furthermore, our results also show that the selective AMPA receptor

antagonist GYKI52466 and NMDA receptor antagonist ketamine had no effect on the formation and activation, excluding the possibility that this signaling was due to activation of AMPA receptor and NMDA receptor, that is, DNQX exerts its inhibitory effects especially through KA receptor. All our data suggest that GluR6 subunit of KA receptor selectively mediates JNK3 activation via the GluR6·PSD-95·MLK3 signaling module.

MLK3 contains an N-terminal SH3 domain, a leucine zipper, a Cdc42/Rac interactive binding (CRIB) motif, and a large proline-rich C-terminal region [7]. In resting cells, SH3 domain binds to a single proline residue between the zipper and CRIB motifs and silences the activity of MLK3 [42]. Therefore, MLK3 represents a serine/threonine kinase that is autoinhibited by an SH3-mediated intramolecular interaction. These inhibitory interactions can be disrupted in response to appropriate stimuli. Previous reports show that SH3 domain is first identified as targeting domains which induce intermolecular association via binding to proline-rich sequences [5,20]. MLK3 clustering is mediated by the interaction of its C-terminal proline-rich region with the SH3 of PSD-95 [31], while C-terminal amino acid sequence ETMA of GluR6 directly binds to PDZ domains of PSD-95, as reported previously [7,8]. It is probable that these bindings relieve SH3-mediated autoinhibition of MLK3. As to the results obtained in this study, the alteration of MLK3 autophosphorylation is parallel to the association of GluR6·PSD-95·MLK3 signaling module, suggesting the formation of GluR6·PSD-95·MLK3 signaling module facilitated MLK3 autophosphorylation. It is known that Thr277 and Ser281 serve as the autophosphorylation residues of MLK3 and, furthermore, that the autophosphorylation is crucial for MLK3 activation and the subsequent phosphorylation of downstream signaling pathway [18]. Consequently, the lever of autophosphorylation indirectly represents MLK3 activity. We speculated the whole process. First, ischemic stress triggered the excessive release of glutamate and induced the formation of GluR6·PSD-95·MLK3 signaling module via activation of KA receptor. During this process, the SH3 domain of PSD-95 bound to the C-terminal proline-rich region of MLK3, which may lead to subsequent remodeling, elimination of its autoinhibition, and facilitation of activation. Subsequently, activated MLK3 disassociated from GluR6·PSD-95·MLK3 complex, reassembled MLK3-MKK4/7-JNK3 signaling module, maybe via JIP-1 scaffold protein, and finally led to JNK activation.

During different reperfusion time points after 15 min of ischemia, the time course of the association of GluR6·PSD-95·MLK3 was consistent with that of MLK3 autophosphorylation, which decreased from 30 min to 3 h then peaked at 6 h and at last reduced after 1 day. At the same time, the interaction between MLK3 and JNK3 had a similar alteration. However, p-JNK3 exhibited two peaks during reperfusion. The early activation occurred at 30 min, whereas the late activation extended from 6 h to 3 days. The first activation of JNK3 at 30 min of reperfusion

might be closely connected with reactive oxygen species because our previous study has shown that antioxidant N-acetylcysteine can inhibit the early activation of JNK3 in response to cerebral ischemia [36]. Regarding the late activation peak, the association of PSD-95 with GluR6 and MLK3, MLK3 autophosphorylation, and JNK3 phosphorylation exhibited common peaks at 6 h of reperfusion, suggesting that the late activation of JNK3 was mediated by GluR6·PSD-95·MLK3 signaling module.

To explore the pathological role of JNK3 activation mediated by GluR6 in brain ischemia, our results show that NS102 significantly increases the number of surviving hippocampal CA1 pyramidal cells compared with single ischemic insult. Consequently, inhibition of kainate receptor, especially GluR6, effectively reduced the JNK activation-induced neuronal degeneration, which may give a possible therapeusis for cerebral ischemia. The late activation of JNK3 was closely connected with the GluR6·PSD-95·MLK3 signaling module. The GluR6·PSD-95·MLK3 signaling module was involved in neuronal cell death, suggesting that KA receptor plays an important role in activation of MAPK pathway and the delayed neuronal degeneration.

In summary, we report the formation and alteration of GluR6·PSD-95·MLK3 signaling module in rat hippocampus by investigating the protein–protein interactions following cerebral ischemia. Brain ischemia induced alteration of GluR6·PSD-95·MLK3 complex, and in turn had an effect on MLK3 autophosphorylation and ultimately changed the JNK3 phosphorylation. GluR6, a subunit of KA receptor, partially contributed to elevation of JNK3 activity in ischemic rat hippocampus. Our present study provided insight into the role of GluR6·PSD-95·MLK3 signaling module in JNK3 activation, the underlying molecular mechanisms, and its specific effect on the delayed neuronal cell death in ischemic stroke; it is also different from the role of Ca^{2+} in classical excitotoxicity especially through NMDA receptor. In view of the important role of JNK pathway in neuronal apoptosis, the clarification of JNK activation mechanism will be especially significant, which provides the valuable clue for valuable drug treatment of cerebral ischemia.

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