Differential Roles of NMDA Receptor Subtypes in Ischemic Neuronal Cell Death and Ischemic Tolerance

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Background and Purpose—Activation of NMDA subtypes of glutamate receptors is implicated in cell damage induced by ischemia as well as for the establishment of ischemic tolerance after ischemic preconditioning in animal models. We investigated the contributions of NR2A- and NR2B-containing NMDA receptors to ischemic cell death and ischemic tolerance in a rat model of transient global ischemia.

Methods—Transient global ischemia was produced in rats by 4-vessel occlusion. Neuronal injury was analyzed by Fluoro-Jade B and Nissl staining. Phosphorylation of CREB was detected by Western blotting and immunohistochemistry. In situ hybridization and reverse transcriptase–polymerase chain reaction were used to evaluate the mRNA level of cpg15 and bdnf.

Results—NR2A subtype-specific antagonist NVP-AAM077 enhanced neuronal death after transient global ischemia and abolished the induction of ischemic tolerance. In contrast, NR2B subtype-specific antagonist ifenprodil attenuated ischemic cell death and enhanced preconditioning-induced neuroprotection. Furthermore, selectively blocking NR2A-, but not NR2B-, containing NMDA receptors inhibited ischemia-induced phosphorylation of CREB and the subsequent upregulation of CREB target genes such as cpg15 and bdnf.

Conclusions—We found that NR2A- and NR2B-containing NMDA receptor subtypes play differential roles in ischemic neuronal death and ischemic tolerance, suggesting attractive new strategies for the development of drugs for patients with stroke. (Stroke. 2008;39:3042-3048.)

Key Words: CREB • ifenprodil • ischemic tolerance • NMDA receptor • NVP-AAM077 • transient global ischemia

Ischemic stroke, which results from cardiac arrest or cerebral arterial occlusion, causes devastating complications and represents a global health problem. Considerable evidence suggests that the release of excess glutamate during and after an ischemic insult leads to glutamate receptor hyperactivity, triggering harmful intracellular effects, including calcium overload and the generation reactive oxygen species. The disruption of cellular homeostasis eventually leads to neurodegeneration. On the other hand, a short period of sublethal ischemia can induce tolerance of neurons to subsequent, more prolonged ischemia. This phenomenon is defined as ischemia tolerance. Currently, the molecular mechanisms underlying both ischemic cell death and ischemia tolerance are largely unknown, but understanding the basis of these phenomena may provide new therapeutic strategies for this devastating neurological problem.

NMDA type of glutamate receptors has been implicated in both ischemic neuronal injury and ischemic tolerance. The noncompetitive NMDA receptor antagonist MK801 attenuates ischemic cell death and blocks the development of ischemic tolerance in gerbils and in cultured cortical neurons; however, evidence from the rat models of ischemia have not supported this. Drugs that modulate NMDA receptor functions have enormous therapeutic potential because of the involvement of these receptors in a wide array of neurological functions. However, the development of effective NMDA receptor-targeting drugs has been hindered by psychomimetic or cardiovascular side effects. Thus, elucidating the molecular mechanisms by which NMDA receptors exert their functions in ischemic tolerance and ischemic neuronal injury may provide clues as to how to design new therapies that prevent the pathological effects of NMDA receptors with fewer side effects.

The NMDA receptor is composed of an NR1 subunit combined with one or more NR2 subunits and, in some cases, an NR3 subunit. The NR2 subunit has 4 isoforms (NR2A to NR2D), which have distinct distributions in the central nervous system. In the adult brain, both NR2A and NR2B are prominent in the hippocampus. Recent studies suggest that NMDA receptor subunit-specific signaling may differentially govern the direction of synaptic plasticity and play differential roles in pathological conditions such as epilepsy...
and stroke.\textsuperscript{17–19} Given the essential role of NMDA receptors in ischemia and the selective enrichment of NR2A and NR2B in the hippocampus, which is sensitive to ischemic insult, we reasoned that NR2A- and NR2B-containing NMDA receptor subtypes might have differential roles in ischemic cell death and ischemic tolerance. Using a well-characterized rat model of transient global ischemia, we found that the NR2B subtype-specific antagonist ifenprodil remarkably attenuated ischemic neuronal death and enhanced ischemic tolerance, whereas the NR2A-selective antagonist NVP-AAM077 exacerbated the neuronal vulnerability induced by global ischemia and abolished the protective effect of ischemic preconditioning.

Materials and Methods

Surgery and Experimental Design
All experiments were performed on Sprague-Dawley (250 to 280 g) rats as previously described.\textsuperscript{20} Rats were purchased from Shanghai SLAC Laboratory Animal Co, Ltd. Rats were anesthetized with 20% chloral hydrate, both vertebral arteries were permanently occluded by electrocauterization at the level of the first cervical vertebra, and both carotid arteries were dissociated. The next day, rats were subjected to 10 minutes of global transient ischemia by temporary bilateral occlusion of the carotid arteries or a sham operation followed by reperfusion for the times indicated. In the ischemic preconditioning group, rats were subjected to 2 minutes of sublethal ischemia 48 hours before a 10-minute lethal ischemia. During the ischemic period, body temperature was maintained between 36.5 and 37.5°C. All procedures were approved by the Animal Experiment Committee of the Shanghai Institutes for Biological Sciences.

Drug Administration
The NR2A antagonist NVP-AAM077 was synthesized by our collaborator, Professor Wen-Hu Duan (Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) as described previously.\textsuperscript{21} The efficacy and specificity of NVP-AAM077 were confirmed in our previous study.\textsuperscript{17} The NR2B-specific antagonist ifenprodil was purchased from Sigma. After anesthesia, rats were fixed in a stereotaxic apparatus and a cannula was lowered into the right lateral ventricle at 0.9 mm caudal to bregma, 1.4 mm lateral to the midline, and 4.1 mm below the surface of the cerebral cortex. Both drugs were diluted in saline and were administered intracerebroventricularly 30 minutes before ischemia. The injection period was 10 minutes for 5 μL of 0.4 mmol/L NVP-AAM077 or 6 mmol/L ifenprodil as described in our previous study.\textsuperscript{17} The control group received the same volume of saline.

Fluoro-Jade B and Nissl Stainings
Fluoro-Jade B and Nissl stainings were performed as described previously.\textsuperscript{17} The number of Fluoro-Jade B-positive cells and Nissl-positive cells were quantified in the CA1 subregion in 2 pairs of 2 consecutive sections of the dorsal hippocampus (at approximately −3.6 and −4.5 mm from bregma) using Image-Pro software 5.0.

Western Blotting and Immunohistochemistry
Approximately 10 to 20 μg of hippocampal homogenate was loaded onto each lane and size-fractionated on 9% SDS-PAGE. After electrotransfer, polyvinylidene fluoride membranes were blocked with 5% milk in 0.05% Tween-20 for 1 hour and then probed with rabbit antiphospho-CREB (1:1000; Cell Signaling Technology). HRP-coupled goat anti-rabbit IgG (1:4000; Amersham) was used as the secondary antibody. The membranes were then stripped and probed with HRP-conjugated anti-GAPDH (1:5000; MultiSciences Biotech) or with anti-CREB antibody (1:2000; Santa Cruz) as a loading control. The bands were revealed by an enhanced chemiluminescence plus Western blotting detection system (Amersham Life Science).

For immunohistochemical experiments, brain slices were permethalized in 0.3% Triton X-100 for 60 minutes and blocked with 10% goat serum for 60 minutes. Slices were then incubated with rabbit antiphospho-CREB (1:200; Cell Signaling Technology) in 10% goat serum in phosphate-buffered saline at 4°C for 36 hours. After thoroughly wash with phosphate-buffered saline, slices were probed with Alex 488 conjugated goat antirabbit secondary antibody at 4°C for 36 hours (1:2000; Molecular Probes). The slices were viewed using a Zeiss 2 photon laser confocal scanning system (Zeiss LSM 510).

In Situ Hybridization
In situ hybridization was performed as previously described.\textsuperscript{17} Polymerase chain reaction products of the full-length open reading frame of rat cpg15 were ligated with pGEM-T Easy vector. The plasmids were linearized for in vitro RNA transcription to prepare antisense and sense DIG-RNA probes using a SP6/T7 transcription kit (Roche). Coronal brain sections were fixed and washed extensively with phosphate-buffered saline. They were treated for 10 minutes at room temperature with 50 μg/mL proteinkase K (Sigma) in PK buffer (5 mmol/L EDTA and 50 mmol/L Tris-HCl, pH 7.2) and washed with phosphate-buffered saline. Then they were treated with 4% formaldehyde for 15 minutes and washed with phosphate-buffered saline again before they were incubated in prehybridization solution for 3 to 4 hours at 60°C. Afterward, the sections were incubated with 0.5 to 1 μg/mL probes in hybridization solution for 12 to 16 hours at 65°C. After hybridization, the sections were sequentially washed, twice in 2× SSC at 60°C for 30 minutes, 2× SSC (including 0.1 g/mL RNase) at 37°C for 30 minutes, 2× SSC at room temperature for 10 minutes, twice in 0.2× SSC at 60°C for 30 minutes, and 0.2× SSC at room temperature for 15 minutes. The sections then were washed in PBT (1× phosphate-buffered saline, 2 mg/mL bovine serum albumin, and 0.1% Triton X-100) for 15 minutes before they were blocked with 5% bovine serum albumin in PBT for 3 hours at room temperature and incubated with preabsorbed antidigoxigenin antibody diluted to a final concentration of 1:3000 with 5% bovine serum albumin in PBT at 4°C overnight. Finally, the sections were washed in PBT and AP buffer (100 mmol/L Tris buffer, pH 9.5, 50 mmol/L MgCl₂, 100 mmol/L NaCl, and 0.1% Tween-20) and then reacted with nitroblue-tetrazolium chloride/5-bromo-4-chlor-indolyl-phosphate (1 μL nitroblue-tetrazolium chloride and 3.5 μL 5-bromo-4-chlor-indolyl-phosphate in 1 mL AP buffer) in the dark for 8 to 10 hours.

Reverse Transcriptase–Polymerase Chain Reaction
For analysis of cpg15 and bdnf expression in vivo, rats were decapitated under deep anesthesia. Brains were rapidly removed, and hippocampi were dissected quickly on ice and homogenized on ice in 1 mL of Trizol Reagent (Invitrogen). RNA was extracted according to the protocol provided by the manufacturer, and the final RNA pellet was suspended in diethylpyrocarbonate-treated water. Reverse transcription–polymerase chain reaction was performed using M-MLV kit (Invitrogen). Primer sequences were cp15: 5'–CCCGGTGCAAATAGCTTAC-3' 5'TATCTTTGGCCCTTCTCTG-3'; bdnf: 5'–CATCCAGTTCCACCAAGGT-3', 5'–CCATGGTGTCGGCACACGT-3'; and glyceraldehyde-3-phosphate dehydrogenase: 5'–CCCCAATGTACCTGGTTGTG-3', 5'–CTCAGTTAGCCAACAGGATGC-3'. Band intensities were quantification with PhosphorImager (Storm 860; Amersham Biosciences, Arlington Heights, Ill) using Image-Quant 5.0 software, and all bands were normalized as percentage of control values.

Statistical Analysis
Statistical data are given as mean±SEM. The significance of differences was determined using analysis of variance followed by post hoc t test using Prism 4.0 software. Statistical significance was defined as P<0.05.
Results

Previous work has demonstrated that NMDA-mediated excitotoxicity is involved in ischemic cell death.1,22-24 To determine whether NR2A- and NR2B-containing NMDA receptors have distinct roles in ischemic neuronal death, we administered the NR2A-selective antagonist NVP-AAM077 (2 nmol) or the NR2B-specific antagonist ifenprodil (30 nmol) by lateral ventricular microinjection 30 minutes before ischemic challenge and assessed neuronal death 3 days after reperfusion (Figure 1A). A single 10-minute ischemia resulted in widespread neurodegeneration in the CA1 region because a large percentage of CA1 pyramidal neurons were positively stained with Fluoro-Jade B (Figure 1B, left panel). Nissl staining revealed a remarkable neuronal loss in the CA1, whereas the CA3 and the DG remained intact (Figure 1B, right panel). Interestingly, pretreatment with NVP-AAM077 (2 nmol) exaggerated the damage in the CA1 and led to cell loss in the CA3 and the DG as well, subfields that are normally resistant to ischemic insult. Conversely, preischemic administration of ifenprodil (30 nmol) significantly reduced ischemic cell damage in the CA1 (Figure 1B-C).

To determine whether the 2 subtypes of NMDA receptors also play differential roles in ischemic tolerance, we admin-istered the selective antagonists NVP-AAM077 and ifenprodil before a brief period (2 minutes) of ischemia 48 hours before the lethal 10-minute ischemia and assessed neuronal death 3 days after reperfusion. A single 5-μL dose of NVP-AAM077, ifenprodil, or saline was delivered by intra-cerebroventricular injection 30 minutes before ischemic preconditioning (Figure 2A). Consistent with a previous finding,25 a 2-minute preconditioning ischemia robustly protected CA1 neurons against the subsequent lethal ischemia as shown by Fluoro-Jade B staining (Figure 2B, left panel) and Nissl staining (Figure 2B, right panel) of adjacent sections. The preconditioning ischemia alone did not induce cell death (data not shown). Inhibiting NR2A subtype receptors with NVP-AAM077 completely abolished the neuroprotective effect induced by ischemic preconditioning and further aggravated neuronal death in the preconditioned group. In contrast, blocking the NR2B subtype with ifenprodil further enhanced the neuroprotective effect induced by ischemic preconditioning (Figure 2D-E).

Previous work has demonstrated that ischemia for 2 minutes induces CREB phosphorylation in the gerbil hippocampus26,27 and suggests that CREB activation and subsequent CREB-dependent gene expression might play an important role in the acquisition of ischemic tolerance. To elucidate whether different subtypes of NMDA receptor also have unique effects on preconditioning-induced phosphorylation of CREB, we administered NVP-AAM077 or ifenprodil 30 minutes before preconditioning (Figure 3). Two minutes of ischemic preconditioning led to strong upregulation of phosphorylated CREB in CA1 pyramidal neurons but not in the CA3 region 30 minutes later compared with the sham-treated group as assessed by immunohistochemistry with an antibody specific to phosphorylated CREB. Blocking NR2A receptors significantly attenuated preconditioning-induced CREB phosphorylation in CA1 pyramidal neurons, whereas blocking NR2B receptors had no major effect (Figure 3A). These results were further confirmed by Western blotting of hippocampal homogenates with antiphosphorylated CREB.

Phosphorylated CREB levels increased 5 minutes and 30 minutes after ischemic preconditioning in hippocampal. However, NVP-AAM077, but not ifenprodil, abolished this upregulation (Figure 3B-E).

To determine whether NR2A or NR2B is involved in posts ischemia induction of CREB target gene expression, mRNA levels of the CREB target gene cpg15 were assayed in rats injected with NR2A or NR2B antagonists 30 minutes before a 2-minute ischemia challenge. We first performed a time course experiment to define the spatiotemporal changes...
in cpg15 mRNA expression after ischemic insult. Within 5 minutes of the 2-minute preconditioning ischemia, cpg15 mRNA was highly upregulated in the CA3 and DG, whereas expression was slightly increased in the CA1. cpg15 expression peaked 15 minutes after the ischemia and gradually decreased to baseline level after 12 hours (Figure 4A). Administering NVP-AAM077 blocked the induction of cpg15, whereas ifenprodil had no effect on preconditioning-induced upregulation (Figure 4B). These results were further confirmed by reverse transcriptase–polymerase chain reaction of hippocampal lysates. Preconditioning-induced upregulation of cpg15 and bdnf, another CREB target gene, were blocked by NVP-AAM077 but not ifenprodil (Figure 4C–D). These results together suggest that NR2A alone is responsible for mediating the upregulation of CREB and its targets after an ischemic insult.

Discussion
Our study presents 3 major findings. First, different NMDA receptor subtypes play different roles in ischemic cell damage and ischemic tolerance. Blocking NR2A-containing NMDA receptors enhanced neuronal death after transient global ischemia and abolished the induction of ischemic tolerance. In contrast, inhibiting NR2B-containing NMDA receptors attenuated ischemic cell death and enhanced preconditioning-induced neuroprotection. Second, NR2A but not NR2B mediates ischemia-induced CREB activation in the hippocampus. Blockade of NR2A inhibited CREB phosphorylation after ischemic preconditioning, but NR2B had no effect. Third, ischemic preconditioning-induced upregulation of the CREB target genes cpg15 and bdnf is NR2A but not NR2B subtype-dependent. Thus, NR2A-mediated CREB-dependent gene expression might provide a molecular basis for ischemic tolerance.

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system. NMDAR-mediated signals are critical for the survival of developing neurons and for several forms of synaptic plasticity.28 On the other hand, excessive glutamate causes excitotoxicity, which has been implicated in a diversity of neurodegenerative disorders. NMDA receptors are believed to be tetrameric protein complexes comprised of NR1 subunits with at least one type of NR2 subunit. Different NR2 subunits confer distinct electrophysiological and pharmacological properties on the receptors and couple with different signaling mechanisms. In mature cultured neurons, NR2A-containing NMDA receptors promote, whereas NR2B-containing NMDA receptors inhibit, the trafficking of GluR1 and contribute differentially to
activation of the downstream MAP kinase signaling pathway.29 Different roles of NMDA receptor subtypes in neuronal plasticity, epilepsy, and ischemic insult have also been described.17–19,30 We demonstrated here that blocking NR2A-containing NMDA receptors enhanced neuronal death after transient global ischemia. In contrast, inhibiting NR2B-containing NMDA receptors attenuated ischemic cell death.

The subcellular localization of NMDA receptors might determine the nature of NMDAR signaling after ischemic reperfusion. NR2A is preferentially located at synaptic sites, and NR2B is enriched in extrasynaptic sites in mature neurons.31,32 Furthermore, synaptic NMDAR activation promotes neuronal survival, whereas extrasynaptic NMDAR activation results in cell death.33,34 However, recent research suggests that the localization of receptors has little influence on the differential roles of NR2A- and NR1/NR2B-containing receptors in promoting cell survival and death.19 We recently found that neither NR2A nor NR2B selective antagonists block acute status epilepticus-induced death of pyramidal neurons in the hippocampus,17 suggesting that both subtypes can be involved in compromising survival after extensive NMDAR activation.

Previous studies have shown that after ischemia or stroke, selectively delayed neurodegeneration occurs in the hippocampus,35 whereas a short duration of ischemia protects cells from degeneration induced by a subsequent lethal insult. The degree of insult might govern different neuronal responses, thus initiating different downstream signaling pathways. A modest ischemic insult, or ischemic preconditioning, triggers a protective cellular program. However, mechanisms underlying the protective effects of preconditioning are poorly understood. That the NMDA receptor noncompetitive antagonist MK801 attenuates ischemia tolerance, whereas an AMPA receptor antagonist has no effect, suggests that activation of the NMDA receptor, but not the AMPA receptor, is involved in the protection of ischemia tolerance.36 Previous studies demonstrate that the NMDA receptor-dependent cellular signaling pathway is crucial in the protective effect of ischemia tolerance and that de novo protein synthesis is also required in this process.37–39 Our results demonstrated that blocking NR2A completely abolished the neuroprotective effect induced by ischemic preconditioning. However, blocking NR2B further enhanced the neuroprotective effect. We propose that combination of highly specific agonists for the NR2A-containing NMDA receptor and specific antagonists for the NR2B-containing NMDA receptor may have great potential for reducing the consequences of ischemia.

The molecular mechanisms underlying ischemic preconditioning have not yet been elucidated. The different roles of NMDA receptor subtypes might be largely dependent on the activation of their downstream signaling pathways. Ischemia induces rapid upregulation of CREB-targeted genes.57 Phosphorylation of CREB in neurons after ischemia or exposure to glutamate is induced by NMDA receptor-gated calcium influx. CREB phosphorylation and subsequent gene expression may play an important role in the acquisition of ischemic tolerance.27 We demonstrated here that blocking NR2A reduced the phosphorylation of CREB induced by preconditioning in CA1 pyramidal neurons, whereas blocking NR2B

Figure 3. Differential roles of NMDA receptor subtypes in CREB phosphorylation induced by ischemic preconditioning. A, Immunohistochemical analysis of phosphorylated CREB 30 minutes after ischemic preconditioning was performed in hippocampal slices from rats treated with vehicle or NMDA antagonists. B, D, Western analysis of hippocampal homogenates from rats treated with vehicle or NMDA antagonists and killed 5 minutes or 30 minutes after ischemic preconditioning. Gels were blotted with antiphospho-CREB and anti-CREB antibodies. Anti-GAPDH was used as loading control. C, E, Quantification of phospho-CREB and total CREB assessed by Western blotting analysis were normalized to the expression level of GAPDH. Bars represent mean ± SEM. *P < 0.01 compared with vehicle; **P < 0.001 compared with vehicle; n = 3 to 4.
had no effect. We further investigated the effect of NR2A and NR2B on a CREB-regulated gene. Cpg15 is an immediate–early gene induced by Ca\(^{2+}\) influx through NMDA receptors and t-type voltage-sensitive calcium channels and is regulated by multiple transcription factors including CREB.\(^{40}\) Soluble CPG15 protects cortical neurons from apoptosis by preventing activation of caspase pathways induced by growth factor deprivation.\(^{41}\) In a mouse model of transient global ischemia, CPG15 expression is upregulated and may function as a new factor involved in neuroprotection after injury. We found that an NR2A antagonist blocked induction of cpg15, whereas an NR2B antagonist had no effect on the upregulation of cpg15 induced by preconditioning. The differential roles of NR2A and NR2B in CREB activation and CREB target gene expression might contribute to differential cell survival and death.


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*Stroke*. 2008;39:3042-3048; originally published online August 7, 2008;
doi: 10.1161/STROKEAHA.108.521898

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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