Molecular Neurobiology

ISSN0893-7648/05/32(2): 145–155/$30.00

AMPA Receptor Subunit GluR2 Gates Injurious Signals in Ischemic Stroke

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Abstract

Ischemic stroke, or a brain attack, is the third leading cause of death in developed countries. A critical feature of the disease is a highly selective pattern of neuronal loss; certain identifiable subsets of neurons—particularly CA1 pyramidal neurons in the hippocampus—are severely damaged, whereas others remain intact. A key step in this selective neuronal injury is Ca^{2+}/Zn^{2+} entry into vulnerable neurons through α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor channels, a principle subtype of glutamate receptors. AMPA receptor channels are assembled from glutamate receptor (GluR)1, -2, -3, and -4 subunits. Circumstance data have indicated that the GluR2 subunits dictate Ca^{2+}/Zn^{2+} permeability of AMPA receptor channels and gate injurious Ca^{2+}/Zn^{2+} signals in vulnerable neurons. Therefore, targeting to the AMPA receptor subunit GluR2 can be considered a practical strategy for stroke therapy.

Index Entries: Hippocampus; CA1 neurons; selective neuronal injury; AMPA receptors; Ca^{21}/Zn^{21}-permeable channels; Ca^{2+}/Zn^{2+} signals; ischemic stroke.

Introduction

Ischemic stroke is caused by occlusion of blood vessels supplying the brain and is the third leading cause of death in developed countries. A critical feature of brain injury following transient forebrain ischemic insult is a highly selective pattern of neuronal loss in the central nervous system (CNS); certain identifiable subsets of neurons are severely damaged, including CA1 pyramidal neurons in the hippocampus, cortical projection neurons in layer 3, and medium spiny neurons in the dorsolateral striatum, whereas other neurons in the brain remain intact *(1–3)*. One step in this selective neuronal injury involves excessive stimulation of glutamate receptor (GluR) channels, allowing inflow of massive amounts of Ca^{2+}, Zn^{2+}, and Na^{+} in vulnerable neurons (reviewed in ref. *4*). Ca^{2+} overload can trigger several downstream lethal

Received December 2, 2004; Accepted March 16, 2005.
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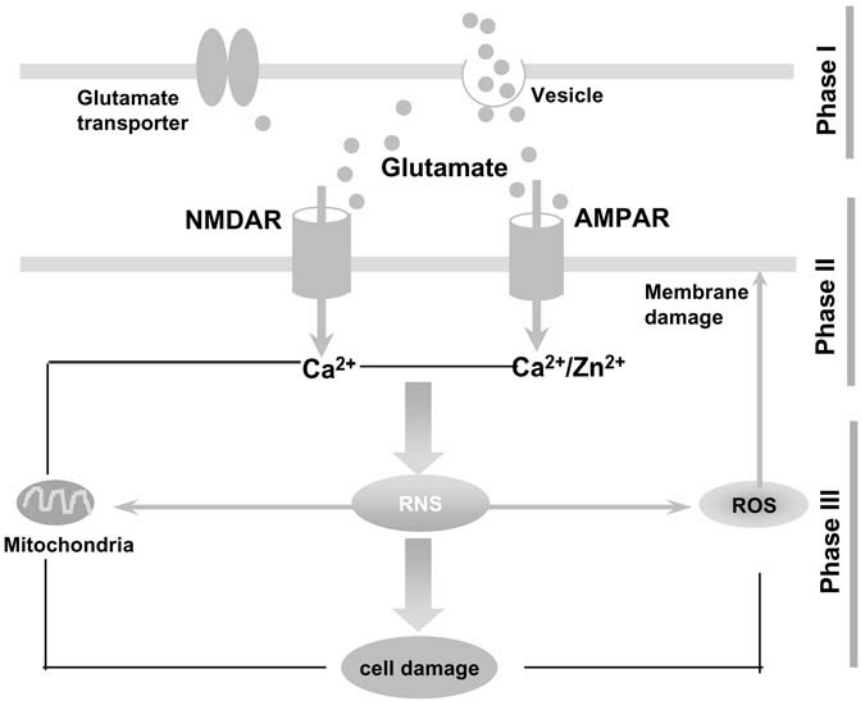

Fig. 1. Glutamate toxicity in ischemic injury. CA1 pyramidal neurons degenerate 3 to 6 d after transient forebrain ischemia. The cellular events underlying this delayed neuronal injury involve three phases. Phase I: during an episode (approx 5 min) of forebrain ischemia, extracellular glutamate concentration in CA1 area of the hippocampus increases because of increased release or decreased re-uptake. Ten minutes after ischemia, glutamate returns to the basal levels. Phase II: occurs 6 to 12 h after ischemia. A critical feature in this phase is the sustained changes of glutamate receptor channel activity, including: (a) AMPA receptor channels become permeable to Ca^{2+} and (b) NMDA receptor channel activity is enhanced. Subsequently, sustained Ca^{2+} inflow leads to mitochondria dysfunction and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Phase III: about 48 h after ischemia, ultimately, neuronal cell is damaged.

reactions, including nitrosative stress *(5–8)*, oxidative stress *(9,10)*, and mitochondrial dysfunction *(11–14)*, as reviewed earlier (*4,15*; Fig. 1). As noted in Fig. 1, the overstimulation of GluRs is the primary intracellular event that induces neuronal death. Therefore, synaptic GluR channels have been considered a promising target for stroke therapy *(16)*.

Glutamate Toxicity in Neuronal Injury

Glutamate plays an essential role in neural development, excitatory synaptic transmission, and plasticity *(17–21)*. Immediately following ischemia, however, glutamate accumulates at synapses *(22–24)*, resulting in extensive stimulation of its receptors, which can eventually be neurotoxic *(4,15,25,26)*. Glutamate activates three classes of ionophore-linked postsynaptic receptors—namely, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and kainic acid receptors. NMDA receptor toxicity is dependent on extracellular Ca^{2+} and, therefore, may reflect a large amount of Ca^{2+} influx directly through the receptor-gated ion channels *(4,15,25–28)*. Because most AMPA receptor channels have poor Ca^{2+} permeability (with exceptions, as discussed later), injury may result primarily from indirect Ca^{2+} entry through

Ca^{2+}-permeable transient receptor potential channel member (TRPM)-7 channels *(29)*, Ca^{2+}-permeable acid-sensing ion channels *(30)*, and voltage-gated Ca^{2+} channels *(31)*. Although excess stimulation of GluRs contributes to ischemic brain injury, blocking them completely could be deleterious to animals and humans because targeting these receptors would also block the physiological action of glutamate in noninjured neurons. Therefore, it may be fruitful to develop a practical approach whereby the pathological effects of glutamate in vulnerable neurons is selectively blocked, leaving the physiological action of glutamate in the CNS unaffected.

NMDA Receptor Channels in Selective Neuronal Injury

Glutamate toxicity largely results from NMDA receptor stimulation *(4,7,15,25,26,32)*. Knockout mice lacking the NMDA receptor *NR2A* gene show decreased cortical infarction after focal cerebral ischemia *(33)*. Systemic administration of an NMDA receptor antagonist protects CA1 pyramidal neurons from ischemic injury *(16)*, suggesting that activation of NMDA receptor channels following ischemia may mediate selective neuronal injury. However, these channels are widely expressed in many types of neurons, including ischemia-sensitive and -insensitive neurons in the CNS *(34,35)*. Therefore, certain pathological signaling molecules that modulate NMDA receptor function in vulnerable CA1 neurons may contribute to selective neuronal loss in the hippocampus of rats following transient forebrain ischemia *(1)*.

NMDA receptor channels are heteromeric complexes consisting of an essential NR1 subunit and one or more regulatory NR2 subunits, NR2A-D *(17,34,35)*. We recently discovered that transient forebrain ischemia causes phosphorylation of NR2A at serine-1232 (phospho-Ser^{1232}) in rat CA1 neurons in vivo *(1)*. We also determined that cyclin-dependent kinase 5 (Cdk5) catalyzes Ser^{1232} phosphorylation and that expression of a C-terminal peptide fragment of NR2A inhibits endogenous Cdk5 (or perturbs the Cdk5-NR2A interaction), thereby abolishing Ser^{1232} phosphorylation and protecting CA1 pyramidal neurons from ischemic insult *(1)*. Because Cdk5-mediated regulation of NMDA receptor channels occurs only in CA1 neurons and not in other areas of the hippocampus, researchers concluded that covalent modification of NMDA receptors by Cdk5 is an essential intracellular event that determines vulnerability of neurons to the insult *(1)*. Therefore, endogenous Cdk5 can be considered a suitable target regarding modification of cell survival during the diseases.

Expression of Ca^{2+}-Permeable AMPA Receptors in Vulnerable CA1 Pyramidal Neurons

Activation of endogenous Cdk5 depends on Ca^{21} entry through AMPA receptors *(1)*, which constitute a major GluR subtype *(19–23)*. Functional AMPA receptor channels are assembled from GluR subunits 1 through 4 *(19–23)*. The Ca^{21} permeability of AMPA receptor channels is determined by the GluR2 subunit *(36–43)*. In the adult CNS, nearly 100% of the messenger RNA (mRNA) encoding GluR2 is edited at the Q/R site corresponding to residue 607, where the genomic glutamine (Q^{607}) codon is converted to an arginine (R) codon *(37–42)*. Edited GluR2(R) subunits form Ca^{21}-impermeable channels, whereas unedited GluR2(Q) channels allow Ca^{21} entry *(36–43)*. In most CA1 neurons, AMPA receptor channels contain the GluR2(R) subunits and, therefore, are impermeable to Ca^{21} entry *(43)*. A recent study showed that transient forebrain ischemia reduces GluR2 mRNA levels in hippocampal CA1 neurons *(44)*. This observation led to the "GluR2 hypothesis," which postulates that reduced GluR2 expression allows Ca^{21} entry through AMPA receptor channels, which, in turn, induces CA1 pyramidal cell death *(44–47)*. The GluR2 hypothesis was proposed based on a causal relationship between CA1 pyramidal cell injury and reduced *GluR2* gene expression following transient forebrain ischemia *(45,46)*.

Therefore, it is important to understand whether a decrease of GluR2 mRNA actually causes AMPA receptor channels in vulnerable CA1 pyramidal neurons to become permeable to Ca^{2+} entry. Ca^{2+} indicator dyes can be used to estimate AMPA receptor-mediated Ca^{2+} transients in CA1 pyramidal neurons. Indeed, an earlier work demonstrated that AMPA caused a larger rise of intracellular Ca^{2+} $[Ca^{2+}]i$ in postischemic neurons compared to nonischemic control neurons *(45)*. In recent studies, we performed whole-cell patch clamp recordings in individual CA1 pyramidal neurons from the rat hippocampus visualized with infrared illumination and differential interference contrast (DIC, infrared-DIC) optics systems (*1,2*). AMPA receptors containing GluR2(R) showed inward currents with high extracellular Na^+, but not high Ca^{2+}, whereas receptors lacking GluR2(R) showed large inward currents with either high Na^+ or Ca^{2+} *(2)*. These data allowed us to determine the permeability of AMPA receptor channels to Ca^{2+} (P_{Ca}/P_{Na}) by calculating the shift in reversal potentials of AMPA receptor-mediated excitatory postsynaptic currents ($EPSCs_{AMPA}$) when the extracellular solution was switched from high Na^+ to high Ca^{2+}. Using this electrophysiological approach, we estimated that the Ca^{2+} permeability of AMPA receptor channels in CA1 pyramidal neurons of rats 12 h after transient global ischemia was 18-fold greater than that in sham controls. These data demonstrate that transient forebrain ischemia induces AMPA receptor channels permeable to Ca^{2+} entry into vulnerable CA1 pyramidal cells.

Inhibition of Ca^{2+}-Permeable AMPA Receptor Channels Protects Vulnerable Neurons From Forebrain Ischemic Insult

Transient forebrain ischemia induces expression of Ca^{2+}-permeable AMPA receptor channels in vulnerable CA1 neurons, which then activates a series of intracellular injurious signals *(1)*. To investigate this possibility, it is necessary to selectively block Ca^{2+} permeability of AMPA receptor channels in rat CA1 vulnerable neurons in vivo. An early work reported that administration of Naspm, an open-channel blocker selective for Ca^{2+}-permeable AMPA receptors *(48,49)*, reduced GluR2 antisense-induced cell death and suggested that cell death induced by multiple injections of GluR2 antisense was mediated by Ca^{2+}-permeable AMPA receptors *(47)*. Recently, we generated virus-based gene expression vectors to directly introduce the *GluR2(R)* gene that is independent of Q/R site editing into the adult rat hippocampus in vivo. Using whole-cell patch clamp recordings, we obtained direct evidence that direct introduction of the *GluR2(R)* gene selective inhibits Ca^{2+} permeability of AMPA receptor channels occurring in vulnerable CA1 pyramidal neurons of adult rats following transient forebrain ischemia *(2)*. This study provides the first direct evidence that selective inhibition of Ca^{2+} permeability of AMPA receptor channels rescues CA1 pyramidal neurons from forebrain ischemic insult *(2)*.

Conversely to CA1 pyramidal neurons, granule neurons in the hippocampus are insensitive to ischemic injury *(1–3)*, and AMPA receptor channels in these cells are Ca^{2+}-impermeable. To further demonstrate whether expression of Ca^{2+}-permeable AMPA receptor channels determine vulnerability of neurons to the insult, we engineered these granule cells to express Ca^{2+}-permeable GluR2(Q) channels by direct introduction of exogenous unedited *GluR2(Q)* gene into the adult hippocampus. Following this manipulation, we found that the granule neurons degenerate following transient global ischemia *(2)*. Similarly to our results observed upon expression of unedited *GluR2(Q)* gene, a recent report showed that delivery of GluR2(Q) complementary DNA into the hippocampus by a liposome-mediated gene transfer increases the sensitivity of neurons in the hippocampus to forebrain ischemia insult *(50)*. Together, it is suggested that the expression of Ca^{2+}-permeable AMPA receptor channels provides a route for toxic Ca^{2+} entry, which then triggers neuronal cell death in the hippocampus *(51–55)*.

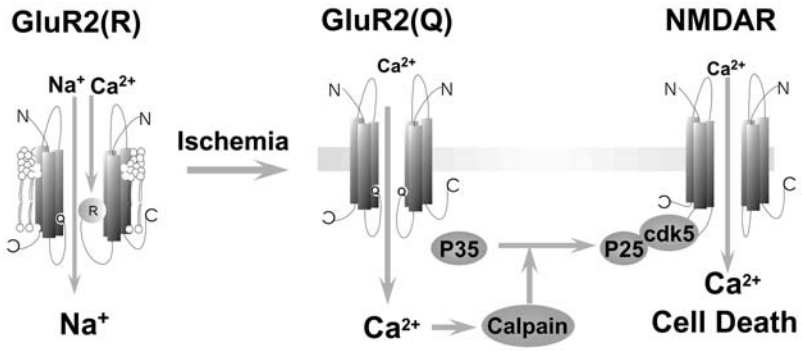

Fig. 2. Induction of Ca^{2+}-permeable AMPA receptor channels primes cell death. AMPA receptor channels in CA1 pyramidal neurons normally contain an edited form of GluR2 subunit (GluR2[R]) and, therefore, are Ca^{2+} impermeable. Following transient forebrain ischemia, GluR2(R) levels in CA1 neurons decreases, resulting in Ca^{2+} entry through AMPA receptor channels (*see also* refs.*45* and *46*). The subsequent p25 accumulation leads to prolonged activation of Cdk5, which phosphorylates the NR2A subunit of NMDA receptors at Ser^{1232} and induces further Ca^{2+} entry through NMDA receptor channels, leading to cell death.

Ca^{2+}-permeable AMPA receptor channels are not only permeable to Ca^{2+} but are also highly permeable to Zn^{2+} *(51–55)* and that a brief ischemic insult in rats induces Zn^{2+} accumulation in vulnerable neurons *(56,57)*. Therefore, Ca^{2+}/Zn^{2+} influx can be considered an initial event for activation of an array of downstream enzymes that degrade membranes and proteins essential for cellular integrity, as illustrated in Fig. 1. Our recent results demonstrate that expression of the *GluR2(R)* gene blocks Ca^{2+} entry through AMPA receptor channels and prevents activation of endogenous Cdk5 in vulnerable CA1 neurons *(1,2)*. Furthermore, Cdk5 activation causes the sustained opening of NMDA receptor channels, resulting in an intracellular Ca^{2+} overload; we propose that a GluR2-gated intracellular signaling cascade determines selective neuronal vulnerability after forebrain ischemia, as described in Fig. 2.

The Q/R Site Editing of GluR2 in Vulnerable CA1 Pyramidal Neurons

As discussed earlier, the Ca^{2+}/Zn^{2+} permeability of AMPA receptor channels is dominated by the GluR2 subunit; an edited GluR2(R) forms Ca^{2+}-impermeable AMPA receptor channels, whereas the unedited GluR2(Q) allows Ca^{2+} entry through the channels *(37–43)*. Accordingly, expression of Ca^{21}/Zn^{21}-permeable AMPA receptor channels in vulnerable CA1 neurons following transient forebrain ischemia can simply result from reduced *GluR2* gene expression, as described earlier. Notably, delivery of GluR2 proteins from intracellular pools to cell surface is required for expression of functional AMPA receptor channels at CA1 synapses *(57,58)*. Therefore, reduction of GluR2 protein levels should decrease the numbers of functional AMPA receptor channels, as illustrated in Fig. 3A.

However, electrophysiological recordings of the miniature $EPSCs_{AMPA}$ from CA1 pyramidal neurons revealed that the mean amplitude of the miniature $EPSCs_{AMPA}$ in rats that were subjected to transient forebrain ischemia was the same as that in sham animals (Fig. 3B,C). These results are consistent with previous reports that, although AMPA receptor channels are permeable to Ca^{2+} *(1,2)*, the $EPSCs_{AMPA}$ are not reduced *(59,60)*. Because two distinct GluR1/GluR2 and GluR3/GluR2 channels are located in CA1 pyramidal neurons *(61–63)*, unchanged amplitudes of synaptic responses

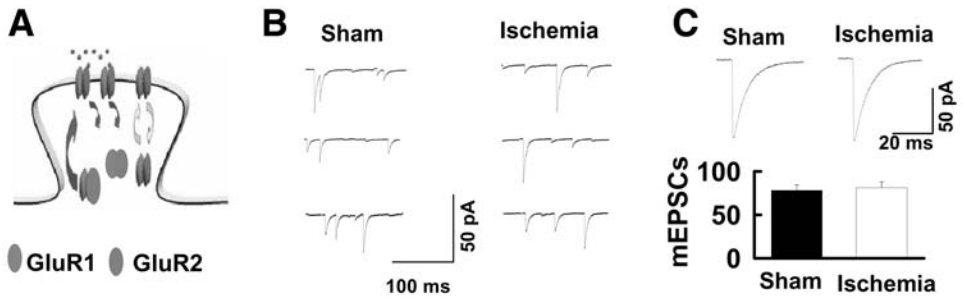

Fig. 3. The mean amplitude of miniature EPSCs$_{AMPA}$ remain unchanged in CA1 pyramidal neurons following transient forebrain ischemia. **(A),** An illustration shows GluR2-dependent translocation of GluR1/GluR2 receptor channels at CA1 synapses. Note: GluR1 is unable to incorporate into cell surface without GluR2. **(B),** Sample traces of miniature EPSCs$_{AMPA}$ at 270 mV taken from CA1 pyramidal neurons in hippocampal slices from rats 12 h after sham or transient forebrain ischemia. The recordings were in the presence of 50 μ*M* of AP5, 10 μ*M* of Bicucculline. **(C),** Averaged traces of the miniature EPSCs$_{AMPA}$ from **(A)** are shown on the top. The mean amplitudes (pA) of the responses are summarized in bar graph. Data are mean 6 SEM (n = 8 cells/four animals).

following reduced *GluR2* gene expression could result from overexpression of GluR1 and GluR3 subunits. To address this question, we analyzed protein levels of AMPA receptor subunits in CA1 area of the hippocampus from rats 12 h after transient forebrain ischemia. Western blots demonstrated that the GluR2 proteins in CA1 dorsal hippocampus were reduced by 38.8 ± 4.9% (n = 6; Fig. 4) compared to sham control rats (*p < 0.01). Unlike the GluR2 subunit, protein levels of GluR1, GluR3, and GluR4 subunits in CA1 neurons following transient forebrain ischemia were insignificantly changed. Together, we suggest that transient forebrain ischemia facilitates the events for incorporation of GluR2 subunits to plasma membrane. Under this circumstance, although *GluR2* gene expression is reduced, the numbers of functional AMPA receptor channels remain unchanged.

Previous studies revealed that Q/R site-unedited GluR2 easily exits the endoplasmic reticulum and becomes the cell surface receptor channels *(64,65)*. These findings implicate that pre-mRNA editing of GluR2 at Q/R site could be impaired in vulnerable CA1 pyramidal neurons following transient forebrain ischemia. Consistent with this hypothesis, recent studies have demonstrated that impaired Q/R site editing contributes to selective motor neuronal death in patients with amyotrophic lateral sclerosis *(66,67)*. Gene-targeting studies demonstrated that the deficits of GluR2 pre-RNA editing at Q/R site reduced GluR2 mRNA levels because of nuclear accumulation of incompletely processed primary GluR2 transcripts *(58)*. Therefore, reduced GluR2 mRNA levels observed in the CA1 area of the rat hippocampus following transient forebrain ischemia may result from reduced Q/R site editing of GluR2 pre-mRNA. To address this question, it is necessary to use single-cell reverse transcriptase-polymerase chain reaction to estimate GluR2 Q/R editing versus pre-mRNA accumulation in the nucleus of individual neurons in the rat hippocampus.

Transcriptional Regulation of GluR2 Expression in CA1 Neurons

Cell-survival-related gene expression has its basis in signaling cascades initiated by the transcription factor cyclic adenine monophospate response element-binding protein (CREB; refs. *68–71*), which is believed to regulate GluR2 promoter activity *(72)*. Therefore, we examined whether CREB activation stimulates expression of the *GluR2* gene and, in turn, inhibits GluR2-gated injurious signals in vulnerable CA1 neu-

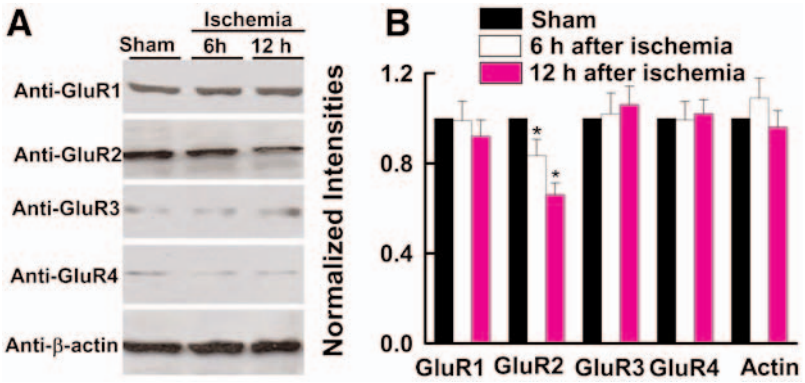

Fig. 4. GluR2 proteins in CA1 hippocampus is reduced following transient forebrain ischemia. **(A),** Representatives of Western blots of the CA1 cell lysates with antibodies, as indicated. **(B),** Summarized data are shown in the bar graph. In this study, the CA1 extracts (20 μg of protein) were denatured with sodium dodecyl sulfate sample buffer and separated by SDS-polyacrylamide gel electrophoresis through a 12% gel; transferred into nitrocellulose membranes; and incubated with rabbit anti-GluR1 (1:500; Chemicon), anti-GluR2 (1:200; Santa Cruz), anti-GluR3 (1: 200; Santa Cruz), antiGluR4 (1:500; Santa Cruz), and anti-β-actin (1:1000; Chemicon). Immunoreactive bands were detected using an enhanced Chemiluminescent kit (Amersham Biosciences) and quantiated using Densitometer Quantity One (Bio-Rad). Each reactive band was normalized its respective sham (defined as 1.0). Data are mean 6 SEM ($n = 4$; $^*p < 0.01$, compared to sham).

rons *(2)*. For this purpose, we expressed a constitutively active CREB, VP16-CREB, in rat CA1 pyramidal neurons in vivo *(2)*. VP16-CREB expression inhibited Ca^{2+}-permeable AMPA receptor channels in vulnerable CA1 pyramidal neurons following transient forebrain ischemia. Although the exact nature of the CREB signals for neuronal survival remains to be determined, expression of GluR2(R) may be one of the CREB pathways responsible for neuronal survival, because the protective role of CREB is abolished by directly introducing unedited GluR2(Q) gene *(2)*. Therefore, on the one hand, CREB increases GluR2 gene expression, thereby increasing the relative level of GluR2(R). The finding that CREB induces expression of brain-derived neurotrophic factor (BDNF) supports this hypothesis *(73,74)*. BDNF binds to the GluR2 promoter that contains the gene silencing transcription factor neuronal repressor element-1 silencing transcription factor (REST)/neuron-restrictive silencer element (NRSE). Additionally, it was reported that forebrain ischemia induces expression of REST and, in turn, reduces GluR2 gene expression *(75)*. Importantly, acute knockdown of the REST gene rescues postischemic neurons from ischemia-induced cell death *(75)*. Therefore, BDNF probably disrupts the interaction of REST/NRSE with the GluR2 promoter, thus stimulating GluR2 gene expression *(72)*. On the other hand, CREB activation may directly facilitate Q/R site editing of GluR2 mRNA, because increased mRNA editing also increases GluR2 mRNA levels by reducing nuclear accumulation of incompletely processed primary transcripts *(76).*

Conclusion

To date, all clinical stroke trials targeting GluRs (AMPA or NMDA) have failed, possibly because receptor antagonists also block the physiological actions of glutamate in noninjured neurons *(77,78)*. This article outlines a potential approach to selectively block the pathological effects of AMPA receptors by targeting GluR2 subunit, thereby inhibiting only Ca^{21}/Zn^{21} permeability. Thus, this approach should not affect the physiological actions of

Ca^{21}/Zn^{21}-impermeable AMPA receptors in noninjured neurons in the CNS. Therefore, this work may define a promising target for stroke therapy. As reported *(66,67)*, a defect in GluR2 editing also contributes to motor neuron death in amyotrophic lateral sclerosis. It is probable that a similar therapeutic approach may apply to this disease.

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