Therapeutic Targets for Cerebral Ischemia Based on the Signaling Pathways of the GluN2B C Terminus

Yongjun Sun, PhD; Linan Zhang, PhD; You Chen, BE; Lijing Zhan, BE; Zibin Gao, PhD

Overactivation of the N-methyl-D-aspartate receptor (NMDAR) after cerebral ischemia is a crucial reason for neuron death. Although NMDAR antagonists have exhibited neuroprotective effects in animal models, it is disappointing that several severe side effects have occurred in patients. NMDAR is a heteromer containing 2 obligate N-methyl-D-aspartate receptor 1 (GluN1) subunits and a variety of GluN2 and GluN3 subunits. The GluN2 subunit, which contributes specifically to neuron death after stroke, has been studied extensively. An opposing action of the GluN2A and GluN2B subunits in mediating cell death and cell survival was observed.1,2 The results indicate that the GluN2A subunit produces prosurvival activity, whereas the GluN2B subunit leads to a prodeath signal. However, von Engelhardt et al3 found that the GluN2A subunit can also mediate NMDA-dependent toxicity in DIV21 cultures. This paradox may have resulted because the pharmacological approach used to study subunit composition was not flawless.4 In view of this limitation and according to the methods of molecular biology, Martel et al4 demonstrated that the C-terminal domains of GluN2B promote neuron death more efficiently than those of GluN2A in cerebral ischemia.5 In short, NMDARs containing GluN2B are more lethal than those containing GluN2A. Prodeath signaling pathways mediated by neuronal nitric oxide synthase (nNOS), death-associated protein kinase 2 (DAPK2), phosphatase and tensin homolog located on chromosome 10 (PTEN), and calcium/calmodulin-dependent protein kinase II (CaMKII) have been linked to GluN2B activation. Therapeutic targets based on these signaling pathways of the GluN2B carboxy terminus (C terminus) will be introduced in this review.

Therapeutic Targets Based on the Signaling Pathways of the GluN2B C Terminus

GluN2B–nNOS Signaling Pathway

The GluN2B–nNOS signaling pathway, which plays an important role in neuron death, is the most widely studied GluN2B pathway (Figure 1). GluN2B/PSD-95/nNOS Complex

The PDZ domains of postsynaptic density-95 (PSD-95) can bind to nNOS and the C terminus of GluN2B. According to the coupling of PSD-95, the production of nitric oxide (NO) can be regulated by NMDAR. NO derived from GluN2B/PSD-95/nNOS signaling not only mediates NMDAR-dependent excitotoxicity but also inhibits regenerative repair via the regulation of histone deacetylase 2 during the recovery stage.6 Blocking NO production derived from nNOS by interfering with the formation of the GluN2B/PSD-95/nNOS complex may be a promising strategy.

Tat-NR2B9c, which comprises the cell-membrane transduction domain of the HIV-1 Tat protein and the C-terminal residues of GluN2B, is the first reported peptide that perturbs NMDAR–PSD-95 protein interaction.7 Tat-NR2B9c can protect cultured neurons from excitotoxicity, reduce focal ischemic brain damage in animals, and improve their neurological function.8 It is worth mentioning that Tat-NR2B9c is safe and effective in treatment of patients with iatrogenic stroke after endovascular aneurysm repair.9 In addition, disrupting nNOS–PSD–95 interaction via ZL006,10 IC87201,11 honokiol,12 4-phenyl-1-(4-phenylbutyl)-piperidine,13 and trimaprisate14 can prevent excitotoxicity and does not influence nNOS catalytic activity.

PSD-95 may be an alternative therapeutic target for excitotoxicity. Aarts et al15 found that Tat-PDZ1-2 can decrease excitotoxicity after NMDA application in cultured cortical neurons. Wang et al16 proved that overexpression of the PDZ1 domain can perturb the binding of PSD-95 to NMDAR, suppress the activity of both NMDAR and nNOS, and thus protect rat hippocampal CA1 neurons against cerebral ischemic injury. It was also reported that the suppression of PSD-95 by antisense oligonucleotides diminishes postsischemic pyramidal cell death in the rat hippocampal CA1 subfield.17 Bach et al18 reported the design and synthesis of a novel dimeric inhibitor, Tat-N-dimer (Tat-NPEG4(IETDV)2), which interacts with PDZ1-2 of PSD-95 and protects against ischemic brain damage.

The formation of the GluN2B/PSD-95/nNOS complex depends on the interaction of the PDZ domains of PSD-95 with GluN2B and nNOS. Most of the compounds uncoupling this...
complex may be ligands of the PDZ domain. On one hand, the human genome contains hundreds of different PDZ domain-containing proteins and all of the NMDAR subunit C termini possess a promiscuous type I PDZ interaction motif; however, above any other PDZ proteins, PSD-95 and nNOS are important for effecting NMDAR-dependent excitotoxicity. In spite of this, removing peroxynitrite, the downstream cytotoxic molecule of nNOS, by pharmacological antioxidants is a promising strategy in cerebral ischemia treatment.

**Downstream Molecules of nNOS**

The C-terminal PDZ ligand of nNOS (CAPON) is a protein specifically associated with nNOS. The C-terminal 13 amino acids of CAPON interact with the PDZ domain of nNOS and the N-terminal PTB domain of CAPON can interact with downstream molecules of nNOS. It was reported that the nNOS–p38MAPK pathway is mediated by CAPON during neuronal death after an excitotoxic stimulus. L-TAT-GESV, a cell-permeable peptide, can compete for the unique PDZ domain of nNOS that interacts with CAPON and can double the amount of survival tissue in a severe model of neonatal hypoxia–ischemia. CAPON may be a new high-specificity target for ischemia.

Dexamethasone–induced Ras-related protein 1 (Dexras1) is a small G protein that is specifically coupled to nNOS via CAPON. Stimulation of NMDA receptors activates nNOS, leading to S-nitrosylation and the activation of Dexras1, which physiologically induces iron uptake. Iron overload results in neuronal damage. Salicylaldehyde isonicotinoyl hydrazone (SIH), a selective cell-permeable iron chelator, markedly protects neurons from cell death induced by NMDA, and the deletion of Dexras1 in mice attenuates NMDA-induced excitotoxicity. Moreover, downregulated Dexras1 is involved in the protective effects of calycosin on cerebral ischemia rats. Thus, drugs that selectively block Dexras1 may be neuroprotective.

Poly(ADP-ribose) synthetase (PARS) is enhanced after focal ischemia in the rat brain. nNOS is responsible for the activation of PARS. Increased NO coming from nNOS can react with superoxide to form peroxynitrite. Peroxynitrite, but not various NO donors, activated PARS and attenuated poly(ADP-ribose) formation in mice deficient for nNOS. The extensive activation of PARS can rapidly lead to cell death through depletion of energy stores and damage to DNA. PARS inhibitors display protective effects against cerebral ischemia.

**Neuronal Nitric Oxide Synthase**

Although nNOS inhibitors can reduce the infarct volume in both permanent and transient models, blockade of NO signaling in rats may impair normal physiological function, such as open field behavior, limb coordination, and fear conditioning. In spite of this, removing peroxynitrite, the downstream cytotoxic molecule of nNOS, by pharmacological antioxidants is a promising strategy in cerebral ischemia treatment.
The p38 stress–activated protein kinase (p38MAPK) is a proposed downstream prodeath effector of nNOS. Cao et al. found that NOS inhibitors reduce both glutamate-induced p38 activation and the resulting neuronal death. Li et al. reported that excitotoxic activation of p38MAPK and subsequent neuronal death were reduced by competition with the nNOS–CAPON interaction and by knockdown with CAPON-targeting small interfering RNAs. nNOS–CAPON uncouplers and p38MAPK inhibitors may become new types of anti-ischemia drugs.

Striatal-enriched phosphatase (STEP) is a brain-specific intracellular tyrosine phosphatase which is related to excitotoxicity. Whole STEP plays an initial role in neuroprotection by disrupting the p38MAPK pathway. However, the degradation of active STEP is associated with the secondary activation of p38MAPK. The application of a cell-permeable STEP-derived peptide, Tat-STEP, which is resistant to degradation of active STEP, inhibits the destructive effect of nNOS. Therefore, the combined application of these types of drugs or their use as an alternative treatment for PSD-95–nNOS uncouplers is supported.

**GluN2B–DAPK1 Signaling Pathway**

DAPK has been identified as a novel Ca²⁺/calmodulin-dependent protein kinase and is maintained at substantial levels in the nervous system. DAPK is activated by dephosphorylation in response to cerebral ischemia. Tu et al. demonstrated that cerebral ischemia recruits DAPK1 into the GluN2B protein complex (Figure 2) and potentiates its activity and that the disruption of this association by GluN2B<sub>ct</sub> reduces damage in the brain. Also, protection of pyruvate against glutamate excitotoxicity is mediated by regulation of the DAPK1 protein complex.

Kang et al. reported that DAPK is regulated by DANGER and that DANGER binds directly to DAPK and inhibits its catalytic activity. DANGER may physiologically regulate the viability of neurons and may represent a potential therapeutic target for stroke and neurodegenerative diseases.

DAPK1 phosphorylates p53 at serine-23, acting as a functional version of p53. The tumor suppressor p53 is a sequence-specific transcription factor that can trigger both transcription-dependent and mitochondria-related apoptosis. Interrupting DAPK1–p53 interaction by the deletion of DAPK1 death domain or application of Tat-p53DM can protect mouse cortical neurons from ischemic damage. Thus, the p53 may be a desirable target for the treatment of ischemic insults.

Protein kinase D (PKD) is a kinase substrate of DAPK. It has been reported that oxidative stress enhances the interaction between DAPK and PKD in 293 cells, which leads to the activation of c-Jun N-terminal kinase (JNK) and programmed necrosis. JNK is an important stress-responsive kinase that is activated by cerebral ischemia. Both D-JNKI-1, a peptide inhibitor of JNK, and SP600125, a small molecule inhibitor of JNK, diminished JNK activity after ischemia and reduced the infarct volume in a dose-dependent manner. Although NMDAR-mediated activation of JNK is PSD-95-independent, this does not exclude a possible connection between GluN2B and JNK. Therefore, GluN2B/DAPK1/PKD/JNK may be a prodeath signaling pathway after stroke.

Autophagy not only exercises important biological functions but also critically contributes to the neuronal fate on cerebral ischemic stress. Zalckvar et al. reported that DAPK1 phosphorylates beclin-1 at Thr119 and promotes the induction of autophagy. Moreover, p53 can also induce autophagy. The upregulated level of autophagy after cerebral ischemia is because of the increased activity of DAPK1. Increasing evidence indicates that the inhibition or induction of autophagy under some conditions may be neuroprotective. Although whether inhibition of autophagy increases or decreases the rate of neuronal death is still under debate, it is certain that autophagy-related proteins may be new therapeutic targets for stroke.

In view of their powerful prodeath effects, targeting DAPK1, p53, or JNK may be more effective than targeting others. However, there are several problems that require attention. One is that DAPK1 and p53 are important tumor suppressors, and enhanced cell proliferation effects induced by the inhibition of DAPK1 or p53 should be carefully studied. Second, the relationship of GluN2B, DAPK1, and JNK should also be confirmed. Finally, it is must be clarified whether an enhanced level of autophagy induces neuron death after stroke.
GluN2B–PTEN Signaling Pathway

PTEN is a tumor suppressor that plays an important role in the regulation of several cellular processes. It has been reported that PTEN physically associates with the C terminus of NR1 in GluN2B-containing NMDA receptors. PTEN also contains a PDZ-binding motif at its C terminus and NMDA receptor activation triggers a PDZ-dependent association between PTEN and PSD-95. GluN2B, PSD-95, and PTEN may form a complex in vivo. nNOS is involved in the S-nitrosylation activation of PTEN after cerebral ischemia in the rat hippocampus. Therefore, PTEN may be activated by the GluN2B signal through nNOS. PTEN can induce neuronal damage after ischemic insults through several pathways (Figure 3): antagonizing the phosphatidylinositol-3 kinase (PI3K) signaling pathway, negatively regulating the membrane expression and function of GABAARs, positively regulating extrasynaptic NMDARs and interfering with nuclear signaling. However, PTEN also induces a neuroprotective effect through activating the GluN2A cell prosurvival pathway. Although it has a neuroprotective effect, the main action of PTEN is to promote neuronal death after stroke. Pretreatment with potassium bisperoxo (1,10-phenanthroline) oxovanadate (bpv),

Table. Experimental Data With Effective Postsischemic Treatment in Vivo

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Potential Drugs</th>
<th>Animals</th>
<th>Model</th>
<th>Effective Administration Time</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disruption of GluN2B–PSD-95 interaction</td>
<td>Tat-NR2B9c</td>
<td>Rats</td>
<td>MCAO</td>
<td>1 or 3 h after stroke</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Macaques</td>
<td>ES</td>
<td>IS</td>
<td>Just after aneurysm repair</td>
<td>12</td>
</tr>
<tr>
<td>Disruption of GluN2B–PSD-95–nNOS interaction</td>
<td>Tat-HA-NR2B9c</td>
<td>Rats</td>
<td>MCAO</td>
<td>4 d after stroke</td>
<td>10</td>
</tr>
<tr>
<td>Disruption of PSD-95–nNOS interaction</td>
<td>ZL006</td>
<td>Mice</td>
<td>MCAO</td>
<td>1 or 3 h after reperfusion</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Honokiol</td>
<td>Rats</td>
<td>MCAO</td>
<td>0, 1, or 3 h after reperfusion</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PPBP</td>
<td>piglets</td>
<td>HI</td>
<td>5 min after recovery</td>
<td>15</td>
</tr>
<tr>
<td>nNOS inhibitor</td>
<td>TRIM</td>
<td>Rats</td>
<td>MCAO</td>
<td>5 or 90 min after stroke</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>7-Ni</td>
<td>Rats</td>
<td>MCAO</td>
<td>5 min after stroke</td>
<td>21</td>
</tr>
<tr>
<td>Disruption of nNOS–CAPON interaction</td>
<td>Tat-GESV</td>
<td>Rats</td>
<td>HI</td>
<td>Just after carotid occlusion</td>
<td>27</td>
</tr>
<tr>
<td>Scaevenging peroxynitrile</td>
<td>Baicalin</td>
<td>Rats</td>
<td>MCAO</td>
<td>At the onset of reperfusion</td>
<td>25</td>
</tr>
<tr>
<td>Reducing degradation of active STEP</td>
<td>Tat-STEP</td>
<td>Rats</td>
<td>MCAO</td>
<td>6 h after stroke</td>
<td>38</td>
</tr>
<tr>
<td>Disruption of GluN2B–DAPK1 interaction</td>
<td>Tat-NR2B13</td>
<td>Mice</td>
<td>MCAO</td>
<td>60 min after stroke</td>
<td>41</td>
</tr>
<tr>
<td>Disruption of DAPK1–p53 interaction</td>
<td>Tat-p53DM</td>
<td>Mice</td>
<td>MCAO</td>
<td>6 h after stroke</td>
<td>47</td>
</tr>
<tr>
<td>JNK Inhibitor</td>
<td>D-JNKI-1</td>
<td>Mice</td>
<td>MCAO</td>
<td>6 or 12 h after stroke</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>SP600125</td>
<td>Mice</td>
<td>MCAO</td>
<td>0, 0.5, or 1h after reperfusion</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Tat-JBD</td>
<td>Mice</td>
<td>MCAO</td>
<td>30 min after reperfusion</td>
<td>52</td>
</tr>
<tr>
<td>PTEN inhibitor</td>
<td>bpv</td>
<td>Mice</td>
<td>MCAO</td>
<td>24 h after stroke</td>
<td>68</td>
</tr>
<tr>
<td>Reducing PTEN nuclear translocation</td>
<td>Tat-K13</td>
<td>Rats</td>
<td>MCAO</td>
<td>2 or 6 h after stroke</td>
<td>65</td>
</tr>
<tr>
<td>Autonomous CaMKII inhibitor</td>
<td>Tat-CN21</td>
<td>Mice</td>
<td>MCAO</td>
<td>1 h after stroke</td>
<td>75</td>
</tr>
</tbody>
</table>

bpv indicates potassium bisperoxo (1,10-phenanthroline) oxovanadate (V); CaMKII, calcium/calmodulin-dependent protein kinase II; CAPON, C-terminal PDZ ligand of nNOS; DAPK1, death-associated protein kinase 1; ES, embolic stroke; GluN, N-methyl-D-aspartate receptor; HI, hypoxia-ischemia; IS, iatrogenic stroke; JNK, c-Jun N-terminal kinase; MCAO, middle cerebral artery occlusion; nNOS, neuronal nitric oxide synthase; PSD-95, postsynaptic density-95; PTEN, phosphatase and tensin homolog; and STEP, striatal-enriched phosphatase.
a potent inhibitor of PTEN, prevents ischemic brain injury. Zhang et al demonstrated that the application of Tat-K13, a peptide that prevents the nuclear translocation of PTEN, even 6 hours after stroke strongly protected against ischemic brain damage. Although delayed administration of a PTEN inhibitor bp as long as 24 hours after ischemia did not reduce infarction during the acute phase, functional recovery was improved. The long time window may be because PTEN inhibition enhances the regenerative ability of neurons. These results suggest that the inhibition of PTEN may represent a novel strategy for the treatment of stroke.

The PTEN inhibitor has an anti-ischemia effect in both the acute and chronic stages. This type of drug may have a wide time window and can be used any time after stroke. Like DAPK1 and the p53 inhibitor, the powerful promotion of cell proliferation should be explored in other tissues.

### GluN2B–CaMKII Signaling Pathway

CaMKII is also involved in pathological excitotoxic glutamate signaling (Figure 4). Glutamate-induced Ca²⁺-influx causes CaMKII to translocate to postsynaptic sites. Specifically, the binding of CAMKII’s T-site to the GluN2B region around S1303 is essential for this type of translocation. Although deletion of CaMKII’s serine 1303 predisposes neurons to increased damage after ischemia, several studies have indicated that inhibiting stimulated and autonomous CaMKII activity attenuates the neuronal cell death induced by excitotoxicity. This contradiction could be explained by the developmental effects caused by the absence of CaMKII. Although the conventional CAMKII inhibitor KN93 attenuated excitotoxicity only when present during the insult, Tat-CN21, derived from the endogenous CaMKII-specific inhibitory protein CaM-KIIN, significantly reduced the infarct size in a mouse stroke model when injected 1 hour after the onset of arterial occlusion. The underlying mechanism is the blockade of the Ca²⁺-independent, autonomous activity of CaMKII generated by GluN2B association or Thr286 autophosphorylation. These results demonstrate that CaMKII autonomy may be a drug target for postsischemic neuroprotection.

Although the autonomous CaMKII inhibitor was proven to be effective in postsischemia treatment, in view of the powerful prosurvival effect of CaMKII (Figure 4), the CaMKII inhibitor may have a narrow time window. The best administration time may be around the time of stroke.

### Perspectives

Because most of the potential drugs with effective postischemic treatment were derived from the GluN2B–NOS signaling pathway (Table), this signaling might be the most important among the 4 prodeath pathways. More to the point, Tat-NR2B9c has passed its phase II clinical trial. Although the other 3 signaling pathways shared relatively small proportion of effective drugs, PTEN and JNK inhibitors were also 2 types of promising potential drugs.

Disappointed results of NMDAR blockers indicate that NMDAR also plays an important role in promoting neuronal survival. It is generally agreed that rather than inhibiting all of the NMDAR signals, selectively preventing the prodeath signal pathway is a good strategy. However, this is not easy to accomplish. There are some controversies regarding the differential attribution of neuronal survival and death to distinct NMDAR subpopulations and locations. Therefore, more attention should be paid to the downstream prodeath proteins of NMDAR. In addition, stroke induces acute and delayed cell damage that lasts for months. Promoting regenerative repair, including neurogenesis and dendritic remodeling, may also be an alternative strategy in the treatment of stroke. Tat-NR2B9c and bpv are 2 successful examples of delayed administration.

Among the drugs with effective postischemic treatment, many are Tat fusion proteins. Tat can increase cellular drug uptake by activating different types of endocytosis pathways, as well as direct translocation. The ability of Tat to deliver macromolecular cargo to the brain will greatly facilitate the development of anticerebral ischemia drugs. However, this Tat-based drug delivery approach is potentially fraught with several scientific and technical problems, which include a lack of cell selectivity, instability, complicated influences by peptide cargo, uncertainty in guaranteeing an effective concentration in its target and immediate degradation after oral administration. The rational design of small molecular drugs is a unique method for overcoming these disadvantages once and for all.

### Sources of Funding

The authors acknowledge support from the National Science Foundation of China (NSFC 81200886, NSFC 81402886), the Natural Science Foundation of Hebei Province (H2014208004), the State Key Laboratory Breeding Base—Hebei Key Laboratory of Molecular Chemistry for Drug and Hebei Research Center of Pharmaceutical and Chemical Engineering.

### Disclosures

None.

### References

Fear conditioning involves activation of an NOS1AP-mediated neurotoxicity in cerebral ischemia-reperfusion injury.


The expression of contextual fear conditioning involves activation of the nNOS-nNOS interaction with P38 MAPK and upregulated miR-375 are involved in protective effects of calycosin on cerebral ischemia/reperfusion rats. *J Neurol Sci.* 2014;339:144–148. doi: 10.1016/j.jns.2014.02.002.


60. Jurado S, Benoit M, Latino A, Knafo S, Petrok CN, Esteban JA. PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression. EMBO J. 2010;29:2872–2880. doi: 10.1038/emboj.2010.160.


Therapeutic Targets for Cerebral Ischemia Based on the Signaling Pathways of the GluN2B C Terminus
Yongjun Sun, Linan Zhang, You Chen, Liying Zhan and Zibin Gao

Stroke. 2015;46:2347-2353; originally published online July 14, 2015;
doi: 10.1161/STROKEAHA.115.009314

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/8/2347

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/