Nitric Oxide Synthase Isoforms Undertake Unique Roles During Excitotoxicity

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Background and Purpose—Excitotoxicity is a component of many neurodegenerative diseases. The signaling events that lead from excitotoxic injury to neuronal death remain incompletely defined. Pharmacological approaches have shown that nitric oxide production is critical for the progression of neurodegeneration after the initiation of excitotoxicity by the glutamate analog kainate. Although nitric oxide additionally triggers blood–brain barrier (BBB) breakdown, the breakdown does not in itself inevitably lead to neuronal cell death, because neuroprotective pharmacological means can be used subsequently to prevent the neural death.

Methods—In this study, we use a genetic approach to analyze the contribution of 3 nitric oxide synthase (NOS) isoforms, neuronal NOS, endothelial NOS, and inducible NOS, to neurodegeneration and BBB breakdown in this setting.

Results—We find that neuronal NOS is critical for the progression of kainate-stimulated neurodegeneration, whereas endothelial NOS is required only for BBB breakdown. Inducible NOS is not required for either event.

Conclusions—The observation that endothelial NOS-deficient mice undergo excitotoxic neurodegeneration in the absence of BBB breakdown unlinks the two processes. These findings suggest that it may be possible to achieve full amelioration of excitotoxic-triggered neurodegeneration through developing isoform-specific inhibitors solely for neuronal NOS. (Stroke. 2007;38:1938-1945.)

Key Words: animal models of human disease ■ blood–brain barrier ■ endothelium/vascular type/nitric oxide ■ excitotoxicity ■ neurodegeneration

Under physiological conditions, most of the glutamate in the central nervous system localizes to presynaptic vesicles and returns rapidly after being released during depolarizing events. However, pathological situations such as hypoxia and ischemia can lead to excess release of glutamate and its accumulation in the extracellular space, which initiates the pathway of neuronal death known as excitotoxicity. One of the events triggered by excessive glutamate release and relevant to excitotoxicity is the production of nitric oxide (NO). NO is produced by nitric oxide synthase (NOS), of which there are three major isoforms named after the type of cell where they were originally reported: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), which was first described in macrophages. The three NOS isoforms are expressed in the central nervous system. NOS activity increases during kainic acid (KA)-induced excitotoxicity; however, the potentially distinct roles undertaken by the individual isoforms in vivo during excitotoxin-mediated neurodegeneration remain incompletely defined.

The specific NOS isoform activated during disease can determine whether the NO produced promotes degeneration or neuroprotection. Activation of nNOS in a model of focal ischemia was reported to promote neurotoxicity, whereas eNOS activation resulted in neuroprotection presumably by affecting blood–brain barrier (BBB) permeability. Cortical neurons from nNOS-/- mice are resistant to NMDA and hypoxic injury. Glia also produce NO primarily through the induction of iNOS. In general, iNOS is considered responsible for the high output of NO in pathological, inflammatory central nervous system states. Microglia are implicated in neuronal death and excitotoxicity, ischemia, and multiple sclerosis potentially through a NO mechanism.

Although NO production is the most widely studied reaction catalyzed by NOS, the enzyme can also generate superoxide and does so most readily when the substrate required for generating NO is exhausted locally. The propensity to produce superoxide differs among the individual isoforms. nNOS is most characteristic of degeneration with the production of superoxide when levels of l-arginine and tetrahydrobiopterin decrease; eNOS does this less readily. Finally, iNOS produces the least amount of superoxide, seemingly because glia (particularly astrocytes) produce enough l-arginine to keep its intracellular concentration high even during stress and injury. During excitotoxicity, the NO produced by nNOS, and to a lesser extent eNOS, can combine with superoxide generated to yield...
peroxynitrite (ONOO−), a reactive compound involved in neurotoxicity in response to KA.7

Another factor that can influence excitotoxicity is BBB permeability. BBB disruptions are associated with central nervous system diseases, including excitotoxicity. Because NO is a vasodilator, it can modulate the BBB; NO can compromise BBB integrity during stroke and glutamate release,22 in hypertension,23 and various in vitro models of neurological diseases.24,25 The relationship and timing of BBB breakdown to neurodegeneration are not clearly established.

To date, investigators have used pharmacological methods that do not definitely distinguish between the isoforms to examine the role of NO in excitotoxicity. Here we use a genetic approach to explore the roles of individual NOS isoforms on excitotoxic death and BBB breakdown after KA injury. Intrahippocampal KA injections were performed on wild-type, nNOS−/−, iNOS−/−, and eNOS−/−deficient mice to assess their susceptibility to neurodegeneration and the timing of BBB breakdown. We find that only nNOS is critical for neurodegeneration; deficiency of eNOS or iNOS does not affect the excitotoxic response. However, eNOS is found to be required for BBB breakdown, revealing that neurodegeneration can proceed in the absence of BBB compromise, thus dissociating the 2 processes in this disease model.

**Materials and Methods**

**Animal Procedures**

All animal experiments followed National Institutes of Health guidelines and were approved by the Department of Laboratory Animal Research at SUNY Stony Brook. nNOS-deficient (nNOS−/−),26 iNOS-deficient (iNOS−/−),27 eNOS-deficient (eNOS−/−),28 and C57Bl6 (wild-type [wt]) mice had ad libitum access to food and water and were placed on 12-hour light/dark schedule. All mice used have been backcrossed for more than 12 generations into the C57Bl6 background.

**Excitotoxic Injury**

Adult mice were injected with KA as described.29,30 At the indicated times, mice were killed, and the brains were removed and sectioned. For infusion experiments, microsomatic pumps delivering L-arginine into the hippocampus were subcutaneously inserted,29,30 as described.

**Nitric Oxide Donor and Peroxynitrite Injections**

Wt or NOS−/− mice were stereotactically injected with the NO donor 2,2′-dihydroxytrinitroso-hydrazine (NOC-18) or the NO and superoxide donor 3-(4-morpholino)isodiyne-imine-hydrochloride (SIN-1) in 500 nL as mentioned previously. If the injection was in combination with KA, NOC-18 or SIN-1 was injected first and allowed to dissipate for 2 minutes; KA was then injected.

**Cresyl Violet Staining**

Coronal sections (20 μm) were stained with cresyl violet to assess neuronal survival, which was quantified using Scion Image beta 4.02. The freehand tool used to define pyramidal neuronal loss on TIFF files of serial sections. The entire length of the pyramidal layer was measured using arbitrary units and the percent loss on the injected side calculated. The process was repeated 5 times per section and averaged. No calculated percentage had a SD more than 2. At least 5 sections were quantified for each experimental point. Six to 8 mice were used for each quantification.

**Blood–Brain Barrier Breakdown**

BBB breakdown was assessed as described.2,31 In brief, KA-injected NOS−/− and wt mice were injected intravenously with a solution of 2% Evans blue and killed 1 day later. The injected and un.injected hippocampi were removed, weighed, homogenized in 0.5% TritonX-100 in phosphate-buffered saline, and centrifuged at 21,000 g for 30 minutes. The amount of Evans blue in the supernatant was quantified at 620 nm, subtracted from the background, and divided by the wet weight. Values are presented as percent of the total signal and represent average values for at least 5 mice per experimental group.

**Immunofluorescence**

Sections (20 μm) from KA-injected wt or NOS−/− mice were fixed in 95% ethanol and then in acetone. After blocking in serum, sections were incubated with 3 μg/mL anti-occludin antibody. Fluorescein isothiocyanate-conjugated secondary antibody was added and the sections were washed and mounted with Vectashield mounting medium.

**Reverse Transcriptase–Polymerase Chain Reaction**

The hippocampus was removed from KA-treated mice and each side was weighed. The tissue was homogenized in TRIZOL, chloroform extracted, and the RNA precipitated using isopropyl alcohol, dissolved in RNase-free water, and stored at −80°C. One microgram of RNA of each sample was used for reverse transcriptase–polymerase chain reaction using the SuperScript II Reverse Transcriptase kit and oligo (dT)12–18 (500 μg/mL).

A 50-μL polymerase chain reaction volume was used per 1 μL cDNA of each sample. Primers were added at a concentration of 25 pmol/μL:

- **Actinsense** 5′-CTCCCCGT ATGGCCTCTGGTC-3′
- **Actinantisense** 5′-TCTACCTGCTGCTGCTGAT-3′
- **iNOSsense** 5′-CCCGTGCAAGCTCTTTCTT-3′
- **iNOSantisense** 5′-GGATTGCAT TTCGCTGTACTC-3′
- **eNOSsense** 5′-GGATATGATGTGGTGTCCCT-3′
- **eNOSantisense** 5′-GCAAACCTGGTAGGTAATTG-3′
- **nNOSsense** 5′-AAGTCGTGAGCAGATGGTG-3′
- **nNOSantisense** 5′-AACATACGAACCGCAATCTCC-3′

The intensity of polymerase chain reaction products was quantified using Scion Image.

**Statistics**

Statistics were performed using one-way analysis of variance followed by a Bonferroni-Dunn test for multiple comparisons within a group or a two tailed t test for comparisons between groups; P<0.05 was considered significant and marked by an asterisk (*) or tau (τ); P<0.01 and P<0.001 were very significant and marked by two (***) or three (****) asterisks, respectively. Error bars indicate the standard error of the mean. In all experiments, n is the number of animals used per genotype or condition (at least 6).

**Results**

**Nitric Oxide Synthase Expression Changes in Response to Kainate**

Intrahippocampal KA injection results in rapid neuronal and glial changes. The stimulated glutamate secretion and Na+ and calcium influx trigger changes in immediate early gene expression and glial responses that include morphological alterations and migration to injury sites. Neuronal death is observed in this model by 12 hours after KA injection;23 by day 1, degeneration of the neuronal layer is apparent using cresyl violet staining as a readout.

Although nNOS and eNOS are constitutively expressed and their activity regulated primarily by increases in intracellular Ca2+, under stress, they can also become transcriptionally upregulated.33 Reverse transcriptase–polymerase chain reaction
Neuronal Nitric Oxide Synthase<sup>−/−</sup> Mice Resist Excitotoxicity

We have reported that NO is an important mediator in KA-induced excitotoxicity.<sup>7</sup> To examine the role of the individual isoforms in this process, we injected KA into the mice lacking the individual NOS genes. The mice were killed 1 day after KA injection and the brains removed, sectioned, and stained with cresyl violet. KA injection in wt mice resulted in the death of pyramidal neurons along the CA1–3 hippocampal subregions, as shown in Figure 2 (wt panels with and without KA injection).<sup>30</sup> KA injection into inOS<sup>−/−</sup> and eNOS<sup>−/−</sup> mice similarly resulted in extensive neurodegeneration. In contrast, KA injection into the nNOS<sup>−/−</sup> mice did not cause the hippocampal neurons to degenerate. This result indicates that KA-induced excitotoxic death is dependent on the presence of functional nNOS but not the other two isoforms.

Neither Nitric Oxide Nor ONOO− Supplementation Restores the Toxic Effects of Kainate in Neuronal Nitric Oxide Synthase<sup>−/−</sup> Mice

We have reported that the susceptibility to KA-induced death in tissue plasminogen activator<sup>−/−</sup> (tPA<sup>−/−</sup>) animals is rescued by the administration of the NO donor NOC-18.<sup>7</sup> An NO donor is a metabolically neutral compound that breaks down over time to yield NO, and the amount injected was designed to approximate the levels of NO generated during excitotoxic events.<sup>7</sup> Our ability to achieve this rescue pointed to a crucial role for NO in the neurodegeneration process and suggested that NO production lies downstream of tPA action. To determine whether the toxic effects of KA could be restored in nNOS<sup>−/−</sup> mice by supplying back NO, NOC-18 was coinjected with KA into the hippocampus. NOC-18 alone had no effect on neuronal viability<sup>7</sup> (Figure 3C and 3G). As had been shown in Figure 2, nNOS<sup>−/−</sup> mice are resistant to KA excitotoxicity, which is shown again here (Figure 3A, 3B, and 3G). Coinjection of NOC-18 with KA did not restore the toxic effects of KA (Figure 3D and 3G).

The downstream metabolite peroxynitrite can mediate the toxic effects produced by NO, whereas NO alone does not suffice (if prevented from combining with superoxide to form ONOO−).<sup>7</sup> It was therefore conceivable that the coinjection of NOC-18 and KA into nNOS<sup>−/−</sup> mice might not elicit toxicity because in the absence of the nNOS gene, no superoxide was generated and hence no ONOO− formed. To test this hypothesis, SIN-1, a compound capable of generating both superoxide and NO on breakdown (essentially an ONOO− donor), was coinjected with KA into the hippocampus of nNOS<sup>−/−</sup> mice.
Rescue of the degenerative response was not observed (Figure 3F and 3G). This experiment indicates that exogenous, diffuse replacement of ONOO$^-$/H$_{11002}^-$ does not suffice to promote neurodegeneration, which raised the question of whether the local production of ONOO$^-$ actually is critical for mediating neuronal cell death in this model system. We next set out to test this issue by using a nonpharmacological method to block the production of ONOO$^-$.

Infusion of l-arginine Is Neuroprotective in Wild-Type Mice Treated With Kainate

The production of ONOO$^-$ appears dependent on the action of nNOS to generate both NO and superoxide. Although nNOS preferentially produces NO exclusively, superoxide is produced when the substrate required to generate NO, l-arginine (l-arg), becomes limiting. This situation is physiologically relevant, because low levels of l-arg occur during neurodegenerative events.$^{34}$ Accordingly, it is believed that on KA injection, l-arg levels become exhausted, leading to ONOO$^-$ formation and neurodegeneration. We set out to test this hypothesis by examining the consequence of increasing the l-arg reservoir. Wt mice were infused with 500 $\mu$mol/L l-arg 2 days before injection with KA. l-arg alone had no effect on neuronal survival (Figure 4). However, infusion of the l-arg before KA injection conferred protection against degeneration (arrows in Figure 4A delineate limited degeneration). To evaluate whether the infusion of l-arg affected ONOO$^-$ production, we immunostained for N-tyrosine, a protein modification mediated by ONOO$^-$/H$_{11002}^-$.

Endothelial Nitric Oxide Synthase as a Mediator of Blood–Brain Barrier Breakdown

We and others have shown that NO can mediate BBB breakdown.$^{7,24,25}$ However, to our knowledge, no study has determined which NOS isoform is critical for this event. To evaluate the requirements for the different NOS isoforms for BBB function, we assessed BBB integrity using 2 methods: immunostaining for occludin, a protein that localizes to endothelial tight junctions in intact BBB, and exclusion of Evans Blue dye. On BBB compromise, the normally punctate occludin staining becomes diffuse as the protein uniformly redistributes across the plasma membrane. As shown in Figure 5A, occludin immunostaining revealed BBB breakdown in wt and iNOS$^{-/-}$ KA-injected hippocampi but intact-appearing BBB in nNOS$^{-/-}$ and eNOS$^{-/-}$ KA-injected mice. We measured diffusion of Evans blue into the hippocampus after intrahippocampal KA injection, which, if detected,
would indicate breakdown of the BBB. The injected and uninjected hippocampi were individually removed, and the extravasated Evans blue quantitated for this brain region (Figure 5B). Compromise of the BBB was again detected in wt and iNOS−/−/−-injected hippocampi. However, both nNOS−/− mice, which were protected from neurodegeneration, and eNOS−/− mice, which were not, failed to exhibit detectable BBB breakdown after KA injection (Figure 5A and 5B). Coinjection of NOC-18 with KA into eNOS−/− animals partially restored BBB breakdown (Figure 5B), confirming a role for NO in this process, but had no effect on BBB in nNOS−/− animals. We find that although eNOS−/− mice are sensitive to KA-induced neurodegeneration, they are resistant to BBB breakdown. These results indicate that BBB compromise and excitotoxin neurodegeneration can be unlinked.

**Discussion**

The cellular source from which NO is produced has been proposed to be responsible in part for determining whether NO has toxic or protective effects during ischemia.

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*Figure 3.* Treatment of nNOS−/− mice with NOC-18 or SIN-1 does not restore KA toxicity. nNOS−/− mice were injected with 1 mmol/L NOC-18 or 300 μmol/L SIN-1 alone or with KA along with either 300 μmol/L SIN-1 or 1 mmol/L NOC-18. Wt mice were injected with KA as a control. Mice were killed at day 1 to assess neuronal survival. n=6 for each condition as summarized in G (***P<0.001).
ously, we showed that NO is a component of the KA-stimulated neurotoxic pathway and that the NO action lies downstream of the function of tPA. We also found that ONOO− promotes neurodegeneration but, in contrast to NO, does not promote BBB breakdown, suggesting that BBB breakdown might not be a required step for neurodegeneration. In this study, in an effort to determine NOS isoform-specific effects, we used individual NOS isoform-deficient animals and analyzed the extent of KA-induced neurodegeneration and BBB breakdown.

Changes in NOS expression have been described in many reports, often in the context of a specific disease. eNOS and nNOS are generally considered to be constitutively expressed. However, our experiments indicate that eNOS and nNOS expression levels change in response to stimuli (Figure 1). Why nNOS expression decreases 1 day after KA injection is unclear. One possibility is that the nNOS-expressing neurons are the pyramidal neurons that specifically degenerate in response to KA. It may not be this simple, however, because it has been reported that nNOS-expressing neurons potentiate neurodegeneration but are highly resistant to degeneration themselves. Alternatively, nNOS may be downregulated by an unknown signaling mechanism in an attempt to rescue the remaining neurons.

Previous work has demonstrated that the pharmacological or genetic disruption of nNOS function results in neuroprotection after NMDA treatment. However, in cell culture models in which nNOS was pharmacologically inhibited, lack of nNOS-derived NO was reported to not regulate KA excitotoxicity. In our model, nNOS−/− mice are dramatically protected from KA neurodegeneration (Figure 2). A possible explanation for this discrepancy may lie in differences in the methods used to elicit excitotoxicity. Cell culture models may not mimic what is occurring in the whole animal, in which the correct cell types are present at the correct cell ratios with the appropriate extracellular matrix support. In other studies that did use mouse disease models, either the KA was injected systemically rather than directly into the hippocampus or the pharmacological inhibitor was applied systemically rather than through a microosmotic pump. Taken together, we feel that the methods used here more directly address the requirement of NOS isoforms in excitotoxicity.

During excitotoxic events, nNOS produces ONOO− when substrates such as L-arginine become exhausted. We previously demonstrated that the resistance to neurodegeneration in tPA−/− mice was reversed by the addition of NO or ONOO−. In this report, we found that the same protocol did not overcome the neuroprotection observed for nNOS−/− mice. We nonetheless showed that the production of ONOO− is likely to be critical for neurodegeneration by demonstrating that when ONOO− is not formed (as a result of supplementation with L-arginine), neuroprotection is observed. How can these findings be reconciled? We propose that these findings suggest that nNOS is not fully inactive in tPA−/− mice, and hence the pharmacological supplementation may be able to rescue a hypomorphic situation.

Figure 4. Infusion of L-arginine protects wt neurons from KA toxicity. A total of 500 μmol/L L-arginine was infused to wt mice 2 days before KA injection. A, Neuronal survival was visualized 1 day later. Infusion of L-arginine followed by injection of phosphate-buffered saline had no effect on neuronal viability. B, Quantification of the neurodegeneration is presented as percent of surviving neurons in the total hippocampus on the injected side in comparison to the noninjected side. Statistical analysis comparison of treated wt mice with wt controls using a two-tailed t test. **P<0.01. n=6 for each genotype. C, Infusion of L-arginine reduces ONOO− production after KA injection. Hippocampal sections were prepared from the mice 1 day after the phosphate-buffered saline or KA injection and immunostained to detect the presence of N-tyrosine, an ONOO−-mediated protein modification.
fails in mice in which NO and ONOO⁻ production is abolished. nNOS is activated by changes in Ca²⁺, which occur after KA injection to a partial degree even in tPA⁻/⁻ mice; hence, the deficiency in NO and ONOO⁻ production is presumably only partial in these mice, potentially enabling diffuse supplementation using an NO or ONOO⁻ donor to restore the degenerative pathway. The difference could also reflect requirements for NO/ONOO⁻ in different subcellular locations; NO/ONOO⁻ provided by the donors would more easily rescue an extracellular deficiency as opposed to an intracellular one. This might be the case for tPA⁻/⁻ mice, if only limited amounts of NO and ONOO⁻ are generated (intracellularly) in them, and these modest amounts do not succeed in diffusing substantially to the extracellular environment.

Although eNOS deficiency does not prevent KA neurodegeneration (Figure 2), it does provide protection against BBB breakdown (Figure 5). It has been suggested that eNOS may be protective during ischemia because it increases cerebral blood flow. The specificity of BBB breakdown was assessed by occludin immunofluorescence. Diminished staining was observed in KA-injected wt and iNOS⁻/⁻ mice (n=7). eNOS⁻/⁻ and nNOS⁻/⁻ mice exhibited normal occludin staining after KA (n=6). B, Wt mice were injected with 2% Evans blue after the KA injection. BBB breakdown was assessed by the amount of Evans blue that leaked into the parenchyma. Each experimental group is represented as percent of total signal for each experiment. The asterisk (*) indicates a significant difference to the respective uninjected side (P<0.05) by a two-tailed t test. The triple asterisk (****) indicates P<0.001. tau (τ) indicates P<0.05 between the NOC-18 and KA eNOS⁻/⁻ coinjected side compared with the wt KA-injected side.

The BBB is also protected in nNOS⁻/⁻ mice (Figure 5). This may result from the lack of neurodegeneration in nNOS⁻/⁻ mice or because nNOS-generated NO is also required to trigger BBB breakdown. There is evidence that like eNOS, nNOS can regulate cerebral blood flow. nNOS may mediate BBB permeability independently from eNOS through indirect signaling events from neurons to BBB.

Our data show that nNOS activity is required for the KA neurotoxic effects, which appear to ensue from ONOO⁻ formation after L-arginine is depleted. NO-mediated BBB breakdown occurs through a different mechanism from that of NO-mediated neurodegeneration, because eNOS⁻/⁻ mice, which exhibit susceptibility to excitotoxic death comparable to wt mice, have intact BBB.

These findings suggest that effective neuroprotection might be achievable using an nNOS-specific inhibitor, which raises the prospects of diminished bystander effects in comparison to a broad-spectrum inhibitor that targets all 3 isoforms.

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