Continuing Postischemic Neuronal Death in CA1
Influence of Ischemia Duration and Cytotoxic Doses of
NBQX and SNX-111 in Rats

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Background and Purpose—Transient forebrain ischemia results in a 24- to 72-hour delayed loss of CA1 neurons. Previous work has not assessed whether insult durations can vary the degree and maturation rate of CA1 injury and whether there are different ultrastructural features of death after brief or severe ischemia. We also tested whether known cytoprotective drugs achieve permanent or transient neuroprotection.

Methods—In the first experiment, ischemia was induced for 5, 15, or 30 minutes with the use of the 4-vessel occlusion rat model with 1- to 28-day survival. Others subjected to 5 or 15 minutes of ischemia and allowed to survive for 14 or 7 days, respectively, were examined with electron microscopy. Finally, we determined whether NBQX (30 mg/kg ×3 at 0 or 6 hours after ischemia), an AMPA antagonist, and SNX-111 (5 mg/kg at 6 hours after ischemia), an N-type Ca²⁺ channel antagonist, provided enduring CA1 protection against 10 minutes of ischemia.

Results—CA1 damage was not detected at 24 hours. Thirty minutes of ischemia produced 47% and 84% CA1 damage at 2 and 3 days, respectively. A 15-minute occlusion yielded 11%, 74%, and 86% loss at 2, 3, and 7 days, respectively. Five minutes of ischemia produced an even slower progression with 24%, 52%, and 59% loss at 3, 7, and 14 days, respectively. Ultrastructural examination after 5 and 15 minutes of ischemia revealed necrosis with no morphological evidence of apoptosis. Both NBQX (P<0.021) and SNX-111 (P<0.001) significantly reduced CA1 death at 7 days (~35%) but not at 28 days (~80%) compared with saline treatment (~79%).

Conclusions—Brief forebrain ischemia results in a slower progression of CA1 loss than more severe insults. Nonetheless, neuronal injury had necrotic, not apoptotic, morphology. NBQX and SNX-111 only postponed CA1 injury. (Stroke. 1999;30:662-668.)

Key Words: calcium channels ■ hippocampus ■ glutamates ■ neuronal death ■ rats

A striking but selective loss of neurons follows brief forebrain ischemia. In particular, the CA1 zone of the hippocampus is remarkably sensitive among vulnerable neuronal groups. Since CA1 cell death has been found to occur over 24 to 72 hours,1–3 there is a widespread reliance on euthanasia times of 4 to 7 days. We tested the assumption that all durations of ischemia result in a similar maturation rate by studying the progression of CA1 damage after 5-, 15-, and 30-minute periods of 4-vessel occlusion in rats.

Most electron microscopic studies of typical CA1 ischemic injury (ie, over 2 to 4 days) indicate a necrotic mode of death. Early changes such as disaggregation of polyribosomes and dilated organelles1,4,5 precede the eventual death of CA1 cells, which exhibits the typical features of necrosis (ie, mitochondrial flocculent densities, clumped chromatin, membrane breaks).4,6 However, studies assessing DNA fragmentation after global ischemia have suggested biochemical evidence for apoptosis.7–11 In addition, findings of CA1 neuroprotection with protein synthesis inhibitors, such as cycloheximide or anisomycin,12–14 suggest pharmacological evidence for apoptosis since this may depend on new protein synthesis.15 Importantly, several studies show that apoptosis-promoting genes, such as Bax, are upregulated after global ischemia,16–18 and there are reports of elevated caspase activation in CA1.19,20 Furthermore, therapeutic strategies such as the use of caspase inhibitors can, at least temporarily, lessen CA1 neuronal loss.19,21 Finally, the antiapoptotic strategy of Bcl-2 overexpression reduces neuronal injury after focal cerebral ischemia.22–24

It has been argued that a brief insult is more likely to result in apoptosis, while severe ischemia produces necrosis (ie, a continuum). For example, mild ischemia of the...
liver results in extensive apoptosis, while severe ischemia causes coagulation necrosis.²⁵,²⁶ Likewise, it has been suggested that apoptosis is more prevalent after mild focal ischemia.²⁷–²⁹ Thus, this study examined not only whether various durations of global ischemia result in a similar maturation rate of CA1 damage but also whether there are different ultrastructural features of CA1 death after brief or prolonged global ischemia.

The cellular and molecular events responsible for selective vulnerability and the delayed nature of CA1 injury are the subject of much speculation. One leading hypothesis purports that excessive release of glutamate with resultant calcium entry and excessive postischemic sensitivity to excitatory neurotransmission are key factors in the pathogenesis of CA1 loss. Indeed, antagonists of the amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F) quinoxaline [NBQX]) have been repeatedly found to reduce CA1 loss.³⁰,³¹ Similarly, the ω-conotoxin SNX-111, which potently blocks N-type Ca²⁺ channels and thereby prevents glutamate release, has also been found to markedly reduce CA1 injury.³²,³³ We explored whether the potent neuroprotective drugs NBQX and SNX-111 would provide enduring CA1 protection. Some of these data have previously been reported in abstract form.

Materials and Methods

Four-Vessel Occlusion Preparative Surgery

Adult male Wistar rats were obtained from Charles River (Montreal, Quebec, Canada) and used at a weight of 175 to 255 g. All experiments, which were approved by a local animal care committee, were in accordance with the Canadian Council on Animal Care Guidelines. Forebrain ischemia was induced by using a modification³⁴ of the 4-vessel occlusion model initially described by Pulsinelli and Brierley.³⁵ Rats were anesthetized with 3% halothane and subsequently maintained with 1% to 2% halothane by an infrared lamp. Rats that became unresponsive or ataxic, or showed no righting reflex were excluded. The following day rats were briefly reanesthetized to open the wound. Both carotid arteries were then occluded (no anesthesia) with 2-0 silk. An 18-gauge needle was used as a guide to pass a 1-0 silk thread through the neck posterior to the trachea, esophagus, external jugular veins, carotid arteries, and vagal nerves. The silk was anterior to the cervical and paravertebral musculature.

Ischemia

The following day rats were briefly reanesthetized to open the wound. Both carotid arteries were then occluded (no anesthesia) with aneurysm clips, during which core temperature was regulated at 37.5 ± 0.5°C by an infrared lamp. Rats that became unresponsive or had initial running behavior, loss of righting reflexes, and dilation of pupils were included, while rats that ceased to remain in coma or developed righting reflexes during ischemia or seizure activity during or after ischemia were excluded.

Experimental Paradigms

Time Course of Cell Death

Rats were subjected to 5 minutes of ischemia and allowed to survive for 1, 2, 3, 7, 14, or 28 days. Rats occluded for 15 minutes survived for either 1, 2, 3, or 7 days, while animals subjected to 30 minutes of ischemia survived for 1, 2, or 3 days. The number of rats included in these groups and the death rate are given in the Table.

Ultrastructure Study

Rats were subjected to 5 (n=5) or 15 minutes (n=5 with 1 death) of ischemia and allowed to survive for 14 or 7 days, respectively.

| Time Course Study in Rats Subjected to 5, 15, or 30 Minutes of 4-Vessel Occlusion Ischemia |
|---|---|---|---|---|---|---|---|
| Ischemia Duration, min | Scheduled Euthanasia Time, d | Rats Entered, n | Deaths, n | Total Surviving to Euthanasia, n |
| 30 | 1 | 5 | 1 | 4 |
| 2 | 9 | 3 | 6 |
| 3 | 13 | 6 | 7 |
| 15 | 1 | 7 | 0 | 7 |
| 2 | 3 | 0 | 3 |
| 3 | 42 | 0 | 42 |
| 5 | 1 | 6 | 0 | 6 |
| 2 | 3 | 0 | 3 |
| 3 | 75 | 0 | 75 |
| 7 | 37 | 0 | 37 |
| 14 | 10 | 1 | 9 |
| 28 | 10 | 0 | 10 |

Mortality was significantly greater after the longer duration of ischemia.

Sections of the anterior dorsal hippocampus at ∼ −3.8 mm to bregma³⁶ were used for transmission electron microscopy, while paraffin-embedded sections at −3.3 mm were used for quantitative cell counts.

Neuroprotection Study

Rats were subjected to 10 minutes of ischemia and subsequently given saline or NBQX (Novo Nordisk) dissolved in water. Repeated 30 mg/kg IP doses were given at 0, 15, and 30 minutes either immediately after ischemia or after a 6-hour delay. Animals were allowed to survive for 7 or 28 days. There were 6 (7-day survival) and 9 rats (28-day survival) in the NBQX-treated groups (no delay), whereas the 6-hour delayed NBQX groups had 5 and 3 rats in the 7- and 28-day survival groups, respectively. Other rats given NBQX were prematurely killed because of obvious sickness, which was thought to be due to renal damage.³⁷ Saline-treated groups (no delay) had 7 and 10 rats, whereas the 6-hour delayed injection group had 11 and 3 rats at 7- and 28-day survival times, respectively. SNX-111 (NEUREX Corporation), a ω-conopeptide, was given at a dose of 5 mg/kg IV at 6 hours after ischemia. SNX-111-treated rats were allowed to survive for 7 (n=11) or 28 days (n=7) after ischemia.

Assessment of Hippocampal Injury

In all cases except the ultrastructure study, rats were perfusion fixed with a mixture of 80% methanol, 10% formaldehyde, and 10% acetic acid after anesthesia with halothane. The brains were left in situ at 4°C overnight. Coronal sections (7 µm) of paraffin-embedded brains were cut at 3.3 mm posterior to bregma³⁸ and stained with hematoxylin and eosin. Normal cells (not eosinophilic) were counted in the entire hippocampal CA1 band and expressed as percentage dead on the basis of previous counts of normal tissue. Data are presented as mean ± SD. Groups were compared by independent t tests with the use of either the pooled or separate version, depending on heterogeneity of variance as determined by Levene’s test.

Rats in the electron microscopy study were perfused with Karnovsky’s fixative (3% glutaraldehyde, 4% formaldehyde) with the brain left in situ overnight before removal. Thin sections of the hippocampal CA1 region were taken at ∼ −3.8 mm and processed for electron microscopy. Ultra-thin sections of CA1, stained with uranyl acetate and lead citrate, were then taken on the basis of toluidine blue–stained semi-thin (1 µm) sections. Several thousand neurons per group were examined with a

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Hitachi M600 electron microscope. The anterior block of brain was transferred to a 10% formalin solution before paraffin embedding, sectioning, and staining with hematoxylin and eosin. The number of viable CA1 neurons was counted at 3.3 mm posterior to bregma.

Results

Time Course Study
Mortality (Table) both during and after ischemia varied between groups and was greatest in the 30-minute ischemia groups (3-day survival group comparisons; $P<0.0001$; Fisher’s exact test). Mortality after 15 minutes of ischemia was also greater than in the rats with 5 minutes of ischemia (7-day survival; $P=0.002$). Some animals were killed because of generalized convulsions, which were most prominent in the 24- to 48-hour postischemic period. Only animals surviving the scheduled time were included in the histology analysis. No significant injury was noted in any of these groups at 1-day survival (Figure 1). However, the maturation rate of CA1 injury was clearly related to the duration of ischemia. At 2 days, significant injury had occurred in the 30-minute group ($P=0.0378$ versus 5-minute occlusion group). While the 15- and 30-minute periods of ischemia resulted in extensive CA1 cell loss by 3 days ($P<0.0001$ versus 5-minute occlusion group), it took 7 days for this injury to mature in the rats subjected to 5 minutes of ischemia. In rats given 5 minutes of ischemia, there was significantly more damage in groups allowed to survive for 7, 14, and 28 days than in those allowed to survive for 3 days ($P\leq0.0003$). There was also a trend for continuing damage after 7 days in the 5-minute occlusion groups ($P=0.0683$).

Ultrastructural Study
In agreement with the aforementioned survival time experiment, a similar degree of CA1 injury was noted in rats subjected to 5 minutes of ischemia with 14-day survival (77.6±17.7% damage) and 15 minutes of ischemia with 7-day survival (80.6±14.7% damage). Light (Figure 2) and ultrastructural (Figure 3) features of ischemic injury in both the 5- (14-day) and 15-minute (7-day) occlusion groups were qualitatively similar. Prelethal signs of injury in many of the remaining 20% of CA1 neurons predominantly included organelle (rough endoplasmic reticulum, mitochondria) dilations and cytoplasmic and intranuclear vacuoles. Lethal damage included mitochondrial flocculent densities, nuclear and plasma membrane breaks, and a clumped chromatin appearance. These changes and the fact that cytoplasmic perturbations preceded nuclear changes indicate necrosis. Furthermore, signs of apoptosis (eg, apoptotic bodies) were never observed with light microscopy or transmission electron microscopy. However, large clumps of chromatin (Figure 2B), which were from clearly necrotic cells (Figure 3D), might be confused with apoptotic bodies when examined with low-magnification light microscopy.

Neuroprotection Study
In the drug study (Figure 4), all saline control groups had a similar, nonsignificantly different ($P\geq0.1460$) degree of CA1 neuronal damage and were therefore combined for statistical comparisons (combined mean, 79%). NBQX given immediately (17%; $P<0.0001$) or at 6 hours (27%
P after ischemia was significantly neuroprotective (versus control) at 7-day survival. However, NBQX given immediately (88%; \( P = 0.0939 \)) or after a 6-hour delay (91%; \( P = 0.1844 \)) did not reduce injury in animals that survived for 28 days. Similarly, postischemic SNX-111 administration reduced CA1 damage at 7- (35%; \( P = 0.0006 \)) but not 28-day survival (80%; \( P = 0.7683 \)). Accordingly, both NBQX and SNX-111 groups that survived for 28 days had significantly (\( P \leq 0.0101 \)) less protection than those assessed after 7-day survival. A similar maturation effect (7- versus 28-day survival) was not found for the 10-minute occlusion groups given vehicle injections.

Discussion
The maturation rate of untreated ischemic injury in the hippocampus has been widely accepted to occur from 24 to 72 hours after ischemia.\(^1,2\) Thus, many laboratories have relied on euthanasia times of \( \approx 4 \) days, assuming that injury would be complete by this time. Our data prove that this assumption is false because the completion of CA1 loss took longer than 72 hours after brief ischemia. Furthermore, the eventual loss of neuroprotection provided by NBQX and SNX-111 indicates that CA1 neurons may die after 7-day survival. In total, these data imply that once a threshold of injury is initiated, CA1 injury may inevitably progress until most or all CA1 neurons are dead.

The morphological features of delayed CA1 neurodegeneration after the 5- and 15-minute periods of 4-vessel occlusion ischemia were typical of necrosis and similar to most published ultrastructural studies in rat and gerbil models of global ischemia.\(^1,4-6,38\) Although it has been suggested that some or all CA1 cell loss is through apoptosis,\(^8,10,39\) we failed to find any definitive ultrastructural or light microscopic features of apoptosis (eg, apoptotic bodies). This confirms most other electron microscopic studies\(^1,4-6,40\) that have not found morphological evidence for apoptosis in CA1. Notably, our results in global ischemia also do not support the hypothesis that milder periods of ischemia result in a greater apoptotic component.\(^28\)

There are several possible reasons for the marked discrepancy between morphological and biochemical studies. First, the widespread reliance on terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining to selectively identify apoptotic cells is incorrect because this technique also labels necrotic and autolytic cells.\(^41-43\) Furthermore, apoptotic-like DNA fragmentation can occur in morphologically necrotic cells.\(^44\) A study by Petito et al\(^45\) suggests that DNA fragmentation in ischemic CA1 neurons occurs after cell death, and therefore DNA fragmentation is not the cause of neuronal death. Petito et al\(^46\) have also shown that glia, especially oligodendroglia, can undergo apoptosis after global ischemia, and this may account for DNA laddering. Regardless, neither the ultrastructural findings of necrosis nor the limitations of biochemical studies prove that ischemic CA1 neuronal loss does not have an apoptotic component, as is suggested, for example, by the fact that caspase inhibitors can reduce CA1 ischemic injury.\(^19,21\)

The cause of slow CA1 sector cell death after initial protection with NBQX and SNX-111 is unknown. However, it has been shown that a brief period of immediate postischemic hypothermia can only delay and not prevent CA1 loss after global ischemia in rats subjected to 2-vessel occlusion.\(^47,48\) Likewise, a delayed but partial loss of neuroprotection has been documented after lengthy periods...
of delayed postischemic hypothermia in gerbils subjected to global ischemia. 

NBQX has been found to induce a protracted but very mild hypothermia that accounts for most, if not all, of its neuroprotection. Thus, the fact that NBQX induces only a very mild hypothermia may explain why this drug is only transiently neuroprotective since greater drops in temperature are needed to persistently salvage CA1 neurons against global ischemia in gerbil and rat. 

Neuroprotection with SNX-111 may also be due to hypothermia, which has not been assessed. Future studies will have to determine whether more prolonged treatments with NBQX and SNX-111, or similar but less toxic drugs, could persistently reduce CA1 loss, but any such effects would have to be dissociated from the confounding protective effects of hypothermia. These compounds may also convey some functional benefit in the absence of histological protection since these 2 end points do not always concur. Finally, the ultrastructural morphology of slow CA1 cell death after initial protection with NBQX or SNX-111 is not expected to differ from that observed after the 5- and 15-minute untreated insults since ultrastructural studies of slowed cell death by brief and protracted hypothermia indicate a morphologically necrotic, and not apoptotic, mode of cell death.

In summary, this study shows that the maturation rate of CA1 death, which has necrotic morphology, is not static but depends on the duration of ischemia. Likewise, the maturation rate was influenced by the neuroprotective compounds NBQX and SNX-111, which did not convey permanent protection. Accordingly, there is a need for prolonged survival times in global ischemia studies to assess true neuroprotection. Further studies are needed to uncover the mechanisms (apoptotic and necrotic) of this slow cell death and to determine whether they differ from the faster CA1 neuronal loss.

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References


The accompanying article by Colbourne et al emphasizes the necessity of allowing an adequate period of time after a global ischemic insult for maturation of neuronal damage. This is of critical importance for hippocampal CA1 neurons because it takes several days for the damage to mature, and the maturation time is proportional to the duration of the ischemic insult. The present study also suggests that studies with neuroprotective drugs in global ischemia need to incorporate longer periods of time after ischemia before damage assessment. The AMPA antagonist NBQX and the N-type calcium channel blocker SNX-111 were previously shown by several researchers to be neuroprotective in global ischemia; however, in the present study all neuroprotection in global ischemia was lost when damage was assessed 28 days after 10 minutes of global ischemia. On the basis of these findings, it may be necessary to extend the time after ischemia before damage is assessed.

Additional studies will be necessary to determine what the length of this extended period needs to be. The authors did not administer the drugs for a period longer than 6 hours, and it would have been ideal if treatments would have been extended over a longer period of time because of the delay in cell death. Extended treatment periods may be necessary to demonstrate efficacy of some therapeutic agents. The authors construct a case for necrosis as the mode of neuronal cell death in their global model. They observe no morphological evidence for apoptotic neuronal cell death. Attempts to classify CA1 neuronal death as either necrosis or apoptosis may be misleading when attempting to understand mechanisms of death of differentiated neurons. These neurons possess some of the phenotypic characteristics of necrotic neurons but, in contrast, possess all of the biochemical and molecular characteristics needed to die by apoptosis. For example, they show caspase activation, increased levels of Bax and decreased Bcl-2, increased Bcl-xs, expression of Myc and p53, and release of cytochrome c from the mitochondria. Although these neurons do not show the classic morphological characteristics of apoptosis, it is rea-
sonable to think that the above biochemical markers of apoptosis have an important mechanistic role in neuronal death. Thus, the morphological features of the dying CA1 neurons are probably not nearly as important as the biochemical mechanisms that cause neuronal death.

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