Ultrastructural Changes of Neuronal Mitochondria After Transient and Permanent Cerebral Ischemia

Nina J. Solenski, MD; Charles G. diPierro, MD; Patricia A. Trimmer, PhD; Aij-Li Kwan, MD, PhD; Gregory A. Helms, MD, PhD

Background and Purpose—Mitochondrial swelling is one of the most striking and initial ultrastructural changes after acute brain ischemia. The purpose of the present study was to examine the role of reperfusion of the cerebral cortex after transient focal cerebral ischemia on neuronal mitochondrial damage.

Methods—Male Sprague-Dawley rats (n=16) were subjected to either temporary or permanent occlusion of the middle cerebral artery and bilateral carotid arteries. Three experimental conditions were compared: group I, permanent ischemia (3, 5, and 24 hours); group II, transient ischemia (2, 24 hours of reperfusion); and sham surgery. Anesthetized rats were killed by cardiac perfusion, and brain tissue was removed ipsilaterally and contralaterally from the ischemic core section of the frontoparietal cortex. Fixed tissue was prepared for electron microscopic examination, and electron microscopic thin sections of random neurons were photographed. Perinuclear neuronal mitochondria were analyzed in a blinded manner for qualitative ultrastructural changes (compared with sham control) by 2 independent investigators using an objective grading system.

Results—Cortical neuronal mitochondria exposed to severe ischemic/reperfusion conditions demonstrated dramatic signs of injury in the form of condensation, increased matrix density, and deposits of electron-dense material followed by disintegration by 24 hours. In contrast, mitochondria exposed to an equivalent time of permanent ischemia demonstrated increasing loss of matrix density with pronounced swelling followed by retention of their shape by 24 hours.

Conclusions—Neuronal mitochondria undergoing transient versus permanent ischemia exhibit significantly different patterns of injury. Structural damage to neuronal mitochondria of the neocortex occurs more acutely and to a greater extent during the reperfusion phase in comparison to ischemic conditions alone. Further research is in progress to delineate the role of oxygen free radical production in the observed mitochondrial damage during postischemic reoxygenation. (Stroke. 2002;33:816-824.)

Key Words: cerebral ischemia ■ cerebral ischemia, transient ■ microscopy, electron ■ mitochondria [ultrastructure] ■ reperfusion injury ■ rats

There is escalating evidence that mitochondria play a key role in both necrotic and apoptotic neuronal cell death after acute cerebral ischemia.1–7 Early classic ultrastructural studies on ischemic neurons concluded that the earliest site of cell damage was at the mitochondria, as evidenced by varying degrees of mitochondrial matrical swelling.8,9 The relationship between these early observations of ischemia-induced mitochondrial swelling and the more current molecular concepts of mitochondrial membrane permeability transition and the opening of mitochondrial permeability transition pore leading to either necrotic or apoptotic cell death is currently being explored intensely.4,6,7,10–12 Current concepts suggest that mitochondrial swelling may be the result of membrane permeability transition initiated by a variety of stimuli. Since the stimuli for membrane permeability transition during ischemia alone versus ischemia with reperfusion differ as a consequence of generation of reactive oxygen during the reperfusion phase, the following study was designed to examine the temporal ultrastructural changes in neuronal mitochondria under these 2 diverse ischemic conditions. Despite direct histopathological evidence for reperfusion injury in the form of severe neuronal and microvascular damage in stroke animal models of focal middle cerebral artery occlusion (MCAO),13 there has been less attention focused on damage to neuronal mitochondria despite their central role in cell metabolism. The present study uniquely focuses on characterizing the temporal changes of neuronal mitochondria under conditions of either transient or permanent MCAO in a clinically relevant model. Data on the temporal course of these ultrastructural changes, on potential heterogeneity of changes, and on the presence of electron-dense deposits (EDD) believed to represent calcium deposits...
may provide insight into the mechanisms of mitochondrial-mediated brain ischemic/reperfusion injury. These data could translate into successful development of neuroprotective pharmacological strategies designed to target the mitochondria as an adjunct to currently established thrombolytic therapies for acute stroke.

Materials and Methods

Production of Reversible Focal Ischemia in the Rat

Adult male Sprague-Dawley rats (Hilltop Laboratory Animal Inc, Scottsdale, Pa) weighing 250 to 350 g were anesthetized, orotracheally intubated, and mechanically ventilated (Harvard Apparatus Co) weighing 250 to 350 g were anesthetized, orotracheally intubated, and mechanically ventilated (Harvard Apparatus Co). A femoral arterial catheter was placed, and blood pressure was continuously monitored. Both common carotid arteries were exposed, and silk threads were placed loosely around the arteries. With the use of a stereomicroscope, the zygomatic arch was removed, and the temporal muscle and mandibular bone were retracted. A temporal bone craniotomy was performed, and the left middle cerebral artery (MCA) segment was exposed. A Sundt microvascular clip (Codman) was gently applied to the MCA distal to the lenticulostriate microvessels, and arterial occlusion was visually verified. Just before clip placement, the cervical common carotid arteries were occluded by repositioning the surgical clip and loosening both carotid snare. A femoral arterial catheter was placed, and blood pressure was continuously monitored. Both common carotid arteries were exposed, and silk threads were placed loosely around the arteries. With the use of a stereomicroscope, the zygomatic arch was removed, and the temporal muscle and mandibular bone were retracted. A temporal bone craniotomy was performed, and the left middle cerebral artery (MCA) segment was exposed. A Sundt microvascular clip (Codman) was gently applied to the MCA distal to the lenticulostriate microvessels, and arterial occlusion was visually verified. Just before clip placement, the cervical common carotid arteries were occluded by repositioning the surgical clip and loosening both carotid snares. Reperfusion was confirmed visually or with the measurement of relative local cerebral blood flow by laser-Doppler imaging (Moor Instruments). Mean arterial blood pressure, heart rate, brain and rectal temperatures, and arterial blood gas were continuously monitored and recorded each hour. Rectal and brain temperatures were maintained at 37.5°C with a heating pad and lamp when necessary. Each hour a 300-μL aliquot of blood was withdrawn from the left femoral arterial catheter for the analysis of PaO₂, HCO₃-, PaCO₂, and pH (278 Blood Gas Analyzer, CIBA-Corning). This reversible focal stroke model has previously been established with confirmation of a reproducible cortical infarction and is an adaptation of the method of Chen et al.14–16

Experimental Paradigm

Three experimental conditions were compared: permanent ischemia, transient ischemia with reperfusion, and sham surgery, as demonstrated in Figure 1. Group I experiments examined the effect of the duration of MCAO on the ultrastructure of the neuronal mitochondria. In groups IA, IB, and IC, rats underwent either 3, 5, or 24 hours of left MCAO, respectively, followed by immediate cardioperfused fixation. Group II experiments examined the effect of either short or long reperfusion time after 3 hours of left MCAO. In groups IID and IIE, rats underwent 3 hours of left MCAO followed by either 2 or 24 hours of reperfusion, respectively, followed by immediate cardioperfused fixation. Rats in group IIC underwent a total of 24 hours of total ischemia with no reperfusion followed by fixation at the 24-hour period; groups IC and IIC are therefore identical.

A third group of experiments examined sham surgery conditions, with each rat undergoing 3 hours of anesthesia, carotid exposure without ligation, and craniotomy without MCAO. Since group I and group II experiments were performed at different times, each group had a separate corresponding sham surgery, with each group (A through E) compared with its respective sham surgery group. In addition, for each group (A through E) and sham, the right contralateral hemisphere served as an internal “control.” The total number of subjects was 16, with 2 rats per group; groups I and II each had separate sham control. At the completion of the experiment, rats were returned to their cages when fully alert and allowed food and water ad libitum until they were anesthetized with pentobarbital and were killed at the time of fixation (see below).

Electron Microscopy

Rat brain tissue was perfused with normal saline solution followed by phosphate-buffered 2% glutaraldehyde and 4% paraformaldehyde. The brain was carefully removed and immersed in the same fixative, and 2-mm coronal slices were prepared. Three approximately 1- to 2-mm cubes of representative brain tissue were obtained ipsilaterally and contralaterally from an identical superficial portion of the ischemic core section of the frontoparietal infarct, as shown in Figure 2. Each tissue sample was then postfixed in 1% potassium ferrocyanide–reduced osmium tetroxide, dehydrated in graded acetone, and embedded in Epon 812. Semithin (0.5 μm) sections were cut from tissue blocks and stained with 0.5% toluidine blue. The most superficial cortical layer (gray matter) was identified by the presence of meningeal tissue for subsequent thin sectioning. The sections were stained with 0.25% lead citrate and 5% uranyl acetate in 50% methanol and were observed and photographed in a blinded manner by an electron microscope technician using a JEOL 100CX electron microscope who was asked to examine each neuron for evidence of nuclear changes
(chromatin dispersion or clumping), for the presence of cytoplasmic changes including macrovacuoles or microvacuoles, and for the overall shape of the neuron (shrunken, swollen) and the appearance of rough endoplasmic reticulum (RER) compared with matched sham controls. Similarly, each investigator was instructed to examine the perinuclear neuronal mitochondria for abnormalities in mitochondrial distribution or shape, matrix density, cristal structure, and appearance of any abnormal structures compared with matched sham controls; each finding was indicated as mild, moderate, or severe depending on its frequency.

All experimental protocols involving the use of animals were approved by the Animal Care and Use Committee of the University of Virginia and were performed in accordance with approval by the American Association for Accreditation of Laboratory Animal Care. All rats were given a lethal dose of halothane inhalant anesthesia for euthanasia consistent with the Panel on Euthanasia of the American Veterinary Medical Association guidelines.

Results

The physiological parameters of arterial blood pressure, heart rate, and temperature as well as arterial blood gases were monitored and kept within normal ranges in each of the experimental animals. A distinct ipsilateral frontoparietal cortical infarct was verified at the time of brain removal of all group I and II rats. Tissue fixation was of excellent quality with no fixative artifacts, as judged by the electron microscopy technical staff.

Sham Surgery Conditions

Neurons in the sham group have a normal-appearing nucleus and cytoplasm without signs of edema or injury, as illustrated in Figure 3. Organelles within the perikaryon appear normal, including the mitochondria, which demonstrate no evidence of swelling or injury; there is a typical homogeneous staining pattern of the matrix without excessive pallor or increased density or any signs of electron-dense precipitants. The surrounding neuropil also appears normal.

Group I: Effect of Permanent Ischemia on Neuronal Mitochondria With Variation in Length of Ischemia (3, 5, and 24 Hours)

For reader convenience, in most cases data from each experimental group are presented in each figure as an electron micrograph montage of a representative ischemic neuron (A) compared with a sham surgery control neuron (B) with additional enlarged areas of interest (C and D).

After 3 hours of severe ischemia, the neuronal cytoplasm is mildly swollen. The mitochondria within the perinuclear region of the perikaryon are moderately swollen, as seen in Figure 4A compared with sham control in Figure 4B and 4D. After this length of permanent ischemia, the swollen mitochondria appear to have an intact inner and outer membrane with some disruption of the cristal structures, as illustrated in the representative enlarged areas of the cytoplasm in Figure 4C. In addition, the RER is significantly swollen, creating numerous cytoplasmic vacuoles.

In comparison to 3 hours of ischemia, after 5 hours of ischemia there is severe neuronal cytoplasmic swelling with significantly increased electron lucency of the cytoplasm and sparse organelles compared with sham control (Figure 5A and 5B, respectively). At this longer length of ischemia the mitochondria are moderately to severely swollen; dispersed around the swollen mitochondria are increasing numbers of round structures of the same size as control mitochondria with poorly defined cristae and increased matrix density, as illustrated in Figure 5C.

In contrast to both the 3- and 5-hour ischemic neurons, by 24 hours the severe cytoplasmic edema is replaced by an overall shrunken appearance of the neuron with now severe edema of the surrounding neuropil (Figure 6A). The mitochondria within these neurons appear remarkably ultrastructurally “intact,” with a fairly distinct outer border, as illustrated in Figure 6C (arrows). In contrast to the early hours of ischemia, now the majority of the mitochondria demonstrate homogeneously increased electron density of the matrix. There also appear to be fewer and less dilated cytoplasmic microvacuoles resembling RER with attached ribosomes than at the shorter periods of ischemia.

The Table summarizes the ultrastructural changes noted in neuronal mitochondria during increasing lengths of permanent cerebral ischemia (3, 5, and 24 hours, respectively). In general, neurons undergoing the shorter lengths of permanent ischemia demonstrate more evidence of overall mitochondrial swelling with disorganized or dilated cristae in contrast to neurons undergoing 24 hours of ischemia. By the 24-hour time point, neurons appeared shrunken with ultrastructurally “intact” mitochondria containing homogeneous electron-dense matrix, in sharp contrast to the electron-lucent matrix associated with swelling at the earlier ischemic time periods.
Group II: Effect of Transient Ischemia on Neuronal Mitochondria With Variation in Length of Reperfusion (0, 2, and 24 Hours)

After 3 hours of ischemia followed by 2 hours of reperfusion, there is severe neuronal cytoplasmic swelling with a paucity of cytoplasmic organelles. Nuclear chromatin dispersion is seen in some neurons (Figure 7A). The surrounding neuropil is grossly swollen and disorganized, similar to that demonstrated at 24 hours of permanent ischemia. In many mitochondria there is frank disruption of the outer membrane with poor structural integrity (Figure 7C and 7D). After this short period of reperfusion, some neuronal mitochondria demonstrate bizarre idiosyncratic dilated cristae within an electron-dense matrix, as seen in Figure 7D. A few neurons demonstrate mitochondria with no discernible cristae but rather an amorphous “ground glass”–appearing matrix containing a few EDD. In contrast to the findings in neurons undergoing a short period of reperfusion, neurons undergoing 24 hours of reperfusion demonstrate a shrunken appearance with poor nuclear membrane integrity (Figure 8A). Cytoplasmic organelles are clumped and degraded. Neuronal mitochondria appear with a dimpled or irregular “crenated” shape rather than the typical rounded or tubular morphology (Figure 8A and 8B). In addition, the matrix is electron lucent with poorly defined cristae; similar to the mitochondria of the earlier reperfusion time point, some mitochondria contain deposits of very electron-dense material within the matrix (Figure 8C). During both short and long periods of reperfusion, the surrounding neuropil is severely disorganized and edematous.

The Table summarizes the ultrastructural changes noted in neuronal mitochondria during increasing lengths of reperfusion (0, 2, and 24 hours, respectively) compared with the contralateral (nonischemic) hemisphere and with sham surgery.

Mitochondria after even a short course of reperfusion appear to be severely damaged morphologically, exhibiting an electron-dense matrix, EDD, and nonuniform cristal swelling within a severely edematous perikaryon. Mitochondria after a longer course of reperfusion are structurally disorganized with irregular shapes and disrupted membranes, and they contain poorly defined cristae within an electron-lucent matrix. The perikaryon is condensed and shrunken consistent with advancing cell death. During both the long and short
reperfusion times, there is significant deposition of very electron-dense material within the mitochondria.

**Group I Versus Group II: Permanent Versus Transient Ischemia**

The temporal differences in ultrastructure of neuronal mitochondria in each group are demonstrated in Figure 9. In general, the mitochondria within neurons undergoing 24 hours of reperfusion demonstrate significant disorganization of cristae, with some mitochondria demonstrating electron-lucent matrix and others containing heterogeneous electron-dense areas within their matrix. In contrast, after 24 hours of permanent ischemia a large proportion of the neurons demonstrate mitochondria with homogeneous electron-dense precipitants within the matrix space. These “dense mitochondrial bodies” appear to have distinct mitochondrial membranes, suggesting some degree of integrity of the outer membrane. Neurons at both 24 hours of ischemia and at 24 hours of reperfusion are shrunken in size, with severe swelling of the surrounding neuropil. The reperfused neurons, however, seem to have increased evidence of nuclear membrane breakdown with chromatin disorganization and severely shrunken appearance, all signs of advanced cell death.

As mentioned previously, neurons undergoing even a short reperfusion time contain highly degraded mitochondria with increased matrix density and occasional irregular cristae swelling. In contrast, the permanently ischemic cells at a similar length of time demonstrate many globally swollen and enlarged mitochondria.

One additional important difference between group I and group II ischemic neurons was that the surrounding neuropil was more disorganized and edematous in the neurons exposed to a short length of reperfusion than those with a similar length of permanent ischemia.

**Contralateral (Right Hemisphere) Control**

The majority of neurons in the contralateral cortex on the nonischemic side appear normal, with typical nuclear, cytoplasmic, and mitochondrial morphology (data not shown). However, approximately 10% of the cells exhibit evidence of chromatin clumping, dispersion, or margined chromatin of unclear significance. In addition, careful examination of the mitochondria in the contralateral permanent 5- and the 24-hour ischemic groups consistently revealed occasional mitochondria with mildly dilated cristae or with increased intracistral spaces, as demonstrated in Figure 10, in comparison to sham control.

**Discussion**

The main finding of the present study is that neuronal mitochondria undergoing transient ischemia versus those undergoing permanent ischemia exhibit significantly different patterns of injury. In contrast to permanent ischemia, the reintroduction of blood flow results in rapid signs of likely
irreversible severe damage, including initially condensed
electron-dense mitochondria with increased electron-dense
deposits within their matrix, and autophagy. By 24 hours of
reperfusion the mitochondria are degenerating, with complete
loss of outer membrane shape and form. Neurons undergoing
increasing lengths of permanent ischemia also demonstrate
significant mitochondrial damage initially in the form of
increased swelling with cristal disruption, intracristal dilation,
and loss of matrix density. By 24 hours of permanent
ischemia, in contrast to reperfused neurons at 24 hours, the
perinuclear mitochondria demonstrate homogeneously in-
creased matrix density with preservation of the typical round
tubular shape and occasional EDD.

Mitochondrial swelling, as first described 80 years ago,
remains one of the most universal ultrastructural changes
after brain ischemia. Although there are numerous classic
histopathological studies and reviews describing neuronal
changes after ischemia, there is a paucity of modern
ultrastructural studies devoted solely to examining morpho-
logical changes of the neuronal mitochondria. The present
study was designed in light of the increasing appreciation of
the mitochondria in determining cell death and of their
potential role in reperfusion injury and the difficulty in
interpreting current available data, which are generated from
a variety of stroke models using different animal species,
different types of ischemia (irreversible versus reversible

<table>
<thead>
<tr>
<th>Condition</th>
<th>Group</th>
<th>Cristal Integrity</th>
<th>Intracristal Spaces</th>
<th>Matrix Density</th>
<th>Vacuoles</th>
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<td></td>
<td>Intact</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>3-h Ischemia*</td>
<td>IA</td>
<td>+ Disruption</td>
<td>+ Dilation</td>
<td>+ Loss</td>
<td>+++</td>
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<tr>
<td>5-h Ischemia</td>
<td>IB</td>
<td>++ Disruption</td>
<td>++ Dilation</td>
<td>++ Loss</td>
<td>+</td>
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<tr>
<td>24-h Ischemia</td>
<td>IC</td>
<td>+ Disruption</td>
<td>+ Dilation</td>
<td>++ Increase/EDD</td>
<td>++</td>
</tr>
<tr>
<td>0-h Reperfusion†</td>
<td>IIC</td>
<td>+ Disruption</td>
<td>+ Dilation</td>
<td>++ Increase/EDD</td>
<td>+</td>
</tr>
<tr>
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<td>++ Disruption</td>
<td>+ Dilation</td>
<td>++ Increase/EDD</td>
<td>+</td>
</tr>
<tr>
<td>24-h Reperfusion</td>
<td>IIE</td>
<td>++ Disruption</td>
<td>+++ Dilation</td>
<td>++ Loss/EDD</td>
<td>++</td>
</tr>
<tr>
<td>Contralateral</td>
<td></td>
<td>Intact</td>
<td>+ Dilation</td>
<td>Normal</td>
<td>None</td>
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*Fixation immediately after 3-h ischemia.
†Fixation after 24-h ischemia.
ischemia and global versus focal ischemia), and different ischemic areas of the brain (hippocampus versus cortex). To the best of our knowledge, there are no studies with the single specific aim to directly compare qualitatively the early ultrastructural changes of neuronal mitochondria of permanent versus transient ischemia in the same clinically relevant focal stroke model.

In our focal transient ischemia model, it appears that reperfusion produces rapid mitochondrial injury in the form of condensation of the mitochondria, with increased matrix density and increased EDD. Although the exact pathophysiology of this latter observation is unknown, it is suggested that calcium dysregulation with subsequent mitochondrial and cellular damage may be occurring rapidly within these reperfused neurons.41 Simon et al,44 in their ultrastructural study using a global forebrain ischemia model, reported that mitochondrial swelling in the hippocampus occurred simultaneously with increased dense calcium pyroantimonate deposition after 30 minutes of reperfusion and with fewer calcium deposits after 120 minutes of reperfusion. It has been suggested that mitochondrial “flocculent densities” may precede the condensation and may be reliable indicators of irreversible injury.45 In the present model it would be important to determine what occurs at even shorter time lengths of reperfusion to characterize very early mitochondrial changes. In particular, it would be important to determine whether prominent mitochondrial swelling precedes the increased mitochondrial matrix density, condensation, or increased EDD. Massive mitochondrial swelling has been reported by Garcia et al25 after 1 hour of MCAO followed by 10 minutes of reperfusion in a cat stroke model.

A second important observation of the present study is the unexpected finding that there appears to be a preservation of neuronal mitochondrial shape even in areas undergoing degradation by 24 hours of permanent ischemia. Interestingly, this observation was preceded by early severe to moderate mitochondrial swelling followed by less evidence of swelling with time. The presence of ultrastructurally “intact” mitochondria at the 24-hour time point does not necessarily imply functional integrity. Review of the literature indicates only brief mentions of similar findings, particularly in nonneuronal cells, including the microvasculature in a monkey permanent MCAO stroke model and in glial cells.36 Garcia et al25 reported that astrocytic mitochondria remain intact or “condensed” despite massive cytoplasmic swelling in their feline model of transient MCAO. These “intact” electron-dense mitochondria are thought to represent calcium-overloaded mitochondria at some stage of degradation or injury. On the basis of the uniformity of the observation, a much less likely explanation is that the more damaged mitochondria were rapidly removed by this time point, giving the false impression that only mitochondria with intact shapes remain. Unfortunately, there is little understanding on how mitochondria are degraded or recycled as part of a reparative process within mammalian neurons, particularly under pathological conditions of focal ischemia/reperfusion. A recent detailed electron microscopy study on delayed hippocampal neuronal cell death during global ischemia reports mitochondrial selective damage and demonstrates various stages of mitochondrial degradation/turndover under conditions of postischemic hypothermia.46

In a comparison of the effect of permanent versus transient ischemia at 24 hours, it appears that neuronal mitochondria at least ultrastructurally “survive” or tolerate permanent ischemia better than an equivalent time of ischemia/reperfusion. One explanation is based on the well-established fact that during reoxygenation the reperfused neuron is exposed to potential toxins, including oxygen free radicals and important
proinflammatory mediators. Furthermore, there is increasing in vivo evidence that oxidative stress associated with reoxygenation generates free radicals, which are believed to be injurious or even lethal to the mitochondria. There is also increasing in vivo evidence that mitochondria play a role in mediating either necrotic or apoptotic neuronal cell death during ischemia/reperfusion. There remains a vigorous debate over the role of mitochondrial membrane rupture and the release of proapoptotic factors, including cytochrome c, to initiate apoptotic cell death. In the present study apoptotic cell death was not specifically studied, and the area sampled was the dense ischemic core rather than the penumbra, the latter region being where apoptotic cell death may occur. Future studies will extend the present study by comparing the temporal course of mitochondrial ultrastructural changes in the core to those in the penumbra during increasing lengths of permanent versus transient cerebral ischemia.

Finally, an important finding in the present study is that there was some evidence of mild dilation of the mitochondrial outermost layers; it is known that different cortical layers may differ in their vulnerability to ischemia, and therefore data could be skewed toward one effect over another depending on the particular layer examined. Finally, the data presented here represent significant trends based on a small but adequate sample size; as with most studies, sampling errors can occur, and therefore confirmation of these findings is needed.

Despite these limitations, this study provides qualitative temporal and comparative data not previously described that underscore the potential role of mitochondria in mediating reperfusion injury in acute ischemic neuronal cell death. The results of the present study allow us to design targeted in vitro methods aimed at explaining the differences between the pattern of injury in the permanently ischemic versus the reperfused mitochondria. Correlative functional studies to better understand the relationship of the observed differences in mitochondrial swelling, condensation, and EDD to reversibility and type of neuronal cell death (apoptotic versus necrotic) and particularly to the role of mitochondrial transition permeability, calcium regulation, and ATP levels during brain reperfusion are needed and are currently in progress in our laboratory.

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