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Dextromethorphan Inhibits Ischemia-induced c-fos *Expression and Delayed Neuronal Death in Hippocampal Neurons*

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Background: Dextromethorphan (DM), a widely used antitussive agent, has been shown to possess both anticonvulsant and neuroprotective properties functionally related to its inhibitory effects on glutamate-induced neurotoxicity. The current study was designed to determine whether DM administration prevents delayed neuronal degeneration in central nervous system areas after global forebrain ischemia and whether this correlates with inhibition of induction of the immediate early gene *c-fos*.

Methods: Mongolian gerbils, anesthetized with 2% halothane in air at 37°C, received either 0.9% sodium chloride (vehicle, n = 9) or 50 mg/kg DM in vehicle (n = 9) by intraperitoneal injection before bilateral carotid artery occlusion. After 1 h of reperfusion under anesthesia, the animals were killed and the brains removed. Immunohistochemistry was used to detect neurons expressing Fos protein. Computer-assisted image analysis quantified changes in the number of labeled neurons as a function of drug treatment. To determine the extent of delayed neuronal degeneration within the hippocampus, other animals were treated with either DM (n = 7) or vehicle (n = 6) before carotid artery occlusion and allowed to survive for 1 week.

Results: Global forebrain ischemia produced consistent patterns of Fos-like immunoreactivity in the hippocampus and neocortex of vehicle-treated animals. DM inhibited the induction of *c-fos* from 65% to 91%. DM also protected against delayed neuronal degeneration in the CA1 region of the hippocampus ($P < 0.001$).

Conclusions: The induction of nuclear-associated Fos protein represents a sensitive marker of cellular responses to ischemia and a method to assay the effectiveness of pharmacologic interventions. DM markedly inhibited ischemia-induced Fos expression and prevented cell death in CA1. DM given before conditions of ischemia or decreased central nervous system perfusion may be highly beneficial. (Key words: Antitussive agents: dextromethorphan. Brain: ischemia. Immediate early gene: Fos protein. Immunohistochemistry.)

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MANY cardiovascular and neurosurgical procedures that include periods of central nervous system (CNS) ischemia or hypoperfusion are complicated by adverse neurologic sequelae resulting in increased morbidity during the postoperative period or even permanent neurologic dysfunction.[1–5] The evaluation of neuroprotective agents, pharmacologic regimens, or methodologies other than hypothermia[6] for use during ischemia or low-flow states has been limited.[7,8] Recently, attention has focused on the role of glutamate-induced neurotoxicity and in particular on activation of the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor, which may be the common pathway by which a variety of pathogenetic processes such as hypoxia, ischemia, or prolonged seizures can produce neuronal cell death.[9]

In support of this contention, selective and competitive NMDA receptor antagonists have been found to protect significantly against ischemic damage.[10] Competitive NMDA antagonists do not readily cross the blood–brain barrier, however, and so are not suitable for parenteral administration. The *noncompetitive* NMDA channel blockers ketamine, phencyclidine, and MK-801, which are relatively lipophilic and readily penetrate the blood–brain barrier, have been shown to have neuroprotective properties after seizures or ischemic events.[11,12] However, because of their putative actions at σ receptors, these compounds possess dysphoric and even psychotomimetic activity. The widely used over-the-counter, nonopioid antitussive agent,

dextromethorphan (DM) has been shown to possess both anticonvulsant and neuroprotective properties presumably related to its inhibition of neuronal glutamate release.[13–18] In addition, DM has been found to possess NMDA-gated channel blocking properties with minimal adverse side effects.[14]

Studies have shown that the immediate-early gene, *c-fos*, exhibits rapid and transient increases in transcription of mRNA in certain regions within the CNS after seizures, brain injury, ischemia, or pain, which has also been shown to correlate with NMDA receptor activation.[19–23] In this regard, the appearance of Fos protein (the protein expressed by the *c-fos* gene) within neuronal nuclei represents a visible marker of adaptive cellular responses to hypoxic or ischemic stimuli within the CNS and supplies an effective method for assaying pharmacologic interventions.

In the current experiments we used immunohistochemical analyses to monitor the induction of nuclear Fos protein primarily within the hippocampal formation as an indication of ischemia-induced expression of the immediate-early gene and to determine the effects of DM on its appearance. The Mongolian gerbil was selected for these experiments because it is a well-established model of ischemic brain injury.[24] In the gerbil, the absence of collateral circulation between the vertebral and carotid arteries permits total forebrain ischemia with bilateral carotid artery occlusion.

The main objective of our study was to evaluate the potential role of DM as a useful therapeutic agent by providing correlative biochemical and anatomic data relating to its neuroprotective effects in an experimental model of brain ischemia.

Materials and Methods

Animal Model

Male Mongolian gerbils weighing 60–70 g were obtained from Tumblebrook Farms (West Brookfield, MA). All experimental protocols were approved by the Tufts University School of Medicine Animal Research Committee. Animals were anesthetized with 2% halothane in air. A recent study demonstrated that halothane anesthesia does not affect *c-fos* expression in rodent brain.[25] Body temperature was measured with a rectal probe and with a thermocouple probe placed in the temporalis muscle, the temperature of which closely approximates that of the brain.[26] Consequently, temperature was maintained at 37–38°C with a heating blanket (Harvard Apparatus, Natick, MA) and thermal lamps.

After induction of anesthesia and 30 min before bilateral carotid artery occlusion, animals were administered either an intraperitoneal injection of 0.9% sodium chloride (vehicle, n = 9) or DM at 50 mg/kg dissolved in vehicle (n = 9). This dose of DM was empirically determined from pilot experiments performed to evaluate maximal inhibitory effects of the agent on *c-fos* induction and is consistent with concentrations of DM used by other investigators in other stroke models.[15–18] After a midline cervical incision had been made, both carotid arteries were occluded for 5 min with microaneurysm clips.[11] The absence of blood flow was verified by visual inspection of the arteries distal to the site of clamping. Afterward, the clips were removed and the arteries inspected for flow and pulsation. After wound closure with silk sutures, anesthesia was maintained with 2% halothane in air during 60 min of reperfusion to ensure adequate time for induction of Fos protein within the CNS.[19–23] Another group of sham-operated controls (n = 9) were anesthetized the same way but did not have carotid artery occlusion. Three sham-operated gerbils received intraperitoneal vehicle; 3 received intraperitoneal DM; and 3 had no injection. Animals were deeply anesthetized with intraperitoneal ketamine and xylazine, 150 and 15 mg/kg, respectively, and were perfused through the left ventricle into the ascending aorta with 50 ml phosphate-buffered saline (PBS), *p*H 7.2, followed by 150 ml 4% paraformaldehyde in 0.1 M sodium phosphate, *p*H 7.2. The brains were rapidly removed and the tissue was postfixed for 4 h in 4% paraformaldehyde, 0.1 M phosphate buffer, *p*H 7.2, and stored overnight in 20% sucrose with 0.1 M PBS, *p*H 7.2, in preparation for immunohistochemical analysis (described below).

Histopathology

For determination of delayed neuronal degeneration within the hippocampal formation and neocortex, 2 groups of animals were pretreated with either vehicle (n = 6) or 50 mg/kg DM (n = 7) dissolved in vehicle followed by 3 min of bilateral carotid artery occlusion. A third group (n = 5) of gerbils were anesthetized and had sham surgery without carotid artery occlusion. Animals were allowed to survive for 7 days before killing. Fixation was performed by transcardiac perfusion as described above. Brains were removed, frozen and, sectioned with a cryostat (Reichert) at −30°C. Loss of CA1 pyramidal cells was verified by histologic staining

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of 6- and 40-μm coronal brain sections of the hippocampus with Cresyl violet.[22] The same regions of each hippocampal field were counted in all animals and corresponded to the same regions used to count Fos-positive neurons. Intact neurons exhibiting a visible nucleus with nucleoli within a 500-μm length of each hippocampal field[27] in the 6-μm sections were counted at 400× by a blinded observer using a light microscope (Standard, Zeiss, Thornwood, NY). Two 500-μm fields were counted within CA1 and CA3, and one field was counted in the dentate gyrus (DG), cingulate cortex (CC), and CA4.[24] Neurons were counted in both hemispheres and averaged. Animals with >10% asymmetry between hemispheres were excluded to eliminate possible variations in collateral circulation or occlusion techniques.

Immunohistochemistry

Immunohistochemical analyses were performed as previously described.[19,28,29] In brief, after postfixation, serial sections (40 μm) were taken from forebrain areas including the hippocampal formation. Free-floating sections were incubated 30 min in methanol–0.3% hydrogen peroxide, followed by 30 min in 10% normal goat serum in 0.1 M PBS at room temperature. Subsequently, tissue sections were incubated with primary antibody for the protein expressed by *c-fos* (donated by Dr. Tom Curran, Roche Institute, Nutley, NJ; final dilution 1:5,000) dissolved in 0.1 M PBS containing 0.4% Triton X-100 by agitating gently for 40 h at 4°C. The antiserum to the protein expressed by *c-fos* is a rabbit polyclonal antibody directed against the protein product of an *in vitro* translated *c-fos* gene.[30] The sections were then washed three times with PBS, 5 min per wash, and incubated with biotinylated goat anti-rabbit serum (1:1,000 dilution in PBS) at room temperature for 2.5 h. For visualization of immunohistochemical staining, sections were processed by the avidin–biotin–peroxidase method (ABC, Vector, Burlingame, CA) with diaminobenzidine as the peroxidase substrate. Additionally, this procedure was modified to include nickel intensification of the diaminobenzidine reaction.[23] Mounted and coverslipped tissue sections were examined with a microscope (Standard, Zeiss), with brown–black reaction product indicating the presence of immobilized antigen. To assess the specificity of immunohistochemical staining, representative tissue sections were processed in the absence of primary antiserum and yielded essentially no cellular-specific reaction product. Furthermore, in every immunohistochemical analysis, brain sections from vehicle-treated animals, DM-treated animals, and sham-operated controls, were processed in parallel to avoid interassay differences in labeling intensity. Finally, immunohistochemically detected nuclear-associated reaction product is referred to as Fos-like immunoreactivity (Fos-LI).

Image Analysis

Immunohistochemical data were analyzed by a blinded observer using an 80386, DOS-based (Microsoft, Redmond, WA) image-processing system.[31] First, nonoverlapping images of forebrain and hippocampal sections were digitized, and stored on magnetic media. Frames were obtained with a monochrome camera (TM-745, Pulnix, Sunnyvale, CA), coupled to a light microscope (Zeiss), and a Frame Grabber/Display board (DT2855 RS-170, Data Translation, Marlboro, MA). Each eight-bit image (640 × 480 square pixels) was viewed at a magnification of 20× and represented approximately a 0.46 × 0.35–mm portion of the anatomic structure. To minimize the variations in field illumination, digitization of all immunohistochemical data was performed at the same light level.

The detection and quantification of Fos-LI–positive nuclei were achieved by first removing the background from each image, locating the resulting objects, and classifying the objects based on a set of predefined limits.[31] Two criteria were established to separate background pixels from those associated with valid objects. First a background threshold based on statistical data obtained from the entire image was established. Next an overall background threshold was established for each image at 1.5 standard deviations below its mean. Pixels with intensities below this value were defined as valid, whereas those falling above this threshold were discarded. Further elimination of background information and separation of defined objects were achieved with the use of an adaptive discriminator.[32] The local mean was calculated for a 15-×-15 kernel centered around each valid pixel whose intensity was greater than the estimated background. The center pixel was subsequently confirmed by comparing its intensity to a threshold equal to the kernel's mean intensity plus its square root. Pixels whose intensities fell above this threshold were defined as background and discarded.

After removal of background information, the resulting objects were located by detecting groups of adjoining pixels. Well-defined Fos-LI–positive nuclei were defined as valid objects based upon their mean

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intensities, sizes, and circumference to area ratios. In contrast, red blood cells, marginally stained nuclei, nonspecifically toned neuronal cell bodies, as well as other artifacts, were rejected. The number of genuine immunoreactive Fos-positive cells was tabulated for each subfield of the examined hippocampal sections. Animals with greater than 10% difference in the number of Fos-positive cells between corresponding areas of the right and left hemispheres were excluded to avoid possible variations in collateral circulation or occlusion techniques. Finally, the accuracy of the automated counting procedure was corroborated by performing manual cell counts of representative sections chosen at random.

Data Analysis

Immunohistochemical data from the treatment groups (sham, occlusion without DM, occlusion with DM) were evaluated by one-way analysis of variance followed by *post hoc* testing by the Bonferroni method (analysis of variance, GraphPad InStat program). The number of Fos-LI–positive nuclei within discrete areas of the hippocampal formation were expressed as mean ± SEM. DM mediated survival of hippocampal and neocortical neurons 7 days after bilateral carotid artery occlusion were expressed as mean ± SEM and evaluated by one-way analysis of variance of the three groups (analysis of variance). A P value < 0.05 was considered to be statistically significant.

Results

Global forebrain ischemia for a period of 5 min followed by 60 min of reperfusion under halothane anesthesia produced consistent patterns of cellular labeling indicating the presence of Fos-LI distributed in the DG; in the CA1, CA3 and CA4 fields of the hippocampus; and in the CC (fig. 1A). Immunohistochemical labeling was exclusively associated with round or oval cell nuclei with graded signal intensities ranging from relatively light to very dense. Pretreatment with 50 mg/kg DM markedly reduced the number of ischemia-induced Fos-LI–positive nuclei in these same hippocampal regions (fig. 1B). The relative absence of Fos-LI–positive nuclei in the hippocampal formation of DM-treated animals was similar to the pattern of labeling observed in these same regions of all of the sham-operated controls.

Computer-assisted counts of Fos-LI–positive nuclei revealed statistically significant decreases in ischemia-induced expression of the immediate-early gene in the DG and hippocampal fields as a function of DM pretreatment at 50 mg/kg. Typically, the number of Fos-LI–positive cells induced by ischemia varied from 30–60, 25–50, 30–50, and 10–20 labeled nuclei per 10× field in CC, DG, CA3, and CA4, respectively. Additionally, the computer-assisted image analysis positively scored lightly labeled nuclei in the CA1 region yielding values of 15–45 labeled cells per 10× field. In gerbils pretreated with DM (n = 9), the number of Fos-LI–positive cells in hippocampal regions was reduced from 65% and 91% in the CC and hippocampal formation, respectively ($P < 0.001$; fig. 2). The number of Fos-LI positive nuclei in the CC and hippocampal formation of DM-treated animals was not statistically different from the number of Fos-LI–positive nuclei in the sham-operated animals (n = 9, fig. 2).

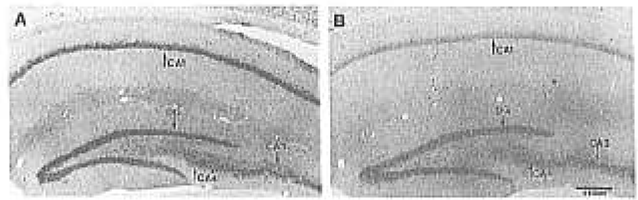

Fig. 1. Photomicrographs showing Fos-like immunoreactivity (Fos-LI) in gerbil hippocampus. (*A*) Global forebrain ischemia for 5 min produced consistent cellular labeling indicating the presence of Fos-LI in the dentate gyrus (DG) and in the CA1, CA3, and CA4 regions of the hippocampus 1 h after global ischemia. (*B*) Pretreatment with 50 mg/kg dextromethorphan markedly reduced the number of ischemia-induced Fos-LI–positive nuclei in these hippocampal regions. Bar = 250 μm.

Bilateral carotid artery occlusion produced consistent degeneration of pyramidal neurons throughout the CA1 region of the hippocampal formation in all vehicle-treated animals (n = 6) 7 days after the ischemic insult (fig. 3A). Although there appeared to be fewer intact cells in DG, CA4, and CC in vehicle-treated animals, compared with sham-operated controls and DM-treated animals, this difference was not statistically significant (fig. 4). There was no detectable ischemic damage after carotid occlusion in cells of the CA3 region although it did cause significant induction of Fos protein as indicated above. DM pretreatment (n = 7) before carotid artery occlusion prevented the destruction and disappearance of neurons in the CA1 region (fig. 3B). The CA1 region of DM-treated animals showed an average of 59 ± 2.4 intact cells, insignificantly different from the number in the sham-operated animals (n = 5) with 61 ± 2.4 intact cells but highly significant compared

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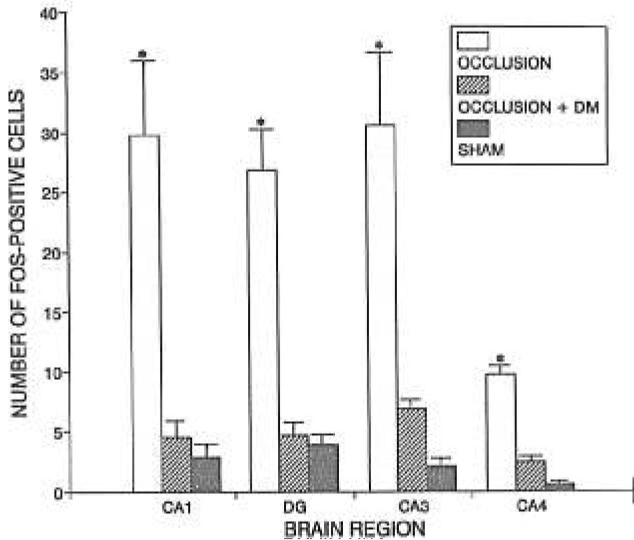

Fig. 2. Reduction of ischemia-induced Fos-LI–positive nuclei in gerbil brain by pretreatment with 50 mg/kg intraperitoneal dextromethorphan. DG = dentate gyrus; CA4, CA3, and CA1 = regions of the hippocampus. Data expressed as mean ± SEM; n = 9. *P < 0.001: significant difference in vehicle-treated animals compared with dextromethorphan-treated and sham-operated animals.

with 9.0 ± 3.1 cells in vehicle-treated animals (n = 6, P < 0.001; fig. 4).

Discussion

In the current study, immunohistochemical analyses were used to monitor the effects of global forebrain ischemia on expression of the immediate-early gene by immunoreactive Fos protein induction in the gerbil hippocampal formation, an anatomic region of the CNS shown to be important for learning and memory. We observed significant decreases in the number of Fos-LI–positive nuclei in all examined regions as a function of DM pretreatment. DM also provided significant protective effects against delayed neuronal death within the CA1 region.

Unlike studies that suggest postischemic inhibition of Fos protein synthesis in CA1 pyramidal cells,[33,34] we found significant Fos protein production in our experiments. This discrepancy is most likely a result of the method used to assay Fos protein. We used a computer based image processing system to count the Fos-LI–positive nuclei. Image analysis with use of a strong discriminator is able to detect even low levels of protein production. Although the cells in CA1 were less in-

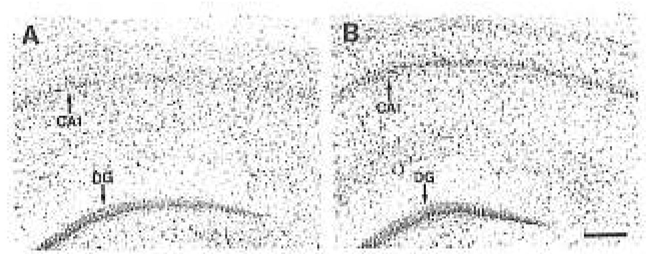

Fig. 3. Photomicrographs of Cresyl violet–stained sections of gerbil hippocampus 7 days after 5 min of bilateral carotid artery occlusion. (*A*) Occlusion without dextromethorphan. There is extensive degeneration of pyramidal neurons throughout the CA1 region of the hippocampus. (*B*) Occlusion after pretreatment with 50 mg/kg intraperitoneal dextromethorphan. Bar = 200 μm.

tensely stained on initial observation through the microscope, the number of ischemia-induced Fos-positive nuclei in CA1 counted by the computer was comparable to the number counted in other regions of the brain and significantly more than the sham-operated animals (fig. 2). The computer program is more objective and consistent than the human-eye "cell-grading" technique used by other investigators.[33,34]

The time course and distribution pattern of Fos protein induction in the current study is consistent with

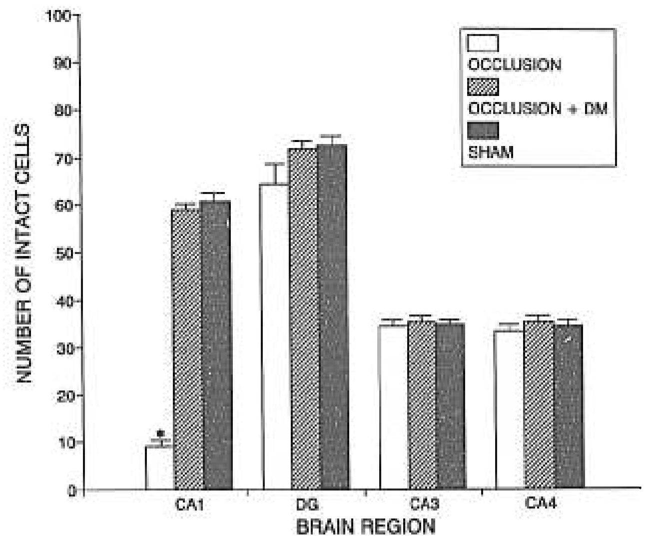

Fig. 4. Reduction by dextromethorphan of CA1 ischemic neuronal damage. *P < 0.001: significant difference in dextromethorphan-treated animals (n = 7) compared with normal saline–treated animals (n = 6). There was no significant difference in neuronal damage between dextromethorphan-treated and sham-operated animals (n = 5, no occlusion). Data expressed as mean ± SEM. DG = dentate gyrus; CA1, CA3, CA4 = regions of the hippocampus.

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the findings of several studies that showed transient and temporal induction of *c-fos* in all regions of the hippocampal formation by immunohistochemical techniques and *in situ* hybridization.[20,35] The expression of *c-fos* in the hippocampus after transient ischemia occurs during the early reperfusion phase and follows a temporal sequence that may reflect the trisynaptic circuitry of the hippocampus (DG → CA3 → CA1). Therefore Fos-LI, which is barely detectable after 15 min of reperfusion, is pronounced after 30–60 min in the granule cells of the DG and at 1–2 h in the pyramidal cells of the hippocampus. We did not observe Fos-LI anywhere in the hippocampal formation after 4 h of reperfusion under halothane anesthesia. Accordingly, we selected 1 h of reperfusion to assay Fos-LI because at this time point Fos protein was visible in all regions of the hippocampal formation. This may account for our observations of Fos protein in the CA1 region, whereas another study that measured Fos 2–4 h after ischemia did not observe significant Fos protein in CA1 neurons.[33] Although the distribution and appearance of Fos-LI is a useful method to assay the effectiveness of pharmacologic interventions, this technique is somewhat limited because Fos is measured at only one time point in each experiment.

It is well known that transient forebrain ischemia causes delayed neuronal degeneration in the CA1 neurons of the hippocampal formation, although the underlying pathologic mechanism for this phenomenon is not known.[24] In CA1 neurons, Fos-LI induction correlated with ischemia-induced delayed neuronal degeneration and both of these processes were inhibited by pretreatment with DM. However, Fos-LI induction in other regions of the hippocampus and neocortex was not correlated with neuronal cell death. Other studies have demonstrated *c-fos* expression in a variety of neurons and glia that survive transient forebrain ischemia, whereas the apparently more vulnerable neurons in CA1, the hilus of the DG, and dorsolateral caudate typically do not.[35,36] Fos protein induction is thought to control the expression of late-response genes, resulting in altered synthesis of neurotransmitters, receptors, and ion channel proteins,[9,21] both as part of normal cell growth and differentiation and in response to noxious stimuli.[19–23] Therefore, the induction of *c-fos* appears to be nonspecific and present throughout the nervous system. Whether within a particular neuron or region of the brain Fos expression is protective or associated with cell death is not clear from these experiments. However, its suppression by DM throughout the entire hippocampal formation serves as a useful method to assay the effectiveness of pharmacologic interventions for ischemia.

Ischemia-induced brain damage is associated with excessive release and impaired cellular uptake of excitatory amino acid neurotransmitters such as glutamate.[17,27,37] Increases in extracellular glutamate concentrations in the hippocampus have been correlated with ischemia-induced delayed neuronal death in CA1.[27,37–42] The mechanism resulting in increased glutamate release has been found to be mediated by depolarization-induced calcium influx through voltage-gated channels.[43] Neuronally released glutamate exerts its toxic effects *via* excessive calcium influx through membrane channels opened when glutamate binds to receptors located on postsynaptic membranes,[13] particularly the NMDA subtype of glutamate receptor.[42] Glutamate, *via* a presynaptic mechanism, stimulates further calcium influx to sustain its continued release.[44]

DM may be functionally classified as a physiologic NMDA antagonist because its major actions are not mediated at well-defined postsynaptic binding sites on the NMDA receptor.[14] Furthermore, DM does not display the noncompetitive binding characteristics of MK-801, ketamine or phencyclidine.[12] Instead a presynaptic mechanism of action has been proposed whereby DM inhibits evoked release of glutamate. For example, DM has been shown to inhibit glutamate release from rabbit hippocampal slices.[18] In addition, specific CNS binding sites for DM demonstrate different distributions from those of postsynaptic glutamate receptors.[14] DM and the calcium-channel blocker cinnazarine strongly compete for the same presynaptic binding site,[45] thereby linking the drug's site of action to calcium channels. Morgan and Curran[46] reported depolarization-induced calcium influx, which is known to increase glutamate release,[43] also induces Fos expression. Ischemia-induced intracellular accumulation of calcium and subsequent cell death has been well documented in the brain.[41,42] Our results demonstrate DM decreases ischemia-induced Fos expression suggesting the drug may inhibit glutamate's mechanism of enhancing calcium influx.

Pharmacologic antagonism of NMDA receptor-mediated neurotoxicity is a novel therapeutic approach in the treatment of cerebral ischemia. We confirm previous results that DM, when given preemptively, is neuroprotective in an *in vitro* model of global forebrain ischemia.[47] DM is more suitable for clinical use than are the noncompetitive NMDA antagonists MK-801,

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phencyclidine and ketamine because it does not have the psychotomimetic properties or other adverse side effects of σ-receptor activity associated with these latter drugs. Even more disturbing are the pathologic changes induced in cerebrocortical neurons by phencyclidine, MK-801, and ketamine.[48] Although structurally related to codeine and morphine, DM has the opposite (*dextro*) steric configuration and therefore does not cause respiratory depression and has no addiction liability or analgesic properties.

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