Role for γ-Aminobutyric Acid in Selective Vulnerability in Gerbils

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We tested the efficacy of various putative neuroprotective agents in the gerbil model of delayed neuronal death. The selective loss of anterior CA1 neurons of the hippocampus 4 days after 5 minutes of bilateral ischemia was complete in >90% of the gerbils examined. We tested 11 agents for their ability to protect against neuronal loss. Only those agents that were associated with the GABAergic system exhibited protection and only when administered before the ischemic insult. The possibility that delayed neuronal death is the result of a primary defect in inhibitory neurotransmission is considered. (Stroke 1989;20:281-287)

Selective vulnerability (SV) of hippocampal neurons was first reported more than 100 years ago,1 and the mechanism for this phenomenon has yet to be elucidated. Many hypotheses have been proposed to explain the delayed neuronal death (DND) that occurs in the CA1 pyramidal cells following an ischemic event.2-10 A more recent hypothesis proposes that certain excitatory amino acids (i.e., glutamate and aspartate) may act as endogenous neurotoxins following seizures, ischemia, and hypoglycemia.11 While extracellular glutamate in low concentrations is toxic to selected neuronal populations,12 it is still unclear whether glutamate antagonists can prevent DND.13-14 Further support of the excitotoxic hypothesis was provided by Rothman,15 who reported that hippocampal neuronal cultures could be protected from anoxic damage by blocking synaptic activity. These results, along with the demonstration that deafferentation of the Schaeffer collaterals blocked postischemic CA1 neuronal death, suggest that synaptic transduction is an important component of SV.16,17

While glutamate may be the toxin, the primary insult that gives rise to glutamate toxicity does not necessarily have to be intrinsic to this system. We questioned whether the insult is an exaggerated excitation secondary to an inhibitory defect within the hippocampus. The major inhibitory pathway within the hippocampus is mediated by γ-aminobutyric acid (GABA).18 Previous studies, both during and after ischemia, have shown marked derangements in both GABA and glutamate metabolism in the CA1 region of the hippocampus.19 While the tissue GABA levels increase during ischemia and only gradually return to control during recirculation, glutamate concentrations do not change during ischemia but decrease during the early stages of recirculation. Thus, a GABAergic lesion might occur during ischemia or shortly thereafter, eventually triggering the excitotoxic phenomenon.

We examined several possible mechanisms of DND by testing specific pharmacologic agents that should counteract the effects of the various purported toxins. Then, we treated gerbils with GABAergic agents and compared the response with that for the first group of putative neuroprotective agents. The complete loss of hippocampal CA1 neurons elicited by ischemia has been shown to be quite reproducible in gerbils and has provided an excellent model for the study of DND.20 The efficacy of each agent to protect CA1 cells was evaluated histologically and by quantification of glycogen. We have previously demonstrated a fourfold increase in glycogen content in the CA1 pyramidal cell region 4 days after an ischemic period, a presumed result of glial enrichment.21 Since it is unresolved when the pathogenic event occurs in SV, we also examined the effect of preischemic and posts ischemic administration of these agents to further elucidate the temporal mechanisms of DND.

Materials and Methods

Female Mongolian gerbils (50–70 g) were fed ad libitum and were anesthetized with 50 mg/kg i.p. methohexital. Under the operating microscope, a
TABLE 1. Agents, Mechanisms of Action, and Doses and Times of Administration in Study of Role of γ-Aminobutyric Acid in Selective Vulnerability in Gerbils

<table>
<thead>
<tr>
<th>Drug (source)*</th>
<th>n</th>
<th>Dose (mg/kg)</th>
<th>Time of administration (min)</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>40</td>
<td>-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GABAergic agents</strong></td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>\</td>
</tr>
<tr>
<td>Pentobarbital (1)</td>
<td>30</td>
<td>50</td>
<td>-60, -15, -4, 0, +0.1, +1, +120</td>
<td>GABA A receptor-effector</td>
</tr>
<tr>
<td>Diazepam (2)</td>
<td>15</td>
<td>10</td>
<td>-5, +5</td>
<td>Benzodiazepine receptor agonist</td>
</tr>
<tr>
<td>Baclofen (3)</td>
<td>9</td>
<td>25</td>
<td>-5, +5</td>
<td>GABA B agonist</td>
</tr>
<tr>
<td>Valproic acid (1)</td>
<td>11</td>
<td>200</td>
<td>-30</td>
<td>GABA transaminase inhibitor</td>
</tr>
<tr>
<td>Muscimol (2)</td>
<td>8</td>
<td>5</td>
<td>-30</td>
<td>GABA A agonist</td>
</tr>
<tr>
<td><strong>Putative neuroprotective agents</strong></td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>\</td>
</tr>
<tr>
<td>Nafidrofural (4)</td>
<td>9</td>
<td>45</td>
<td>-30</td>
<td>Cerebral vasodilator</td>
</tr>
<tr>
<td>Deferoxamine (3)</td>
<td>8</td>
<td>200</td>
<td>-30</td>
<td>Iron chelating agent</td>
</tr>
<tr>
<td>Phenytoin (5)</td>
<td>8</td>
<td>25</td>
<td>-30</td>
<td>Antiepileptic</td>
</tr>
<tr>
<td>Nimodipine (6)</td>
<td>13</td>
<td>1</td>
<td>-20, +300</td>
<td>Calcium antagonist</td>
</tr>
<tr>
<td>Etomidate (1)</td>
<td>13</td>
<td>5</td>
<td>-5</td>
<td>Cerebral depressant</td>
</tr>
<tr>
<td>Ketamine (5)</td>
<td>13</td>
<td>50</td>
<td>-5</td>
<td>Possible N-methyl-D-aspartate antagonist</td>
</tr>
</tbody>
</table>

All agents were injected intraperitoneally. Pretreatment is indicated by minus sign, posttreatment by plus sign, and numeral represents minutes before or after onset of occlusion.

*Numbers in parentheses designate the sources of the various compounds: (1) Abbott Laboratories, North Chicago, Illinois; (2) Sigma Chemical Co., St. Louis, Missouri; (3) CIBA-GEIGY Corp., Summit, New Jersey; (4) Liph, Lyon, France; (5) Parkes-Davis, Morris Plains, New Jersey; (6) Miles Pharmaceuticals, New Haven, Connecticut.

midline cervical incision was made and both common carotid arteries were looped but not occluded. Several hours after the gerbils had recovered from anesthesia, both common carotid arteries were occluded using microaneurysm clips. After 5 minutes the clips were removed and circulation was reestablished. The various agents tested, their mechanisms of action, and the doses and times of administration are shown in Table 1. Control gerbils received saline.

Rectal temperatures of the gerbils were maintained between 36° and 37° C with heat lamps. After 4 days of recirculation, the gerbils were frozen in liquid nitrogen, the brains were removed at -20° C, and the frozen tissue was sectioned on a microtome cryostat. Coronal 20-μm sections were taken from the dorsal hippocampus at the level of the habenula and the choroid plexus of the third ventricle. Representative sections were stained with thionin, and the CA1 region of the hippocampus was examined by light microscopy to determine complete loss, partial loss, or complete sparing of the neurons (Figure 1). In our studies, partial sparing of the CA1 neurons was the exception, and generally the entire CA1 population was either spared or lost. Experiences from our study and two others indicate that protection is essentially an all-or-none phenomenon; therefore, counting the neurons in the CA1 region was not considered necessary. When partial sparing did occur, there was a typical histologic pattern characterized by an increasing loss of neurons from the lateral CA1 region to the subiculum. We use protection to refer to the ability of an agent to significantly reduce the number of gerbils exhibiting complete loss of CA1 neurons. Quantitative assessment of neuronal loss was confirmed in the pentobarbital experiments by measuring glycogen concentration in the cell body layer (i.e., stratum pyramidale) of the CA1 region. Sections of lyophilized cortex and the CA1 and CA3 cell body layers were dissected, and microquantitative analysis of glycogen content was performed on 1-μg pieces of tissue, as described by McCandless et al.

Results

The typical response of untreated gerbils to bilateral carotid artery occlusion was characterized by the early onset of eyelid ptosis and behavioral depression. After the first minute, there were variable periods of random motor behavior, chirping, and opisthotonos interspersed with periods of quiescence. This stereotypical behavior did not persist into the recirculation period. It is unlikely that the seizure-like activity was responsible for the DND since this behavior was evident only when the electroencephalogram of the ischemic regions was isoelectric. This is consistent with the conclusion of Cohn that the observed hyperexcitability both during and after ischemia originates in nonischemic regions such as the brainstem or spinal cord. Control gerbils were alert 30 minutes after the occlusion and remained at their neurologic baseline for 1-4 days after ischemia. In the treated gerbils the behavior was similar, except that activity in gerbils treated with pentobarbital, diazepam, baclofen, muscimol,
etomidate, or ketamine was depressed, but to varying degrees. It also should be noted that the administration of diazepam, muscimol, or ketamine caused clonic jerks on handling before occlusion.

Complete CAI cell loss occurred in 98% of the control gerbils (Figure 2). Pretreatment with pentobarbital, diazepam, or baclofen provided complete CAI sparing in, respectively, 93%, 100%, and 88%. While these gerbils did not display complete CAI sparing, there was evidence of partial sparing, a response rarely seen in control gerbils or after treatment with agents other than pentobarbital. The incidence of loss of CAI neurons in gerbils treated 120 minutes after ischemia was indistinguishable from that of control ischemic gerbils. Partial sparing of the CAI neurons was particularly evident in the gerbils treated with pentobarbital either just before or shortly after the onset of ischemia. There was partial sparing in 31% of the gerbils treated 1 minute before clip application, in 50% of those treated 1 minute after clip application,

The effect of timing of treatment was first examined with pentobarbital (Figure 4). Gerbils treated 60, 15, or 4 minutes before occlusion demonstrated significant CAI cell protection in, respectively, 93%, 100%, and 88%. While these gerbils did not display complete CAI sparing, there was evidence of partial sparing, a response rarely seen in control gerbils or after treatment with agents other than pentobarbital. The incidence of loss of CAI neurons in gerbils treated 120 minutes after ischemia was indistinguishable from that of control ischemic gerbils. Partial sparing of the CAI neurons was particularly evident in the gerbils treated with pentobarbital either just before or shortly after the onset of ischemia. There was partial sparing in 31% of the gerbils treated 1 minute before clip application, in 50% of those treated 1 minute after clip application,

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and in 15% of those treated 1 minute after clip removal (6 minutes after occlusion). Complete CA1 loss in these three groups occurred in, respectively, 15%, 50%, and 85% of the gerbils. Treatment after occlusion with either diazepam or baclofen provided protection in <10% of the gerbils (Figure 5).

A significant fourfold elevation in glycogen content (p<0.05) was seen in the stratum pyramidale of the hippocampal CA1 region 4 days after 5 minutes of bilateral cerebral ischemia (Figure 6). This increase was prevented by pretreatment with GABAergic agents. Of interest was a concomitant 2.5-fold rise in CA3 cell body glycogen concentration after ischemia, and this increase in glycogen content was also prevented by pretreatment with GABAergic agents. These agents administered to gerbils after occlusion did not prevent the threefold increase in CA1 cell body glycogen content and the modest elevation in CA3 somal glycogen concentration. In those gerbils that displayed partial sparing histologically, a glycogen gradient was noted in serial samples from the subiculum (44.6), medial CA1 neurons (42.1), lateral CA1 neurons (17.1), CA2 cells (9.2), and CA3 cells (19.0 nmol/mg dry wt).

Discussion

Many laboratories have used this model of DND for testing the efficacy of agents in the treatment of stroke.22,23,27 The loss of CA1 neurons 4 days after 5 minutes of bilateral ischemia has proven to be reproducible, providing a clear-cut end point for assessing efficacy. The proliferation of glia concomitant with the loss of neurons in the CA1 cell region is supported by an increase in glial fibrillary acidic protein-like immunoreactivity (data not shown), and this apparently accounts for the elevation of glycogen content since glia have higher glycogen stores than neurons.28 A reduction in glycogen accumulation after 4 days of recirculation appears to be a reasonable approach for the quantification of drug efficacy.

A number of theories have been proposed for the mechanism of brain damage following an ischemic episode.2-10 We tested several agents to determine the degree of protection from the deleterious effects resulting from free radical formation, hypoperfusion, elevated tissue calcium concentration, and seizures. These agents were generally ineffective in preventing the loss of CA1 neurons. The inability of the anticonvulsant phenytoin to provide protection is indirect evidence that DND does not result from seizures, as has been suggested.29 Since SV following seizures is generally most profound in the CA3 region,30 the presence of complete CA1 neuronal loss with near-complete sparing of the CA3 cells...
further supports the concept that seizures are not involved in this model of ischemic CA1 DND.

The mechanism for CA1 neuronal protection may be central nervous system (CNS) depression. Other agents, such as ketamine and etomidate, offered moderate protection, and both resulted in CNS depression, as did most GABAergic agents. Ketamine is a purported glutamate antagonist, acting specifically at the N-methyl-D-aspartate receptor; ketamine has also been reported to potentiate GABA.31 Etomidate, a short-acting hypnotic agent, works through a barbiturate-like action.32 The effective agents may not work at a specific site; rather, they may exert their actions through generalized CNS depression. While we cannot preclude a role for CNS depression in the protective effects observed, the ineffectiveness of certain depressants such as methohexital, halothane, and nitrous oxide provides indirect evidence that such is not the case.

The physiologic role of GABA in controlling neuronal excitability has been extensively investigated, and the changes in GABA during and after ischemia are sufficient to cause CNS depression or excitation.34 While the action of GABA varies depending on the area of the neuron examined, the net effect is an inhibition of neuronal firing. The five GABAergic agents we tested apparently act on different sites of either the GABA receptor-chloride ionophore complex (Figure 7) or the GABA B receptor. Barbiturates, particularly pentobarbital, prolong the opening of the chloride ionophore channel and enhance GABA and benzodiazepine receptor binding.35 Diazepam works directly on the benzodiazepine receptor, which is located on the GABA receptor complex, to open the chloride channel.36 Muscimol is a GABA A agonist but is thought to have some toxic effects. Baclofen is a selective GABA B receptor agonist. GABA B receptors are distinct from GABA A receptors and are thought to modify both calcium uptake and the release of neurotransmitters such as glutamate and norepinephrine.33 Valproic acid is a GABA transaminase inhibitor that may increase the concentration of endogenous GABA by preventing its catabolism.37

All the GABAergic agents we tested provided either moderate or complete protection against DND; the most effective agent was pentobarbital. Several laboratories have described barbiturate protection in the gerbil model of DND, but certain aspects of their data were contradictory to those we found. For example, Kirino et al demonstrated a posts ischemic effect of 40 mg/kg i.p. pentobarbital when administered after the clips were removed; halothane was administered until clip application. Hallmayer et al could elicit a protective effect with pentobarbital pretreatment only at 25 mg/kg, not at 50 mg/kg; at the lower dose, the gerbils also received halothane. Some of the time and dose differences could be explained by the use of halothane in these experiments. It is possible that halothane and pentobarbital act synergistically to increase the time during which the latter is effective. Alternatively, halothane might delay or prolong an ischemia-induced event, allowing for a posts ischemic effect of pentobarbital. In contrast, our data suggest that the protective effect of pentobarbital occurs only when the gerbil is treated before ischemia and that there is an only partial effect when pentobarbital is given during or immediately after ischemia. The limited period of pentobarbital efficacy before ischemia suggests that there is a GABAergic-related event occurring either during or shortly after ischemia that ultimately leads to DND. Similar temporal restrictions were observed with two other GABAergic agents, diazepam and baclofen.

If GABA is responsible for the eventual loss of CA1 neurons, such a mechanism would have to take into account the following information. First, the levels of GABA in the CA1 cell body layer are higher than those in either the cortex or the CA3 cell body layer, both during and after ischemia.19 Second, the efficacy of the GABAergic agents is much greater when given before ischemia. Third, the effect of the agents is not limited to only the GABA A receptor. Fourth, neither the GABA A receptor nor the cells containing glutamic acid decarboxylase show evidence of permanent posts ischemic damage after the insult, suggesting a transient, readily reversible event.38,39 Fifth, the damage to CA1 neurons is mediated by glutamate, but later during recirculation. And sixth, the action of these agents is exerted when the tissue is electrically quiescent. It remains to be answered how the GABA system could be perturbed during ischemia in such a way as to permit an imbalance in the excitatory and inhibitory input in the CA1 sector to be expressed hours after the insult.

It is unlikely that disinhibition secondary to a loss of GABA input is responsible since the levels of GABA are significantly higher in the CA1 region during ischemia.19 However, preconditioning synaptosomes with a number of GABA agonists diminishes the chloride flux elicited by GABA.40 Down-regulation of the GABA receptor could explain the increased expression of the glutaminergic pathway, but the temporal disparity between the two events suggests that such a mechanism is not reasonable. Alternatively, these agents may protect the GABA receptor-chloride ionophore complex from effects normally induced by ischemia. By preventing this
presumed alteration of the GABA receptor following ischemia, the entire sequence of events leading to DND would be avoided. While this possibility remains as speculation, the protective effect of agents that modify the GABA receptor is convincing, and further studies will target on the mechanism of this phenomenon.

In conclusion, we have demonstrated that in gerbils, GABAergic agents protect against the delayed death of the CA1 hippocampal neurons elicited by ischemia. These agents prevent cell loss only when given before ischemia, which suggests that the triggering event occurs during ischemia and not during recirculation. Since these agents work at different sites of neural transmission, they may be used as probes to identify the mechanism underlying DND.

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