A Reversible Type of Neuronal Injury Following Ischemia in the Gerbil Hippocampus


SUMMARY The Mongolian gerbil is known to develop delayed neuronal death in the hippocampus following brief forebrain ischemia (Brain Res 239: 57-69, 1982). The effect of pentobarbital on this slow process of neuronal damage was examined. Immediately following 5 min of bilateral carotid occlusion, pentobarbital (10, 20, or 40 mg/kg) was injected. The control animals received saline injection. Seven days following ischemic insult, animals were perfusion-fixed and the neuronal density in the hippocampal CA1 subfield was counted. Most of the neurons in the CA1 sector survived ischemic insult when pentobarbital was given, whereas most of control group neurons were lost without the treatment. The average neuronal density of 20 mg/kg group was 168.2 ± 12.3 (SEM) per 1 mm linear length of the CA1 subfield. The density in 40 mg/kg group was 181.1 ± 14.9. The neuronal density in the whole control group was 34.3 ± 5.1. The density of unoperated normal gerbils was 212.3 ± 3.9. This result indicates that the neuronal damage of "delayed neuronal death" is reversible. On the other hand, when pentobarbital was injected 1 hr following ischemia, it showed no effect. The cell change in the CA1 sector, reversible at the initial stage, seems to rapidly become irreversible, while neurons still retain intact morphologically.

Stroke Vol 17, No 3, 1986

NEURONS IN THE BRAIN play a primary role in the neural function. Neurons, except on rare occasions, lose their mitotic capacity during development. Damages against the brain cause persistent disability. To preserve neurons, therefore, is a matter of great clinical concern in patients who suffer from such destructive lesions as cerebral anoxia or ischemia. It is yet to be known if any modality of treatment can save neurons following ischemia. If a drug, given after ischemic insult, still could show a beneficial effect on the survival of neurons, its clinical usefulness may be invaluable.

The early reports on the effect of barbiturates in reducing brain damage after anoxia or ischemia encouraged further experimental and clinical studies. In experimental focal ischemia, there are copious reports on the beneficial effects of barbiturates. In experimental animals, barbiturates improved the neurological deficit scores, the final outcome, or reduced the size of infarction. The experiments using models of global brain ischemia showed the promising effects of barbiturates. Recent studies, however, seem to dampen the initial enthusiasm. Barbiturate-loading had no protective effect on cerebral anoxia or ischemia. The possible adverse effect was also indicated especially in cases of overdosage. Clinical experiences seem to suggest that barbiturates show beneficial effects when used immediately after ischemic insult in selected cases under strict intensive care. The practical usefulness of barbiturate therapy, thus, is still limited. There exist great medical and social needs for potential resuscitative drugs which can benefit anoxia or ischemia patients.

There is increasing evidence that individual neurons or neuronal groups in the central nervous system have their own specific biochemical, pharmacological, or physiological properties. This inhomogeneity of the brain is also reflected in the development of disease processes in the central nervous system. Also, drug effects against various pathological processes may be inevitably related to the inhomogeneous nature of the brain. The specific action of a drug may be examined more efficiently by restricting the area of study to a specific region of the brain rather than dealing with the brain as a whole. The method of concentrating attention on a well-defined, localized neuronal group in the brain may be warranted as a strategy of research in the study of pharmacological mechanism against cerebral ischemia. One potential method is to find out an in vivo system in which a clear-cut drug effect is shown while the animal is not disturbed by complicated focal or general problems such as ischemic edema or cardio-pulmonary depression. In this system, the following conditions should also be satisfied: the drug effect should be focussed on a specific group of neurons, the effect should be reproducible and predictable, and the animals should survive extended period following insult since final outcome should be judged in a chronic, stable stage.

The Mongolian gerbils (Meriones unguiculatus) were introduced as an experimental animal for cerebral ischemia by Levine and Payan. They noticed that unilateral ligation of the carotid artery in the neck produces cerebral infarction. Later, it has come to be realized that occlusion of the carotid arteries on the both sides brings about uniform forebrain ischemia, during which the value of blood flow is close to zero. Brief bilateral carotid occlusion in the gerbil was shown to produce typical neuronal damage in the hippocampus. More than 90% of the animals subjected to 5 min of bilateral forebrain ischemia demonstrate uniform destruction of the CA1 neurons. The neuronal damage in the CA1 sector evolves very slowly and progressively until it results in massive cell destruction 4 days following ischemia. No definite
ischemic neuronal damage is seen outside the hippocampus. There is no effect of ischemic brain edema microscopically in this model. Although gerbils are known to develop "epileptic" abnormalities during and after ischemia, five minutes of occlusion usually does not cause "epileptic" movement in the gerbil. The survival rate of gerbils following 5 min of ischemia is almost 100%, fatal outcome being only exceptional. The operative procedure of occluding both of the carotid arteries, when experienced, takes only 2 to 3 minutes. These features of this model suggest that the gerbil hippocampus offers an excellent in vivo system in which we can study the specific effect of drug treatment following cerebral ischemia. Delayed neuronal death observed in the CA1 subfield of the hippocampus is not a change inherent only in the gerbil. Slow cell death in the CA1 sector has also been known in the rat.15-17

In this report, we describe the effect of pentobarbital on the survival of neurons in the hippocampal CA1 subfield following brief transient ischemia in the gerbil. To evaluate the drug effect, neuronal cell density in the CA1 sector was used as an index of the effect on neuronal survival.

Materials and Methods

Adult Monglian gerbils weighing 60-80 g were used. Animals were anesthetized with 2% Halothane and the right and left common carotid arteries were exposed through midcervical vertical skin incision. Then anesthesia was discontinued and the carotid arteries on both sides were occluded with aneurysm clips (Sugita temporary clip type 07-940-51) for 5 min. Immediately following 5 minutes of occlusion, gerbils were injected with a given amount (10 mg/kg, 20 mg/kg, 40 mg/kg) of pentobarbital (Nembutal, Abbott Laboratories) intraperitoneally. Each pentobarbital group had corresponding control groups which received the same volume of saline injection. Separately, two groups of delayed drug administration were prepared. Animals in these groups were given pentobarbital (40 mg/kg) 60 or 120 min following clip removal. To circumvent the effect of differences among litters, gerbils were divided in each experiment randomly into pentobarbital groups and corresponding control groups. Each gerbil was randomly coded and processed thereafter only by this code number. The number of gerbils used in this experiment is shown in table 1 and table 2.

After the operation, the gerbils were kept in warmed cages under illumination of infrared lamp. Rectal temperature was monitored. When animals recovered full consciousness and started moving around, they were returned to their cages and permitted free access to food and water.

One week following ischemia, the gerbils were fixed by transcardiac perfusion. Under deep pentobarbital anesthesia, 500 ml of 3.5% formaldehyde in 0.1M phosphate buffer (pH = 7.3) was perfused at a pressure of 130 cm H2O. The animals were kept in a refrigerator overnight and the brains were dissected out the following day. Two-millimeter-thick coronal sections were cut, dehydrated through graded series of ethanol, soaked in xylene and embedded in paraffin. As the normal controls, 8 unoperated adult gerbils were perfusion-fixed in the same method. These specimens were used to evaluate the normal range of the neuronal cell density in the dorsal hippocampus.

Five-micrometer-thick sections, which contained dorsal hippocampus (fig. 1) located 0.5-1.0 mm posterior to the most rostral tip of the hippocampus or 1.4-1.9 mm posterior to the bregma,18 were prepared on a sliding microtome and stained with hematoxylin and eosin or cresyl echt violet and luxol fast blue. Since the neuronal change is similarly seen throughout the rostral-caudal extent of the dorsal hippocampus,14 one section from each animal was used for counting. These sections were examined by one of the authors (A.T.) without knowing the amount and the timing of the drug given. Photographs of left and right dorsal hippocampi (fig. 1) of each specimen were taken using Polaroid type 667 films at a magnification of 30x. The medial and lateral border of the CA1 subfield were marked on the photographs by felt-tipped pen. The medial border was defined at the margin where typical radiation pattern of the dendrites in the stratum radiatum became vague. The lateral border was identified where stratum lucidum below the stratum pyramidale appeared evident. Therefore, a small portion of the CA2 sector was included in the CA1 subfield defined as stated above. The total linear length of the CA1 sector was measured by means of a digitizer (Graphitec Co.). The number of living neurons in the stratum pyramidale within the CA1 subfield was counted using Olympus Vanox photomicroscope at a magnification

<table>
<thead>
<tr>
<th>TABLE 1 Neuronal Cell Density Per 1 mm Linear Length of the CA1 Subfield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal gerbil</td>
</tr>
<tr>
<td>n = 8</td>
</tr>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>Pentobarbital 10 mg/kg</td>
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<tr>
<td>n = 8</td>
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<tr>
<td>Pentobarbital 20 mg/kg</td>
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<tr>
<td>n = 9</td>
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<tr>
<td>Pentobarbital 40 mg/kg</td>
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<td>n = 10</td>
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</table>

*Statistically significant (p < 0.01).  
SEM = standard error of the mean.  
The values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>TABLE 2 Neuronal Cell Density Per 1 mm Linear Length of the CA1 Subfield in the Group of Delayed Pentobarbital Injection (40 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>Pentobarbital 60 min</td>
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<tr>
<td>n = 12</td>
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<tr>
<td>Pentobarbital 120 min</td>
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<tr>
<td>n = 10</td>
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</tbody>
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of 400x. Neurons which had shrunken cell bodies with surrounding empty spaces were excluded.

Based on these data, the neuronal density of the CA1 sector, i.e., the number of CA1 neurons per 1 mm linear length of the stratum pyramidale observed in each 5 μm section, was calculated. The average of right and left neuronal densities was regarded as the neuronal cell density of each gerbil. The values of neuronal density were expressed as the mean value ± the standard error of the mean (SEM). Statistical analysis was done using Wilcoxon's rank sum test.

**Results**

In 8 unoperated normal gerbils, the average neuronal cell density of the CA1 sector was 212.3 ± 3.9/mm, ranging from 180.6/mm to 231.5/mm. Considering the normal variance of cell densities, hippocampus with a neuronal density higher than 160/mm was assumed to be normal or minimally damaged (grade 0). Neuronal cell densities less than 160/mm were divided into 4 grades and thus each neuronal density fell into one of the five grades; i.e., grade 0 (>160/mm), grade 1 (120–160/mm), grade 2 (80–120/mm), grade 3 (40–80/mm), and grade 4 (<40/mm). This grading was used to demonstrate the pattern of distribution of the cell densities within each group (figs. 2–4). Statistical analysis was performed using the actual data of neuronal cell density.

In the gerbils subjected to 5 min of ischemia and injected with saline, an extensive cell loss in the CA1 sector was observed. The pattern of neuronal damage was identical to what has been previously described. In the saline groups as a whole (n = 49), the average neuronal density in the CA1 sector was 34.3 ± 5.1/mm. In these control groups, 36 (73.5%) fell into the group of grade 4, 5 (10.2%) into grade 3, 4 (8.2%) into grade 2, 1 (2.0%) into grade 1, and 3 (6.1%) belonged to the grade 0 group. This grading was used to demonstrate the pattern of distribution of the neuronal densities in the CA1 sector (fig. 2). The neuronal cell density of the saline-injected control group showed a density of 52.8 ± 13.6/mm. The drug effect in these groups was not statistically significant.

When gerbils were given pentobarbital, 10 mg/kg (n = 9), immediately following 5 min of occlusion, the average neuronal density in the CA1 sector was 105.8 ± 13.3/mm. The corresponding control (n = 8) showed a neuronal density of 63.5 ± 16.3/mm. The drug effect in these groups was not statistically significant.

In the animals treated with pentobarbital, 20 mg/kg (n = 10), immediately following ischemia, there was a preservation of the CA1 neurons compared to the corresponding controls (n = 9). The animals treated with pentobarbital demonstrated just an opposite pattern of distribution of the neuronal densities in the CA1 sector (fig. 3). The neuronal cell density of the pentobarbital group was 168.2 ± 12.3/mm, where the control group showed a density of 52.8 ± 13.6/mm. The drug effect was statistically significant (p < 0.01).

Pentobarbital administration in a dose of 40 mg/kg also improved the survival of CA1 neurons. The pentobarbital-injected gerbils showed quite a reversed distribution pattern of the neuronal densities (fig. 4). The average density was 181.1 ± 14.9/mm in 10 treated animals, whereas it was 29.5 ± 10.8/mm in the corresponding controls (n = 10). The difference between
that the metabolic demand unrelated to neural function. They considered that barbiturates reduce neuronal firing and suppress metabolic rate by this property and metabolism following pentobarbital loading decreased in the intact area but was not reduced in the lesion site. Frey and Agranoff recently demonstrated the suppressive effect of pentobarbital on cerebral glucose metabolism using labeled 2-deoxy-D-glucose autoradiography. They showed a generalized suppression of glucose metabolism in normal animals under pentobarbital anesthesia. In animals subjected to unilateral ibotenic acid lesion, the glucose metabolism following pentobarbital loading decreased in the intact area but was not reduced in the lesion site. They considered that barbiturates reduce neuronal firing and suppress metabolic rate by this property and that the metabolic demand unrelated to neural function is not altered. This fact may suggest the relationship between barbiturate effect on ischemic brain and suppression of neuronal excitability.

Our preliminary data have shown that other drugs such as nizofenone (Y-9179) or diazepam (unpublished data) also have comparable favorable effects on the CA1 neurons in the same gerbil model. In view of the fact that all of the drugs above have sedative action, “sedation” of neurons following ischemia seems to be critical for their survival. Similar beneficial effects by other drugs may indicate that the mechanism of barbiturate treatment is a nonspecific one.

Using cultured rat hippocampal neurons, Rothman studied neuronal vulnerability to anoxia. He found that, before the establishment of synapses between cultured neurons, they were less susceptible to anoxia. However, as soon as neurons started to communicate by synapses, they became vulnerable to oxygen deprivation. At this stage, MgCl₂ was added to block synaptic activity and then he noticed that neurons could survive anoxic insult. This result seems to suggest that synaptic activity is inevitably related to the neuronal vulnerability to anoxia or ischemia. It is yet to be shown whether neuronal excitation critically worsens the state of cellular energy reserves and causes cell death. It is obvious that energy failure exerts a predominant initial influence on neurons following transient ischemia. Events after restoration of blood flow, however, are also important even if the energy state is already restored at this stage. Neuronal firing itself may be detrimental for cellular survival after a period of ischemic insult even if it is not necessarily accompanied by energy failure.

Delayed neuronal death in the CA1 neurons observed following 5 min of ischemia in the gerbil is a slow alteration and is not similar to acute ischemic cell death which is noticed shortly after relatively severe ischemic insult. It takes almost 2 days to detect definite morphological changes in the CA1 neurons which herald delayed extensive neuronal death. During this period, there is no impairment of energy metabolism that can account for the extensive cell loss. Sustained electrical activities were recorded from the gerbil CA1 area for up to 24 hr following 5 min of ischemia. These experimental data may suggest that the CA1 neurons are still alive 24 hr following brief ischemia. In the CA1 neurons, an unknown noxious factor which is not directly derived by energy crisis, may exert continuous influences on neurons and may ultimately cause cell death.

As candidates of these noxious factors, putative amino acid transmitters such as glutamate or aspartate have been considered. These amino acids are known to cause neuronal damage because of their “excitotoxic” property. Calcium ion has been postulated as a final common denominator of ischemic cell damage, and the events before and after Ca²⁺ entry are now being studied.

The results described here have demonstrated two aspects of the resuscitation of neurons following brief ischemia. One is optimistic, the other is pessimistic.
Barbiturates, given following restoration of blood flow, favorably alter the course of cell change which, without barbiturates, results in extensive loss of neurons in the hippocampus. This finding encourages further trial of drug treatment of cerebral ischemia. Delayed pentobarbital administration, however, hardly showed any beneficial effects. When the gerbil brain was fixed 60 or 120 min following 5 min of ischemia, it was extremely difficult to detect any structural abnormalities by light or electron microscopy. There was no morphological evidence of a progressively destruc-
tive process, and energy metabolism and electrophysiological activity were restored in these neurons. Even at this stage, pentobarbital injection was already too late to be effective. Bodsch and Takahashi studied protein synthesis following ischemia and showed that synthetic activity in the CA1 neurons was already suppressed 2 hr following brief transient ischemia. The metabolic state of the CA1 neurons seems to be irreversibly disturbed far earlier than any morphological changes are noticed. Neurons may be irreversibly damaged far before disturbance of structural integrity becomes evident. This fact, if it is true, suggests a pessimistic view of ischemia treatment. Neurons may be salvageable following ischemic insult but the possibility of saving damaged neurons may be limited to certain special situations.

The result presented here may indicate, as was already known, that barbiturate therapy against cerebral anoxia or ischemia in patients can be effective if instituted immediately following insults and if the insults are not severe ones. We suggest that future experiments should clarify a more effective mode of therapy and find a way to salvage damaged neurons more efficiently following brain anoxia or ischemia.

Acknowledgments

The authors wish to express their appreciation to Dr. H. Tamura, Laboratory Animal Center, Teikyo University, for the supply of gerbils, to Drs. T. Tanishima and O. Gotoh for their formative discussions and to Miss N. Tomukai for her skillful technical assistance.

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A reversible type of neuronal injury following ischemia in the gerbil hippocampus.
T Kirino, A Tamura and K Sano

Stroke. 1986;17:455-459
doi: 10.1161/01.STR.17.3.455
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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