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 remain intact morphologically.

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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 cardiopulmonary 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 Mongolian 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 (Graphtec Co.). The number of living neurons in the stratum pyramidale within the CA1 subfield was counted using Olympus Vanox photomicroscope at a magnification

### Table 1: Neuronal Cell Density Per 1 mm Linear Length of the CA1 Subfield

<table>
<thead>
<tr>
<th>Group</th>
<th>Control group</th>
<th>Pentobarbital group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal gerbils</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>212.3 ± 3.9</td>
<td>n = 8</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>63.5 ± 16.3</td>
<td>105.8 ± 13.3</td>
</tr>
<tr>
<td>n = 8</td>
<td>n = 9</td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>52.8 ± 13.6</td>
<td>168.2 ± 12.3*</td>
</tr>
<tr>
<td>n = 9</td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 mg/kg</td>
<td>29.5 ± 10.8</td>
<td>181.1 ± 14.9*</td>
</tr>
<tr>
<td>n = 10</td>
<td>n = 10</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant (p < 0.01).

SEM = standard error of the mean.
The values are expressed as mean ± SEM.

### Table 2: Neuronal Cell Density Per 1 mm Linear Length of the CA1 Subfield in the Group of Delayed Pentobarbital Injection (40 mg/kg)

<table>
<thead>
<tr>
<th>Injection</th>
<th>Control group</th>
<th>Pentobarbital group</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>15.0 ± 4.8</td>
<td>46.1 ± 8.9</td>
</tr>
<tr>
<td>n = 12</td>
<td>n = 9</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>22.3 ± 8.9</td>
<td>14.8 ± 5.4</td>
</tr>
<tr>
<td>n = 10</td>
<td>n = 9</td>
<td></td>
</tr>
</tbody>
</table>

SEM = standard error of the mean.
The values are means ± SEM.
FIGURE 1. The dorsal hippocampus of the gerbil. Seven days following 5 min of ischemia, the neurons in the CA1 sector (between the arrowheads) were extensively destroyed. DG: dentate gyrus. Hematoxylin an eosin. X 20.

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 3 animals (6.1%) out of 49 total saline group, the difference of neuronal cell density between right and left hippocampi was greater than 1 in terms of the grades above mentioned.

In the animals treated with 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.

When gerbils were given 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

FIGURE 2. Neuronal densities in the saline-injected control groups as a whole (n = 49). Most of the hippocampi showed massive loss of neurons.

FIGURE 3. Immediately following 5 min of ischemia, pentobarbital (20 mg/kg) was injected. Seventy percent of the gerbil hippocampi appeared intact or minimally damaged.
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that the metabolic demand unrelated to neural function

They considered that barbiturates reduce neuronal fir-

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metabolism following pentobarbital loading decreased

deoxy-D-glucose autoradiography. They showed a

on cerebral glucose metabolism using labeled 2-

ral suppression of glucose metabolism in nor-

dmal animals under pentobarbital anesthesia. In animals

favored effect in the circumscribed region in the

CA1 neurons. The hippocampi of 70% of animals fell into the

grade 0 group.

There was no statistical difference between 60 min and

the treated and control groups was statistically signifi-

cant (p < 0.01).

There seemingly was a difference of neuronal densi-
ties among the saline control groups (table 1). These
differences were not statistically significant. Rectal
temperature was 35.6 ± 0.2 (SEM)°C in the control
groups and 35.4 ± 0.2 (SEM)°C in the pentobarbital-
jected groups. There was no statistical difference in
rectal temperature monitored following ischemic insult.

When pentobarbital was given 60 min or 120 min
following 5 min of ischemia, there was no improve-
ment of the neuronal cell density in the CA1 subfield.
There was no statistical difference between 60 min and
120 min groups (table 2).

Discussion

Pentobarbital showed a definite, reproducible and
favorable effect in the circumscribed region in the
hippocampus following brief bilateral forebrain ischemia.
Without drug treatment, most of the CA1 neurons
died. When pentobarbital was injected immediately
following ischemic insult, these neurons survived the
insult in most part. The gerbil hippocampus proved to
be a distinct structure in which a clear-cut drug effect is
seen following ischemia without being disturbed by
other complicated factors outside the hippocampus.

The precise mechanism of the barbiturate effect as is
shown in this report is not fully understood. 19 Barbitu-
rates are known to reduce the metabolic demand of
neurons. 20 It is conceivable that barbiturates protection
is due to this suppression of energy metabolism and to
the restoration of the balance of supply and demand of
high energy metabolites. Frey and Agranoff 21 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 nor-
mal 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 fir-
ing 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 sup-
pression of neuronal excitability.

Our preliminary data have shown that other drugs
such as nizofenone (Y-9179) 22 or diazepam (unpub-
lished 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 barbit-
urate treatment is a nonspecific one.

Using cultured rat hippocampal neurons, Rothman 23
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 depriv-
ation. At this stage, MgCl 2 was added to block syn-
aptic 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 pre-
dominant initial influence on neurons following tran-
sient 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 accompa-
nied by energy failure.

Delayed neuronal death in the CA1 neurons ob-
served 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. 24 It takes almost 2 days to detect defi-
inite 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. 25 Sustained
electrical activities were recorded from the gerbil CA1
area for up to 24 hr following 5 min of ischemia. 26
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. 27, 28 These amino acids are
known to cause neuronal damage because of their "exi-
totoxic" property. 29 Calcium ion has been postulated as
a final common denominator of ischemic cell dam-

Summary table 1: Neuronal density in the hippocampus of gerbils following 5 min of ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Neuronal density (/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline injected)</td>
<td>0-40</td>
</tr>
<tr>
<td>Pentobarbital (40 mg/kg) injected</td>
<td>40-80</td>
</tr>
<tr>
<td>Pentobarbital (40 mg/kg) injected + nifedipine</td>
<td>80-120</td>
</tr>
<tr>
<td>Pentobarbital (40 mg/kg) injected + nifedipine, 10 min after ischemia</td>
<td>120-160</td>
</tr>
<tr>
<td>Pentobarbital (40 mg/kg) injected + nifedipine, 30 min after ischemia</td>
<td>160-200</td>
</tr>
</tbody>
</table>

Figure 4. Pentobarbital (40 mg/kg) injection immediately following 5 min of ischemia definitely improved the survival of CA1 neurons. The hippocampi of 70% of animals fell into the grade 0 group.
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 destructive 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 results 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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