Behavioral Performance of Rats Following Transient Forebrain Ischemia

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SUMMARY Rats subjected to transient forebrain ischemic injury by the method of four vessel occlusion (4-VO) develop irreversible injury to select populations of vulnerable neurons which include pyramidal cells in the CA-1 region of the hippocampus. This brain area is thought to be crucial for learning and memory. Rats subjected to 30 minutes of 4-VO, and then cerebral reperfusion were tested on a radial 8-arm maze task after they had recovered. The data shows that both 4-VO and control animals improve their performance over trials, but that 4-VO rats are impaired on "working" and "reference" tasks. The data suggest that 4-VO rats' impaired "working" performance is permanent, compared to their transient "reference" impairment. Alterations in sensorimotor activity could not account for these performance deficits since control and 4-VO rats demonstrated equivalent choice time per maze arm. Performance deficits in rats following forebrain ischemic injury may be similar to some of the cognitive deficits found in humans survivors of cerebral hypoxia-ischemia.

THERE ARE MORE SURVIVORS of cardiac arrest, and global cerebral ischemia continues to remain a major cause of persistent morbidity. Some patients surviving acute cardiac arrest will have, on arousal, an amnestic syndrome which may be permanent and characterized by impaired learning and memory for events that occur after the injury, and loss of memories of premorbid events. Despite their memory impairment, amnesic patients retain their general intelligence as measured by standard neuropsychological test batteries, and some patients with amnesia are able to learn and remember tasks that are highly practiced. In patients with amnesia after global cerebral ischemia who have come to post-mortem examination, neuro-pathologic analysis reveals brains that have escaped focal cerebral infarction but which show loss of specific populations of neurons known to be highly vulnerable to global ischemic injury. Neurons sensitive to ischemia include, among others, neocortical neurons in layers 3, 5, and 6, medium-sized striatal neurons, cerebellar Purkinje cells, and most importantly, neurons in the CA-1 zone of hippocampus, an area of brain thought to be critical for learning and memory. The purpose of the present study was to determine whether ischemic damage to these neurons in the rat, and in particular damage to the CA-1 hippocampal neurons, would result in deficits of learning and memory similar to that seen in humans.

Rats subjected to controlled forebrain ischemia by the method of four vessel occlusion (4-VO) develop reproducible and quantifiable morphologic brain injury. The most severe damage occurs in the CA-1 zone of the hippocampus and the dorsolateral striatum, with additional, but less extensive damage, in neocortical layers 3, 5, and 6, cerebellum, and focally in the thalamus. Using the 4-VO method, a majority of animals survive indefinitely after 30 minutes of forebrain ischemia, and it is therefore possible to characterize and quantify their behavior weeks after recovery from the acute insult.

Several behavioral paradigms are used to analyze learning and memory deficits in animals. We chose a radial 8 arm maze task because investigators have demonstrated that rats with hippocampal ablation perform differently on two aspects of this task. By baiting 5 of the 8 arms only once for each trial, it is possible to measure "reference" performance-entering a baited arm, and "working" performance-entering a baited arm only once. The reference aspect of maze performance requires the animal to learn that only 5 of the arms have a food reward, and that these 5 remain constant over all experimental trials. Investigators have shown that this aspect of the task requires learning the repetitive association between constant extra-maze visual cues and a baited arm. These stimulus-response associations remain fixed over all experimental trials. On the other hand, the working component of maze performance is defined by stimulus-response associations that change from trial to trial. During training, the animal learns that an arm is baited only once for each trial, so that after an arm is entered and food is obtained, the animal should not re-enter the arm on subsequent choices because food is no longer available. For the working aspect of the task, the animal must remember which arms have been explored during a single trial. Accordingly, we tested whether rats subjected to 30 minutes of forebrain ischemia (4-VO) could learn the "reference" and "working" aspects of an arm maze.

Methods

Surgical Procedure

Male Wistar rats (Hilltop, Scottsdale, Pennsylvania) weighing between 250 and 300 grams were subjected to severe forebrain ischemia by a modification of the 4-VO method. Briefly, rats were anesthetized by the inhalation of 0.5% penthrane mixed with 30% oxygen and 70% nitrogen. Atraumatic claspers were placed...
loosely around each common carotid artery without interrupting carotid blood flow. At the same time, the vertebral arteries were permanently occluded by electrocautery at the first cervical vertebra. A 15 cm length of #0 surgical silk was passed through the animal’s neck so as to lie posterior to the trachea, esophagus, external jugular veins, and common arteries, but anterior to the cervical muscles. The animals were allowed to recover from anesthesia for 24 hours during which time food was withheld. Animals subjected to the surgical procedure and allowed to recover from anesthesia are indistinguishable from control animals by electroencephalographic, physiologic and subjective behavioral criteria.14 Forebrain ischemia in the awake rats was produced by tightening the carotid artery clamps and then tightening the external surgical suture which encircled the cervical muscles. Animals that did not become unresponsive within 1 minute following this procedure were excluded from the study. The carotid artery clamps were released and the neck suture was cut following 30 minutes of occlusion. Body temperature was maintained at a minimum of 37°C by a heating lamp connected to a rectal thermistor until thermal homeostasis was restored. Control rats were either subjected to penthrane anesthesia and skin incisions (sham-controls) or to penthrane anesthesia and occlusion of the vertebral arteries by electrocautery (vertebral cautery controls).

Animals were allowed to recover for a one month period of observation prior to behavioral testing. During this time the animals subjected to forebrain ischemia gained weight so that at the end of two weeks there was no difference in weights between control and operated rats. It was also impossible to distinguish operated from unoperated rats by observation of feeding, grooming, or exploring habits.

Behavioral Apparatus and Procedure

Rats were tested in a radial 8-arm maze similar to an apparatus previously described.14 Each arm (60 × 10 cm) projected from an octagonal center platform (65 cm wide) and had clear plexiglass rails (3 cm high) on all sides. There was a recessed food hole (3.4 cm in diameter × 1 cm deep) at the end of each arm. The surface of the maze consisted of white formica and was 50 cm above the floor. Stationary extramaze stimuli in the testing room included an operant behavioral apparatus, chairs, a coat rack, and a chemical fume exhaust hood.

One month postoperatively the 4-VO rats (n = 9), vertebral artery cauterized controls (n = 11), and sham operated (skin incision only) rats (n = 18) were placed on a partial food deprivation schedule and by trial 11 they reached 80% of their ad lib weight. Subsequently they were fed 12–15 gm/day to maintain them at this weight.

Maze adaptation started after 1 day of food deprivation. Individual rats were allowed to explore the 8 arm maze for 15 minutes on 3 consecutive days. Noyce food pellets (94 mg) were randomly scattered over the entire maze surface. On day 4, food pellets were scattered only over five of the eight arms. The five baited arms were randomly assigned for each individual rat. On day 5, one pellet was placed in the food hole at the end of each of the 5 assigned arms. The number of baited arms and the spatial location of the baited arms relative to the room remained constant for individual rats over 65 daily trials. This provided the reference aspect of maze performance. The working aspect consisted of remembering which arms were visited on a specific trial.

After adaptation, rats were given 1 training trial each day during which a single food pellet was placed in the food cup of the 5 arms assigned to be baited. For each trial, a rat was placed on the center platform facing a randomly selected arm and allowed to make arm choices until either all 5 pellets were taken, a total of 16 choices were made, or 10 minutes elapsed. Immediately after a trial the rat was returned to its home cage, and given its additional food for the day.

For behavioral analysis of maze performance, an initial entry of a food baited arm was considered a correct choice, an initial entry of an unbaited arm was considered a reference memory error, and reentry of a previously chosen arm was considered a working memory error. Thus, a rat could make a reference error, a working error, or a combined reference and working error. Trials 1–10 were not included in the analysis because all the animals had not reached 80% of their ad lib weight, and because not all animals were making 16 choices or obtaining the five food pellets within 10 minutes. By trial 11, all rats reached the 80% weight criteria, and all rats were completing trials within 10 minutes. All rats were assigned a code number so that behavioral training was blind. All trials occurred between 7 AM and 11 AM.

Neuropathologic Procedure

After a 1 month period of recovery from 4-VO, and 70 days of behavioral training, all 4-VO rats and 5 vertebral artery cauterized control rats, and 5 sham operated control rats were anesthetized and their brains were perfusion-fixed with FAM (40% formaldehyde, glacial acetic acid, methanol, 1:1:8) as previously described.14 Briefly, FAM was perfused via the ascending aorta after the cephalic circulation was washed out with heparinized, physiologic saline. Brains were removed and stored in FAM until they were embedded in paraffin. Paraffin sections (7 um) were taken at the level of the anterior commissure and anterior hippocampus. Brain sections were stained with hematoxylin and eosin, and graded for damage as previously reported.14 The sections were graded with the light microscope by a neuropathologist who had no knowledge of the experimental conditions. Ischemic neuronal damage was graded on a scale of 0–3 with 0 = normal brain, 1 = a few neurons damaged (as few as one neuron damaged), 2 = many neurons damaged, and 3 = majority of neurons damaged.

Behavior

The behavioral analysis was based on two types of errors: A reference error occurred when an animal entered an unbaited arm; a working error occurred when
Finally, there were no differences between 4-VO and control rats in sensorimotor activity as measured by the mean time that an animal spent in an arm (time per trial/total number of arm choices) \( F(1,36) = 0.98, p > .30 \). The amount of time per arm choice decreased over trials 1–65 for both groups \( F(64,2304) = 12.19, p < 0.001 \), and there was no interaction between group and trials \( F(64,2304) = 0.99, p > 0.50 \). An analysis was also carried out for trials 1–10 on the number of trials (±SEM) in which an animal completed a trial within 10 minutes. On trial 1–10 there was no difference (t test, \( p > .25 \)) in the number of completed trials by 4-VO \( (7.2 ± 1.2) \) and control rats \( (6.6 ± 0.5) \). Taken together these results show that 4-VO rats were as active as control rats in the maze during initial trials, and as quick as controls in making arm choices.

**Neuropathology**

The distribution and severity of ischemic neuronal injury are presented in table 1. Quantification of neuronal damage in this study was accurate only in brain regions showing moderate to severe injury; that is,
grade 2 to 3 damage. The 3 month survival period after forebrain ischemia allowed sufficient time for the process of gliosis to obscure lesser grades on neuronal damage. Therefore, ischemic damage in brain regions which in prior studies of acute ischemic injury showed mainly grade 1 damage,\textsuperscript{13} for example neocortex and thalamus, may have gone undetected in the current chronic surviving animals. The brain regions with the most severe damage were the dorsal-lateral striatum and the CA-1 zone of the hippocampus. Two animals showed small foci of neuronal damage in the ventral nucleus of the thalamus (one bilateral, one unilateral) and one animal showed bilateral damage to the lateral geniculate nucleus. The lesions were characterized by loss of neurons, and dense proliferation of polymorphic, plump gemistocytic astrocytes. Rare residual neurons were encrusted by calcium and there were free extracellular calcium deposits. Endothelial cell change and vascular proliferation were not apparent.

**Discussion**

This study reports the quantitative behavioral analysis of the performance of 4-VO rats in a radial 8 arm maze. The results suggest that reference performance of 4-VO rats improves to equal control animals' performance, but working performance of 4-VO rats remains permanently impaired. Working performance requires that the animal distinguish among the particular events that occur during each trial. The animal must remember which arms were explored and not re-enter those arms. The poor working performance of 4-VO rats begins immediately, and may indicate a sensorimotor deficit or a non-specific cognitive impairment in addition to a memory deficit. However, the equivalent choice time per arm between 4-VO and control animals mitigates against a sensorimotor impairment, and our data that show improvement over trials suggests that the operated rats can learn. Whether there is an additional cognitive deficit remains to be explored. In any case, compared to control animals 4-VO animals have a persistent difficulty remembering where they have been during any one trial. The significant statistical interaction on the working task between the groups may be due to the floor effect caused by the small number of errors made by control rats. To suggest that the 4-VO animals might eventually perform working aspects as well as controls, would discount the trend of the last twenty trials.

A distinctly different aspect of the radial maze task, reference performance, requires that the animal learn that food reward is found only on certain arms. The five baited arms for each rat do not change, and these maze arms remain fixed with respect to other objects in the testing room. Others have shown that reference performance depends on the invariant association of a baited arm with extra-maze visual cues.\textsuperscript{18-22} Although 4-VO rats make significantly more reference errors than controls, their reference performance improves over trials, and by trial 65 is identical to controls. These results suggest that 4-VO rats can learn and remember highly practiced invariant aspects of the task, however, the lack of a statistical interaction between group and trial postpones a definitive conclusion regarding the apparent transient reference deficit.

Impaired acquisition of working tasks with preserved reference performance has been demonstrated in rats with CA-3 hippocampal ablation after kainic acid injection, and in rats with transection of the fimbria-fornix.\textsuperscript{21, 28} More extensive lesions in rats produced by aspiration of the neocortex and hippocampus caused impaired acquisition of reference tasks.\textsuperscript{26} In our study, after prolonged testing, the 4-VO animals demonstrate a persistent impairment in working performance out of proportion to impairments in reference performance. In this respect, the 4-VO animals perform similarly to the rats with CA-3 ablation. However, the neuropathologic analysis of the 4-VO rats shows predominantly CA-1 neuronal damage. It may be that a small but critical number of CA-3 were damaged in the 4-VO animals, but were undetected in the chronic state by our morphological analysis. Alternatively, it may be that both CA-1 and CA-3 are necessary for acquisition of a working memory task. Finally, although ischemia selectively damages vulnerable neurons, and the damage is widespread when compared to kainic acid injection or restricted surgical ablation, ischemia after four vessel occlusion is more similar to human global cerebral ischemia.

The dissociated loss of working memory out of proportion to reference memory for the 4-VO rat might have implications for human amnesic syndromes, particularly for patients with amnesia after global hypoxia-ischemia.\textsuperscript{6, 7} Poor memory for recently acquired information is one part of the memory deficit common to a human amnesic syndrome.\textsuperscript{27, 28} Patients with amnesia cannot report events, or items in those events, minutes after they experience them. However human amnesic syndromes are rarely, if ever, complete; that is, amnesics retain general intelligence and some ability to learn.\textsuperscript{6, 15} It may be, as some have suggested, that reference and working performance in rats are functionally distinct psychological memory processes, and

### Table 1: Severity and Distribution of Ischemic Brain Damage

<table>
<thead>
<tr>
<th>Neocortex</th>
<th>Vertebral cauterized rats</th>
<th>Sham operated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>anterior</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>posterior</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA-1</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>CA-2</td>
<td>0.3 (± 0.2)</td>
<td>0</td>
</tr>
<tr>
<td>CA-3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Striatum</td>
<td>2.6 (± 0.1)</td>
<td>0</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.2 (± 0.1)</td>
<td>0</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brainstem</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
that they are similar to human memory processes. While this notion is controversial, and more animal experiments need to be performed, it could be argued that for the 4-VO rats in this experiment, the acquisition of the reference memory component is similar to partial learning abilities in amnesic patients. The persistent deficits in working memory for the 4-VO rats may be similar to the persistent memory deficit for recent episodes in amnesic patients. The continued study of this animal model of global ischemic damage will allow cellular investigation and pharmacologic manipulation that may lead to insights into the amnesia that occurs after global hypoxic-ischemic cerebral injury.

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