Rats exposed to transient forebrain ischemia by the method of four-vessel occlusion demonstrate reproducibly severe injury of the hippocampal CA1 neurons and less severe injury of the dorsolateral caudate nuclei. This pattern of ischemic damage in the rats has been associated with permanent memory impairment. Survivors of cardiac arrest may have an amnesic syndrome characterized by profound deficiencies in new learning but relatively preserved general intelligence and remote memory. In a well-studied patient with amnesia after cardiac arrest, the neuropathologic injury was confined bilaterally to the CA1 region of the hippocampus.

These clinical observations provided the rationale for extending the characterization of the behavior of rats exposed to four-vessel occlusion in mazes which require spatial memory to find food rewards. If rats are trained before ischemia and then tested, their behavior in radial mazes (in which only some arms are baited with a food reward) permit the analysis of two kinds of performance that probably rely on different types of memory. The first type, called reference memory, depends on the constant pattern of the baited arms; for any particular rat the pattern of baited arms never changes, and learning and remembering which arms of a maze are baited depends on invariable spatial cues. The second type, called working memory, depends on variable spatial information; while exploring the maze a rat is prohibited from always choosing successive arms in exactly the same order. So, to avoid reentering arms, a rat depends on variable spatial information to keep track of which arms have already been entered. In eight-arm radial mazes in which five arms were baited, the reference memory of rats with ischemia was always comparable to that of controls. Working memory was permanently impaired when the rats were not trained before ischemia or when the rats were trained to only asymptote performance (typically 50 trials) before ischemia. However, when trained for 70 trials before ischemia (some 20 extra trials after asymptote performance), rats eventually per-
formed the working memory task after ischemia as well as controls. In general, ischemic rats learn and remember reference information yet have difficulty learning and remembering working information.

These data also raise the question of the mechanism of the recovery of working memory. While there is evidence that CA3 neurons recover over a period of days after acute ischemia, there is no evidence that neuronal recovery continues for weeks. Alternatively, there is evidence that the duration of training and the difficulty of the task affect rodent and primate behavior after hippocampal injury. Therefore, the current experiments test whether additional preocclusion training trials alter the recovery of reference or working memory after ischemia in rats in a 12-arm radial maze.

Materials and Methods

We used male Wistar rats (Hilltop, Scottsdale, Pennsylvania) weighing 200–250 g at the start of the experiment. The rats were individually housed throughout the experiment, were provided ad libitum access to water, and were maintained at 80–85% of their original weight.

The rats were trained before and tested after ischemia in a 12-arm radial maze. Each 60×10 cm arm projected from a 75-cm-wide center platform and had 20-cm-high clear Plexiglas rails on all sides. The maze arms for the first day of adaptation. On Day 2 the food pellets were scattered at the end of each arm, and on Day 3 the food pellets were placed in the food hole at the end of each arm. The guillotine doors were raised and lowered two or three times per minute during each adaptation session.

For training, the rats were given one trial each day; a single food pellet was placed in the food hole of seven baited arms, which remained constant relative to room cues throughout the experiment. The rats were allowed to choose arms until all seven food pellets were taken, until 16 choices were made, or until 10 minutes had elapsed. The rats were confined by the guillotine doors to the center platform for approximately 3 seconds after each arm choice. Immediately after a training trial, the rat was returned to its home cage and given its additional food for the day. The rats received 36 training trials in Experiment 1 and 80 training trials in Experiment 2. The rats were then subjected to forebrain ischemia and given 30 days to recover before being returned to the maze for 48 (Experiment 1) or 68 (Experiment 2) testing trials. Testing trials were identical to training trials except for the last 20 trials in Experiment 2, in which the rats were confined to the center platform for 10 seconds after each arm choice.

At the conclusion of training, the rats were rank-ordered on the basis of maze performance. From every three consecutively ranked rats, two were subjected to transient forebrain ischemia by a method previously described and modified, and the third (control) rat received a sham operation. Briefly, on Day 1 the rats were anesthetized by inhalation of 2% halothane mixed with 30% oxygen and 70% nitrogen, the common carotid arteries were encircled with Silastic ligatures, and the vertebral arteries were permanently occluded by electrocautery. Control rats were subjected to halothane anesthesia, skin incisions, and carotid manipulation. The rats recovered for 24 hours. On Day 2 forebrain ischemia was produced in awake rats by tightening the carotid artery ligatures. Rats that lose their righting reflex within 1 minute after carotid clamping and for the subsequent 30 minutes have been shown to have their cerebral blood flow (CBF) reduced to <10 ml/100 g/min, approximately 10% of that in control rats. CBF of >10 ml/100 g/min does not reproducibly impair the righting reflex and produces variable ischemic injury. Rats that lost their righting reflex for 30 minutes were classified as ischemic; rats that retained their righting reflex were eliminated from testing.

The carotid artery ligatures were released after 30 minutes. Body temperature was maintained at 37°C by a heating lamp connected to a rectal thermometer. Previous behavioral assessments have detected no differences between control rats and those exposed to vertebral cautery alone. The rats were allowed 30 days of postoperative recovery. After 3 weeks there was no difference in weight between controls and ischemic rats. It was not possible to distinguish ischemic rats from controls by observing their feeding, grooming, or exploring habits.

After the completion of the testing trials, each rat was anesthetized, and its brain was perfused with a solution of formaldehyde, glacial acetic acid, and methanol (1:1:8) as previously described via the ascending aorta after the brain circulation was washed out with heparinized physiological saline. The brain was removed and imbedded in paraffin. Sections (10 μm) were taken at the level of the dorsal caudate nucleus (bregma −0.3 mm), the anterior hippocampus (bregma −3.3 mm), and the posterior hippocampus (bregma −5.3 mm) and were stained with hematoxylin and eosin. Four consecutive coronal sections were taken at each of the stated coordinates and graded for damage on a 0–3 scale (0, normal brain; 1, as few as one neuron damaged; 2, many neurons damaged; and 3, majority of neurons damaged) as previously reported. For each group, the grades for each location in each hemisphere were averaged, and standard error of the mean (SEM) was calculated.
For statistical analysis of both training and testing, the first entry of a baited arm was considered a correct choice, the first entry of an unbaited arm was considered a reference error, and reentry of a previously chosen arm was considered a working error. All trials were run between 8 AM and 6 PM. The results of six individual trials were averaged as a block, and then repeated-measures analysis of variance (ANOVA) using treatment group (ischemic rats vs. controls) and block as independent variables was performed on each type of error for the last two training blocks and all testing blocks. When the assumptions for the ANOVA of the testing blocks were not met as indicated by the sphericity test, degrees of freedom were adjusted using the Greenhouse-Geisser ε value and performance was evaluated using an adjusted F test. The group×trial interactions were analyzed using the post hoc Tukey-Kramer modification of the honestly significant difference test for pairwise comparisons with unequal sample sizes. The possibility that ischemia had nonspecific effects on motor behavior was examined by an ANOVA of the speed of maze exploration (total time per trial divided by the number of choices). To analyze more thoroughly the effect of ischemia on working memory, the distributions of the initial error and of the subsequent errors within a trial were tabulated and two ANOVAs were performed. The first analyzed the choice on which the rats make their first working error, and the second ANOVA compared the pattern of choices on which all subsequent working errors were made.

Results

In Experiment 1, on training trials there were no significant differences in working or reference errors between groups (21 ischemic rats and 33 control rats; \( F_{1,52}=1.1, p>0.25 \) for working memory and \( F_{1,52}=2.3, p>0.10 \) for reference memory). There were no significant trial effect for either working or reference error (\( F_{1,52}<1.00 \) in both) and no significant group×block interaction (\( F<1.00 \) in all cases). Thus, there was no difference between groups in any aspect of preoperative maze performance.

Figure 1 shows the mean number of working and reference errors made by Experiment 1 rats in the combined last two blocks of the training trials and for all eight blocks of the testing trials. The analysis of training trials demonstrated that the working memory of both controls and ischemic rats improved over the testing trials (\( F_{4,218}=26.9, p<0.001 \)). However, ischemic rats made significantly more working errors than did controls (\( F_{1,52}=20.7, p<0.001 \); a significant group×block interaction was detected (\( F_{4,218}=3.3, p<0.025 \)). Reference memory also improved over the testing trials in both controls and ischemic rats (\( F_{4,218}=71.2, p<0.001 \)). No significant difference in reference errors was detected between groups (\( F_{1,52}=3.7, p>0.05 \)), but there was a significant group×block interaction for reference error (\( F_{4,218}=7.8, p<0.01 \)). Analysis of the group×block interactions showed that the ischemic rats made significantly more working errors than the controls on all blocks of the testing trials (\( p<0.05 \)). However, the ischemic rats made significantly more reference errors than the controls on only the first three testing blocks (\( p<0.05 \)), and the two groups did not differ significantly during the last five testing blocks.

In Experiment 1, analysis of maze exploration speed showed that ischemic rats chose arms more rapidly with additional testing trials (\( F_{7,364}=20.2, p<0.001 \)). However, there was no significant difference in speed between groups (\( F_{1,52}=2.81, p>0.1 \) and no significant group×block interaction (\( F_{7,364}=1.4, p>0.15 \)). Thus, motor abnormalities cannot account for the impaired memory performance of the ischemic rats.

The distribution of initial and subsequent working errors in the testing trials for the ischemic and control rats in Experiment 1 is given in Figure 2.
Ischemic rats made an initial working error significantly sooner than the controls ($F_{1,718}=11.1, p<0.01$). The choice on which the controls made their initial error was 8.1±0.9, in contrast to 7.6±0.1 for the ischemic rats. However, there were no significant differences between groups in the distribution of subsequent errors ($F_{1,718}<1.0$). As expected, ischemic rats made many more working errors than controls.

In the ischemic rats of Experiment 1, the most common postischemic injury (occurring in 19 of the 21 rats) was severe bilateral anterior dorsal CA1 damage; in the other two rats there was lesser CA1 damage. The neuropathologic grade in this area over 42 hemispheres was 2.74±0.11. Analysis of the posterior dorsal CA1 area was similar; neuropathologic grade there was 2.43±0.16. Grade in the dorsal caudate nucleus averaged 1.62±0.12. Four ischemic rats had moderate unilateral cortical damage and small focal unilateral damage in the thalamus; there was slight CA2 and CA3 damage in four other ischemic rats. There was no damage in the controls.

In Experiment 2, on the training trials there was no significant difference in working or reference error between groups ($F_{1,33}>1.00, p>0.8$ for working error and $F_{1,33}<1.00, p>0.6$ for reference error). There was no significant block effect for working or reference error ($F_{1,33}=1.00, p>0.4$ for both) and no significant group×block interaction ($F<1.00$ in all cases). Thus, there was no difference between groups in any aspect of preoperative maze performance.

For the first 48 testing trials in Experiment 2, both the working and reference memory of the 28 controls and the seven ischemic rats improved ($F_{3,92}=28.6, p<0.001$ and $F_{4,161}=46.1, p<0.001$, respectively; Figure 3), but ischemic rats made significantly more working errors than controls ($F_{1,33}=6.1, p<0.025$). The difference in reference errors between groups was not significant ($F_{1,33}=1.37, p>0.20$). A significant group×block interaction was detected for both working and reference errors ($F_{3,92}=10.5, p<0.001$ and $F_{4,161}=7.62, p<0.01$, respectively).

Analysis of the group×block interactions for the first 48 testing trials in Experiment 2 showed that ischemic rats made significantly more working and reference errors than controls during only the first two blocks ($p<0.05$). Thus, ischemic rats given 80 training trials demonstrated recovery of both reference and working memory. ANOVA of the speed of maze exploration for these two testing blocks showed that ischemic rats became quicker at choosing arms ($F_{1,33}=17.7, p<0.001$). However, there was no difference in speed between groups, and no significant
group × block interaction was detected (F_{1,33} < 1.00, p > 0.30 in both cases).

For the final 20 trials in Experiment 2, the rats were delayed on the central platform for 10 seconds after each choice. ANOVA showed both groups improved in both working and reference errors (F_{1,99} = 11.5, p < 0.001 and F_{1,99} = 5.8, p < 0.01, respectively). There was no difference between groups for either working or reference errors, and no significant group × block interaction was detected (F < 1.00 in all cases).

In Experiment 2, all seven ischemic rats had moderate to severe bilateral anterior dorsal CA1 injury, and the neuropathologic grade in this area averaged 2.06 ± 0.30. There was less damage in the posterior dorsal CA1 region; the grade there was 1.43 ± 0.36. In the dorsal caudate nucleus the grade was 1.38 ± 0.22. One ischemic rat had moderate unilateral cortical damage. There was no damage in the controls.

Discussion

Results from these experiments continue to develop the functional analysis of a rat model of ischemic brain injury. In Experiment 1, preserved reference memory performance stands in contrast to the selective and permanent impairment of working memory performance. Ischemic rats learned and remembered reference memory responses dependent on variable spatial information that was useful across all trials. However, ischemic rats had difficulty learning and remembering working memory responses dependent on invariable spatial information that was used only within a trial. Ischemic rats made increasing numbers of working errors on later choices in a pattern that was comparable to that of controls. These results suggest that ischemic rats initially perform almost as well as controls but cannot sustain this level of performance. The increase in working errors commensurate with the increasing number of choices can be taken to indicate that the working memory of ischemic rats is weakened but not completely destroyed. The buildup of working errors within a trial is similar to the performance of animals with large fimbria-fornix transections.20 A “quantitative” weakening of working memory in ischemic rats and rats with fimbria-fornix hippocampal disconnection suggests that brain-damaged rats encode and retrieve working memory information in a manner qualitatively similar to that of normal rats, but with significantly diminished efficiency (see Reference 21 for a review). Regardless of the precise behavioral mechanism, we tested whether the impaired working memory could be modified in Experiment 2. Those results demonstrate that working memory may be improved by reinforcing or strengthening the correct choice with additional training trials. Therefore, the working memory impairment in radial mazes of rats exposed to ischemia is quantitatively small, but under specific conditions it is significant and reliable. With attention to the details of training and design, the radial maze may provide a generally useful measure of behavioral outcome in rat models of ischemia.

In several studies, the effect of training rats before ablationing the hippocampus has been to decrease the severity of the memory impairment.17,22-24 Nevertheless, the results of the current study raise the question of whether this degree of ischemic injury and functional impairment have clinical relevance. Ischemic hippocampal injury occurs in patients, perhaps most often because of the selectively vulnerable nature of neurons in the subregions of the cornu ammonis.25 Although circulatory insufficiency may cause stroke and cerebral infarction of the hippocampus,26 more typically cardiac arrest with the attendant precipitous decrease of CBF causes ischemic neuronal necrosis in the CA1 regions of the hippocampus.25,27 Based on a wealth of clinical and experimental evidence, the hippocampus is believed to be crucial for memory function. It has been shown that some survivors of cardiac arrest may have an amnesic syndrome, a disorder of learning and memory in which patients have a particularly difficult time learning and remembering new, unique, or variable information.8-10 On the contrary, however, these amnesic patients may remember information learned before the injury, usually activities of a repetitive nature such as dressing or multiplication tables; these patients may have some ability to learn certain restricted forms of new information.28-30 Postmortem studies of postarrest amnesic patients have shown a pattern of severe bilateral CA1 neuron loss.6-8 Recently, investigators have described a patient with recurrent severe hypotension and multiple episodes of cardiac arrest who had an antero-grade amnesia for 5 years before his death; on postmortem examination the most severe injury was found in the CA1 hippocampus bilaterally.8 This clinical information suggests that ischemic injury to an apparently restricted area may cause a crippling functional breakdown.

Our results are consistent with previous data on the speed with which rats run a maze and suggest that ischemic rats do not have motor abnormalities.4,5,11-13,31 Although radial arm maze testing does not permit precise measurement of a rat’s speed on each part of the maze, in general, ischemic rats explored the maze as quickly as controls. Our results differ from those of other work that demonstrated abnormalities of motor performance in rats exposed to forebrain ischemia by the method of four-vessel occlusion.32 In those experiments, motor performance of rats exposed to ischemia was significantly depressed 24 hours after exposure but was comparable to that of controls by 48 hours. In view of this transient deficit, it is likely that the absence of motor impairment in our experiments may be due to the 30-day recovery between occlusion and testing.

An important but unresolved question is whether there is a more specific correlation between the
regions of ischemic damage and impaired working memory. Transient forebrain ischemia causes a complex pattern of chronic hippocampal injury, and immunohistochemical techniques may lead to more complete identification of the damage. 33–35 Also, transient forebrain ischemia damages other areas in addition to the hippocampus. 1,2,4,11–13 However, results from other experiments suggest that ischemic hippocampal injury impairs working memory. Experiments that directly compared the performance of animals with bilateral dorsal CA1 destruction after ibotenic acid with that of animals exposed to ischemia demonstrate that both groups have normal reference memory but impaired working memory. 36 Other researchers have shown that working memory is impaired by ablation or neurotoxin focal injury of the hippocampus or by fimbria-fornix transection. 14,20,37 Finally, experiments have demonstrated that bilateral radiofrequency lesions of the caudate nucleus do not impair working memory but do impair reference memory. 38,39 This memory impairment is opposite the performance of animals exposed to ischemia. Thus, in spite of the preliminary characterization of the ischemic injury after four-vessel occlusion, our behavioral data suggest that ischemic hippocampal injury is sufficient to impair working memory.

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