SPATIAL LEARNING DISABILITY IN RATS WITH FOCAL CEREBRAL ISCHEMIA RECovers BY REPEATING MORRIS WATER MAZE TESTS

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ABSTRACT
The effect of repeated Morris water maze tests on the ability of place navigation was examined in rats with permanent unilateral occlusion of the middle cerebral artery (MCA) and/or the ipsilateral common carotid artery (CCA), in relation to choline acetyltransferase (ChAT) immunoreactivity in the basal magnocellular nucleus of Meynert (NBM). The Morris water maze task was conducted 2, 4, 6 and 8 weeks after MCA or MCA/CCA occlusion; each test included two trials per day for 4 days, and the mean latency for the rats to reach an invisible platform below the water surface was measured. MCA- or MCA/CCA-occluded rats subjected to repeated water maze tests exhibited only transient disability of place-navigation; the mean latency of MCA- or MCA/CCA-occluded rats became close to that of sham-operated rats by 6 to 8 weeks after ischemia. Despite the time-dependent recovery of place-navigation activity in MCA- or MCA/CCA-occluded rats, ChAT neurons in the NBM, which are considered to be involved primarily in spatial learning ability, were fewer on the ischemic side than on the non-ischemic side throughout the examination period. Moreover, hemispheric atrophic changes were prominent at later stages after MCA or MCA/CCA occlusion. These findings suggest that early water maze training facilitates the functional restoration of MCA- or MCA/CCA-occluded rats, and that the recovery of place-navigation activity in the ischemic rats cannot necessarily be explained by a reduction in the ischemic injury of ChAT neurons in the NBM.

Cerebral infarction induces a variety of signs and symptoms including hemiplegia, reduced locomotion and cognitive disturbances. For studies of the mechanism(s) underlying ischemic neuronal death and in a search for new methods or drugs for the treatment of brain ischemia, several animal models have been developed. Rats with permanent middle cerebral artery (MCA) occlusion appear to be useful since they invariably develop infarcts in the striatum and/or cerebral cortex and mimic, at least in part, human patients with cerebral infarction (17), although the extent of the ischemic lesion varies with the strain, arterial pressure, blood glucose concentration and age of the rats (5). Since ischemic lesions in the striatal or corticostriatal region of rats with MCA occlusion frequently include the cholinergic pathway from the basal magnocellular nucleus of Meynert (NBM) to the frontoparietal cortex (7), disruption of the acetylcholine neuron system which is primarily involved in space learning activity (11) may lead to a cognitive deficit in MCA-occluded rats.

Rats with transient MCA occlusion, when examined once with the Morris water maze (12) 2 or 3 months after ischemia, have been reported to exhibit space-navigation impairment (1). However, it remains to be determined whether or not impairment of spatial learning in rats with MCA occlu-
sion persists for such a long period if the space-navigation task is repeated soon after ischemia. Moreover, previous studies have shown that chemical lesions of the NBM do not necessarily induce a persistent disturbance in spatial learning (6, 10, 13, 14), and ischemic lesions of the NBM, even if they persist, may affect spatial learning only transiently. Taken together, a study on the time course of space-learning ability and cholinergic neuron changes in MCA-occluded rats would provide information about the effect of repeated water maze tests on space-navigation activity and the link between place-navigation tasks and cholinergic neurons in the NBM. Therefore, the present study examined the time course of space-learning ability in rats with permanent MCA occlusion, which are commonly used as a model of brain ischemia (17). The results of the Morris water maze tests were correlated with the localization of choline acetyltransferase-like immunoreactivity in the NBM.

MATERIALS AND METHODS

Animals and Surgery

Fisher male rats were housed at constant temperature (22°C) with a 12–12 h light-dark cycle and given food and water ad libitum. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Fisher rats weighing approximately 200 g were anesthetized with 2% halothane in a mixture of 70% N2O and 28% O2. An MCA was exposed through a transorbital approach and coagulated at the level of the olfactory tract (MCA-occluded group, n = 18) (17). In another group of animals, the left MCA and the left common carotid artery (CCA) were occluded simultaneously (MCA/CCA-occluded group, n = 18). Eighteen rats were sham-operated.

Behavioral Procedures

Twenty-four rats (8 in each group) were used. The experiment was carried out in a circular swimming pool (140 cm in diameter, 50 cm in height), which was filled with water kept at 22°C and made opaque by the addition of milk. Four points on the rim of the pool were designated arbitrarily: north, south, east, and west, dividing the surface of the pool into 4 quadrants: north-east (NE), north-west, south-east and south-west. Spatial cues were placed in the experimental room and remained at fixed positions throughout the experiment. The movements of the animals were monitored and recorded with a video-camera.

During the test, the rats could escape onto a transparent perspex platform (10 cm in diameter) hidden 2 cm below the surface of the water. The platform was located in the NE quadrant, and starting points were chosen south and north, or west and east. Each trial ended when the rat had climbed onto the platform and stayed there for 15 s, or when it could not reach the platform within 90 s.

The test was performed 2, 4, 6 and 8 weeks after operation. Each test included two trials per day for 4 days, and the mean latency of finding the invisible platform as well as the swimming speed of individual animals was measured each day. Finally, the rats were killed, and their brains were examined histologically.

Succinate Dehydrogenase (SDH) Histochemistry

Six rats (2 in each group) were anesthetized with pentobarbital (40 mg/kg i.p.) 7 days after operation and perfused transcardially with 500 ml of ice-cold 10% glycerin solution. The brain was removed quickly and frozen. Frozen sections 40 μm thick were mounted on poly-L-lysine-coated slides and dried immediately at 37°C. The sections were incubated for 40–50 min with a solution consisting of 0.55 mM nitroblue tetrazolium and 0.05 M sodium succinate in 0.05 M phosphate buffer (PB) (pH 7.4) at 37°C. The reaction was stopped by immersion in 10% buffered formalin for at least 1 h. The slides were then coveredslipped with glycerin.

Choline Acetylttransferase (ChAT) Immunohistochemistry and Cerebral Atrophic Change

Two, 4 and 8 weeks after operation, animals (n = 4 in each group) were anesthetized with pentobarbital (40 mg/kg i.p.) and perfused transcardially first with 50 ml of saline, then with 500 ml of 4% paraformaldehyde-0.075% glutaraldehyde-0.2% picric acid in 0.1 M PB (pH 7.4). After perfusion, the brain was removed and cut into 45 μm sections with a microslicer. One third of the sections were stained with cresyl violet, and another one third with hematoxylin and eosin (HE). The remaining sections were stained with monoclonal antibody against ChAT (Sigma, St. Louis). The
sections were [1] washed with 0.02 M phosphate-buffered saline (PBS) for 30 min, treated overnight with a PBS solution containing 5% bovine serum albumin (BSA) and 5% normal swine serum (NSS) at 4°C, and incubated for 2 days at 4°C with the anti-ChAT antibody diluted 1:2,000 with PBS containing 5% BSA, 1% NSS, and 0.1% Triton X-100; [2] rinsed 3 times in PBS (10 min each time); [3] incubated overnight with biotinylated anti-mouse swine IgG (DAKO) diluted 1:500 with the same solution; [4] rinsed three times in PBS (10 min each time); [5] incubated overnight with peroxidase-conjugated streptavidin (DAKO) diluted 1:600 with PBS containing 5% BSA and 0.1% Triton X-100; [6] rinsed twice in 0.1 M PBS, once in 0.05 M Tris-HCl buffer (TB) (pH 7.6), and once in 0.1 M TB (pH 7.6); and [7] subjected to a modified version of the cobalt-glucose oxidase-diaminobenzidine (Co-GOD) intensification method (16).

Neurons with ChAT immunoreactivity in both NBMs of five serial coronal sections were counted with an image analyzer. Then the left to right ratio was calculated. In addition, the areas of the left and right cerebral hemispheres in six consecutive coronal sections stained with HE were measured by a computer digitizer for calculating of the left to right ratio. Values are represented as mean ± standard error.

Differences in response latency, swimming speed, ChAT neuron number and area of cerebral hemisphere among the MCA-occluded, the MCA/CCA-occluded, and the sham-operated groups were analyzed statistically with the two-tailed Mann-Whitney’s U test.

RESULTS

Place Navigation Task

There was no difference in swimming speed among the three groups 2 to 6 weeks after ischemia or sham operation (Fig. 1).

Two weeks after operation, almost all the rats in each group failed to escape onto the platform within 90 s on the first trial day, and the latency of reaching the platform did not differ among the 3 groups. By the third trial day, the latency of the sham-operated animals had decreased gradually, but that of the MCA-occluded or MCA/CCA-occluded rats was relatively unchanged and significantly longer than that of the sham-operated rats (MCA occlusion, \( P < 0.01 \); MCA/CCA occlusion, \( P < 0.05 \)) (Fig. 2A).

Four weeks after operation, it took significantly longer for the MCA/CCA-occluded rats to escape onto the platform than it did for the sham-operated rats on the first, third and fourth trial days (MCA/CCA-occluded rats on the first, third and fourth trials, \( P < 0.01 \)) (Fig. 2B). The mean response latency was higher in the MCA-occluded group than in the sham-operated group throughout the trial period, but the difference was not statistically significant.

Six weeks after operation, the mean escape latency was still longer in the rats with MCA/CCA occlusion than in the sham-operated rats, but the difference between the two groups was not significant (Fig. 2C). The escape latency of the MCA-occluded rats was similar to that of the sham-operated rats (Fig. 2C).

Eight weeks after operation, all three groups
Fig. 2 Water maze task. A: Two weeks after operation; by the third trial day, the latency of sham-operated rats decreased gradually, but that of MCA-occluded and MCA/CCA-occluded rats were relatively unchanged and significantly prolonged in comparison with that of sham-operated rats. *P < 0.05, **P < 0.01, significantly different from the corresponding sham-operated group. B: Four weeks after operation; it took significantly longer for MCA/CCA-occluded rats to reach the safe platform on the first, third and fourth trial days. **P < 0.01 significantly different from the corresponding sham-operated group. C: Six weeks after operation; the mean escape latency in rats with MCA/CCA occlusion was still longer than that in sham-operated rats, but the difference was not significant. The escape latency of the MCA-occluded rats was similar to that of the sham-operated rats.
exhibited similar values (around 15 s) of response latency in the place navigation task.

Thus, disability in space-learning was noted only transiently after brain ischemia and recovered almost completely 6 weeks after MCA occlusion and 8 weeks after MCA/CCA occlusion, possibly as a result of repeated water maze tests.

**SDH Histochemistry**

SDH activity decreased more in the left corticostriatal region of MCA/CCA-occluded rats than in the left subcortical striatal region of MCA-occluded rats (Fig. 3). Differences in SDH activity between the right and left NBM were not detected in the brain sections examined.

**ChAT Immunohistochemistry**

Frontal sections from the brains of both occlusion groups were examined by ChAT immunohistochemistry and showed a decrease in the number of ChAT-positive neurons in the left NBM ipsilateral to the lesion (Fig. 4). These changes were more prominent in the MCA/CCA-occluded rats.

The ratio of left to right cholinergic neurons in the NBM of both occlusion groups was significantly lower than in the sham-operated animals 2, 4 and 8 weeks after operation (MCA occlusion, postoperation week 2, 4, 8: $P < 0.05$, respectively; MCA/CCA occlusion, postoperation week 2, 4, 8: $P < 0.05$, $P < 0.05$, $P < 0.01$, respectively) (Fig. 5). The sham-operated rats did not exhibit any significant left-right differences in ChAT-immunoreactive neurons within the NBM throughout the examination period. Moreover, no significant time-dependent changes in the ratio of left to right cholinergic neurons were noted in the NBM of the MCA-occluded or of the MCA/CCA-occluded rats.

**Cerebral Atrophic Changes**

Coronal sections from the brains of both occlusion groups showed atrophic changes in the left (ischemic) cerebral hemisphere as revealed by dye staining (Fig. 6). These changes were more prominent in the MCA/CCA-occluded rats.

The ratio of left to right hemisphere areas in the brains of both occlusion groups was significantly
Fig. 4  Photomicrographs of ChAT immunoreactivity in the NBM 8 weeks after sham-operation (A, B), MCA occlusion (C, D) and MCA/CCA occlusion (E, F). A, C, E, right side; B, D, F, left (ischemic) side. Bar = 200 μm

lower than in the brains of the sham-operated group 4 and 8 weeks after operation (MCA occlusion, postoperation week 4, 8: \( P < 0.05, P < 0.01 \), respectively; MCA/CCA occlusion, postoperation week 4, 8: \( P < 0.05, P < 0.01 \), respectively) (Fig. 7). The sham-operated rats did not exhibit any significant left-right differences throughout the examination period. During 4 to 8 weeks after operation, no significant time-dependent changes in the atrophy ratio were noted in the two occlusion groups.
Fig. 5 The ratio of left to right ChAT-positive neurons in the NBM 2, 4 and 8 weeks after operation. *P < 0.05, **P < 0.01 significantly different from the corresponding sham-operated group.

Fig. 6 Photomicrographs showing ischemic forebrain lesions 8 weeks after MCA occlusion (A) and MCA/CCA occlusion (B). Cerebral atrophic changes were more prominent in the MCA/CCA-occluded rats.
DISCUSSION

Although MCA-occluded rats have been shown to exhibit certain disturbances of motor function until 4 weeks after operation (18), the present study did not show any differences in swimming speed among MCA-occluded, MCA/CCA-occluded and sham-operated animals throughout the examination period. Perhaps the activity of central neurons needed for swimming is less severely affected by MCA or MCA/CCA occlusion than that of other neurons relevant to more complex movements. The intact swimming activity of MCA- and MCA/CCA-occluded rats enabled us to use the Morris water maze (12) to examine the time-course of space-learning ability in ischemic rats. At 4 weeks after operation, the mean response latency of rats with MCA or MCA/CCA occlusion was longer than that of sham-operated animals throughout the first to fourth trial days. Moreover, the mean response latency of MCA/CCA-occluded rats on the first trial day at 4 weeks after operation was longer than that on the fourth trial day at 2 weeks after operation. On the other hand, the mean response latency of sham-operated animals remained around or less than 30 s since the fourth trial day at 2 weeks after sham operation. These findings suggest that the animals with MCA/CCA occlusion suffered from the impairment of not only acquisition but also retention of spatial memory during 2 to 4 weeks after operation. By 6 to 8 weeks after operation, the animals with MCA or MCA/CCA occlusion had recovered space-learning ability, possibly as a result of water maze tests repeated at early postischemic stages. The transient impairment of space-navigating ability is also noted in animals with chemical lesions of the septal nuclei, hippocampus or NBM (6, 10, 13, 14).

Since the ability of place navigation in MCA- or MCA/CCA-occluded rats is reduced for only 2 to 4 weeks after operation when the water maze test is repeated, the effectiveness of certain treatments for ischemic rats must be evaluated 2 to 4 weeks after operation. Thus, the Morris water maze experiment has a limitation when it is used as a tool for the assessment of the effects of neuroprotective drugs. Nevertheless, it offers an advantage over other methods in the study of the neuronal basis of learning and memory, with respect to its ability to distinguish deficits in memory formation from those in sensory, motor and motivational processes (11).

Since some of the behavioral abnormalities elicited by experimental lesions of the NBM correlate well with cortical ChAT activity (8) and can be corrected by treatment with cholinergic agonists or grafts (2, 4, 9, 15), learning and memory are assumed to involve cholinergic neurons in the NBM with neural projections to the cerebral cortex. Accordingly, we tried to correlate posts ischemic behavioral deficits with a decrease in the number of ChAT-positive neurons in the ischemic NBM. However, while the decrease in the number of ChAT-positive neurons in the left ischemic NBM persisted 2 to 8 weeks after MCA or MCA/CCA occlusion, the decrease in the number of ChAT-positive neurons of the left hemisphere was not significant. The data suggest that the impairment of learning ability in rats with MCA/CCA occlusion is due to the decrease of the number of cholinergic neurons in the NBM.
occlusion, space-learning ability recovered by 6 weeks after operation. This suggests that the recovery of place-navigation ability after brain ischemia, as revealed by the Morris water maze experiment, cannot be solely explained by changes in ChAT-positive neurons within the NBM. In support of this speculation, several neurotransmitters in addition to acetylcholine in different neuronal circuits have been postulated to participate in the central processes of spatial learning (3, 11). Moreover, the progressive atrophic changes in the ischemic hemisphere during 4 to 8 weeks after MCA or MCA/CCA occlusion appear to be irrelevant to the postischemic restoration of space-learning ability. It is likely that central neurons not damaged by ischemic insult exhibit a marked plasticity to compensate for the functional deficits of animals with MCA or MCA/CCA occlusion.

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