Long-Term Spatial Cognitive Impairment After Middle Cerebral Artery Occlusion in Rats: No Involvement of the Hippocampus

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Summary: The behavioral and neurochemical changes in the chronic phase of permanent occlusion of the right middle cerebral artery (MCA) in rats were investigated. One month after MCA occlusion, 23 rats were unable to solve a radial eight-arm maze task during an entire 1-month period, whereas seven rats were able to solve this task. Three months after occlusion, 19 MCA-occluded rats failed to solve the task successfully again for at least 1 month (the cognitively impaired rats), whereas 11 MCA-occluded rats were able to solve it (the cognitively unimpaired rats). The rats that underwent behavioral testing were examined for any changes in the acetylcholine (ACh) levels in the hippocampus using HPLC with electrochemical detection or the formation of long-term potentiation (LTP) in the population spike of the hippocampal CA1 field. The immunohistochemical distribution of either the microtubule-associated protein 2 (MAP2) or glial fibrillary acidic protein (GFAP) in the hippocampus of the cognitively impaired rats was also studied. In the cognitively impaired rats, neither the suppression of the induction of LTP, nor the degradation of MAP2, nor the increase in the GFAP immunoreactivity was observed in the hippocampus. The levels of ACh in the hippocampus did not change significantly among the cognitively impaired, unimpaired, and the sham-operated rats. These results suggest that MCA occlusion is capable of producing long-term spatial cognitive disturbance in rats without any evidence of neurobiological damage in the hippocampus.

Key Words: Focal cerebral ischemia—Memory—Long-term potentiation—Microtubule-associated protein 2—Glial fibrillary acidic protein.
impairment of spatial cognition after permanent MCA occlusion might be mainly due to neuronal lesions in the cerebral cortex and the striatum. However, a dysfunction of the hippocampal neurons might also occur without any detectable histological alterations.

It has been reported that a dysfunction of the septohippocampal cholinergic system caused memory impairments in various spatial learning tasks (Abe et al., 1993; Dunbar et al., 1993) and also caused a reduction in the levels of acetylcholine (ACh), especially in the hippocampus (Pepeu et al., 1989). On the other hand, many researchers have shown that hippocampal long-term potentiation (LTP) might reflect the activation of physiological mechanisms that underlie some forms of spatial learning (Morris et al., 1986; Grant et al., 1992). Rats exposed to transient forebrain ischemia have shown severe injury to the hippocampal CA1 neurons as well as an impairment of spatial cognition (Volpe et al., 1990; Gionet et al., 1991; Yamamoto et al., 1993). Thus we can speculate that the decrease in the level of ACh and LTP may reflect the deterioration of the hippocampal function associated with spatial learning. In addition, hypertrophy of astrocytes, as reflected by an increase in glial fibrillary acidic protein (GFAP), and a degradation of microtubule-associated protein 2 (MAP2) are considered also to be sensitive and reliable markers of subtle and morphologically undetectable neuronal damage during the early stages of cerebral ischemia (Kuwaki et al., 1989; Inuzuka et al., 1990; Schmidt-Kastner et al., 1990; Rischke and Krieglstein, 1991). Therefore, in this study, we investigated the behavioral, neurochemical, neurophysiological, and immunocytochemical changes in the chronic phase of a permanent occlusion of the right MCA in rats, while focusing on either the alteration of the levels of ACh and LTP in the CA1 field or the distribution pattern of MAP2 and GFAP in the hippocampus of the cognitively impaired rats.

MATERIALS AND METHODS

Surgical preparation

This study was approved by the Animal Research Committee of the Teikyo University School of Medicine and the Fukuoka University. The study was carried out using 51 male Slc Wistar rats (Shizuoka Lab. Animal Cent., Shizuoka, Japan), aged 15 to 20 weeks and weighing 310 to 370 g. The rats were housed in group cages under 12:12 h light:dark conditions and were given free access to food and water.

Thirty-two rats were anesthetized with 2% halothane, and the proximal portion of the right MCA was permanently occluded by the microsurgical technique that was modified from our original method developed by Tamura et al. (1981a) for the purpose of chronic experiments (Yamamoto et al., 1988). The stem of the MCA was electro-cauterized just medial to the olfactory tract and was cut to ensure a complete vascular occlusion. Ten rats also received a sham operation as described previously by Tamura et al. (1981a). The remaining nine rats were used as the intact rats. Behavioral experiments were started 1 month later when the neurological damage to the brain seemed to have stabilized.

The radial eight-arm maze task

One month after occlusion, the behavioral test was carried out with a radial eight-arm maze task (the first session), described in detail elsewhere (Okada et al., 1995). In short, maze adaptation was performed 1 day before the first session. Ten rats were allowed to explore the eight-arm maze only once for 10 min. Food pellets were randomly scattered over the entire maze surface. After adaptation, all rats were trained once a day for 30 consecutive days, during which a single food pellet was placed in each of the food cups of the eight arms. For each trial, a rat was placed on the center platform facing a randomly selected arm and was allowed to make arm choices until either all eight pellets had been eaten or 10 min had elapsed, whichever came first. Immediately after the trial, the rat was returned to its home cage and was given its additional food for the day. The initial entry of an arm was scored as a correct choice, whereas a reentry to an arm previously visited was scored as an error. The choice accuracy was then evaluated according to two indices of maze performance: (a) the number of correct choices during the first eight choices, and (b) the number of errors during a trial. The acquisition of spatial cognition was defined as the ability to reach at least seven different arms in the first eight choices and all eight within the first nine choices for three consecutive trials within a 30-day period. During the training period, each rat was placed on a partial food-deprivation schedule designed to maintain the body weight between 350 and 370 g. The body weight was checked before feeding, and ~15 g of food pellets (Kyudo Co. Ltd., CE-2) was provided each day. The rats had free access to water.

After an interval of 1 month, all the rats were tested again for the radial eight-arm maze task once a day until they acquired spatial cognition (the second session) or for 1 month. As described in the previous article (Okada et al., 1995), we divided the MCA-occluded rats into two groups according to the results of the second session. One group was the cognitively impaired rats that were not able to solve the radial maze task; the other was the cognitively unimpaired rats that were able to solve the task in the second session.

Measurement of ACh

Eleven cognitively impaired rats and 10 cognitively unimpaired rats were used. Ten sham-operated rats were used as control. The rats were placed in a restraining device designed to fit inside the animal chamber of a Toshiba microwave applicator (Muromachi Kikai Co. Ltd.). The device carrying the rat was positioned inside the animal chamber of the microwave apparatus. Microwave power (5 kW) was focused onto the rat’s head and then applied for 1.7 s. The rat was removed from the microwave apparatus, and the brain was removed, frozen, and punched out by the stainless tube (1 mm diameter). As indicated in Fig. 1, the dorsal hippocampal (DH)
and ventral hippocampal (VH) regions ipsilateral and contralateral to occluded MCA were collected. Brain tissue fragments weighing 1–2 mg were homogenized in ice-cold 15% 1 N formic acid, 85% acetone containing ethylhomocholine (an internal standard) with ultrasonic cell disrupter (Microson, Heat System-Ultrasonic Inc.). The homogenate was centrifuged, and the supernatant was transferred to a clean tube to which 1 ml diethylether was added. The mixture was then vortexed vigorously and kept on ice. After centrifugation, the organic phase was discarded, and the sample was then evaporated under a stream of scrubbed nitrogen. The residue was stored at −50°C until analysis. The sample was dissolved in 100 μl of distilled water, and the levels of ACh were measured using HPLC with electrochemical detection and an immobilized enzyme column according to the method of Murai et al. (1989). The data were expressed as the mean ± SD.

Measurements of LTP

Four cognitively impaired MCA-occluded rats and intact animals with different three ages (2, 12, and 27 months) maintained under conventional conditions were used. The number of the 2-, 12- and 27-month rats were all three. In each animal, consecutive transverse slices (400 μm thick) were made from each side of the dorsal hippocampus, and one to three slices were used for the LTP measurement. The detailed procedures of slice preparation and the methods of recording have been described elsewhere (Nakanishi et al., 1991). A single slice was placed in an interface-type recording chamber at a constant bath temperature of 36°C. Electrical stimulation was applied through a bipolar stainless steel electrode placed on the Schaffer collateral pathway. The extracellular field potentials were recorded with a glass electrode filled with perfusate and were placed in the pyramidal cell layer of the CA1 region. Before and after the tetanus was applied at 100 Hz for 1 s, a normal test stimulation with an intensity, 5–10 V, 200 μs duration) was applied at 0.1 Hz. The population spike amplitude was measured from the most negative peak to the line joining the pre- and postspike population excitatory postsynaptic potential. The population spike amplitude was measured as the mean ± SD.

Immunohistochemical procedure

Three cognitively impaired MCA-occluded rats were used. The detailed immunohistochemical procedures have been described previously (Nakanishi et al., 1993). Shortly thereafter, the animals anesthetized with sodium pentobarbital (40 mg/kg) were perfused with chilled isotonic saline and then perfused with chilled 4% paraformaldehyde. After perfusion, the brain was removed, fixed in the same fixative for 24 h at 4°C and eight to 10 sections (40 μm thick) from each side of the dorsal hippocampus were prepared by a cryostat. Each floating section was stained by antibody against GFAP (1:2000 dilution) or MAP 2 (1:1000 dilution) with the avidin–biotin–peroxidase complex method using the Vectastain kit (Vector Laboratories). For quantitative analyses, the number of MAP2-positive proximal dendrites intersecting a line (520 μm) in CA1 region and GFAP-positive cells in the CA1 region (0.18 mm² area) per section of each hemisphere were counted with the aid of a drawing tube.

Statistics

The differences in both the number of correct choices and the total errors were evaluated by split plot analysis of variance (ANOVA) using general linear model (GLM) procedure of SAS. The statistical significance of the differences in the ACh levels among the cognitively impaired, cognitively unimpaired, and sham-operated rats was determined by the Dunn test. The mean LTP magnitude obtained in the ipsilateral and contralateral hippocampal slices from each animal was averaged, and the statistical significance of differences was evaluated by Dunnett’s t test. The mean number of MAP2-positive proximal dendrites and GFAP-positive cells in the CA1 region of the ipsilateral and contralateral hippocampus from each animal was averaged, and the statistical significance of the difference was also analyzed by Dunnett’s t test.

Chemicals

Ethylhomocholine was obtained from the Eicom Corp. Antibodies recognizing MAP2 and GFAP were purchased from Amersham and DAKO, respectively. All other reagents were of the highest grade available.

RESULTS

The radial eight-arm maze task

Two MCA-occluded rats died during the period of the behavioral experiments and were eliminated from the data. When the training period for the MCA-occluded rats was started at 1 month after MCA occlusion (the first session), 23 rats failed to achieve 7 of 8 or 8 of 9 correct choices for three consecutive trials during an entire month. On the other hand, in addition to the intact and sham-operated rats, the remaining seven MCA-occluded rats were able to achieve this criterion. Figure 2 shows the acquisition performance of the first session among the MCA-occluded, sham-operated, and intact rats. The number of correct choices increased significantly over days, F(29, 1247) = 3.76, p < 0.001. An ANOVA on the number of correct choices confirmed a significant difference among these three groups, F(2, 86) = 31.80, p < 0.05. There were no significant differences in the number of correct choices between the sham-operated and the intact rats. The MCA-occluded rats showed less accuracy on maze performance than did the sham-operated rats, F(1, 37) = 142.83, p = 0.053. There was no significant Group × Days interaction in the number of correct choices. The number of errors decreased over days, F(29, 1247) = 1.41, p = 0.073. An ANOVA on the total number of errors confirmed a significant difference among these three groups, F(2, 86) = 107.78, p < 0.01. There were no significant differences in the errors between the sham-operated and the intact rats. The MCA-occluded rats made more errors than either the sham-operated, F(1, 37) = 114.64, p = 0.059, or...
FIG. 1. A schematic representation of the rat brain in the coronal sections demonstrating the four areas sampled for the measurement of the ipsilateral and contralateral levels of acetylcholine (ACh) in the hippocampus. Brain tissue was sampled from two coronal sections at −3.3 mm (plate 1) and −4.8 mm (plate 2) anterior from bregma, respectively, according to the atlas of Paxinos and Watson (1982). DH, dorsal hippocampus; VH, ventral hippocampus.

After a 1-month interval, all the rats were then retrained (the second session). Nineteen MCA-occluded rats were not able to acquire spatial cognition (the cognitively impaired rats), whereas 11 MCA-occluded rats were able to acquire spatial cognition (the cognitively unimpaired rats). In this study, 19 cognitively impaired rats were from the 23 rats that failed to perform the first session. The details of these results were described in the previous article (Okada et al., 1995).

Alterations of the levels of ACh in the hippocampus

As shown in Table 1, compared with those in the sham-operated rats, the levels of ACh in the hippocampus did not change significantly in either the cognitively impaired or unimpaired rats. Compared with those in the intact rats, the ACh levels of the ipsilateral VH region in the sham-operated rats were higher, but a significant difference was not observed.

LTP formation

Figure 3 shows representative population responses to Schaffer collateral stimulation before and 60 min after tetanic stimulation obtained in slices of the dorsal hippocampus from the MCA-occluded rat that showed significant cognitive impairment. In the hippocampal slices ipsilateral to the infarct, stimulation of the Schaffer collateral pathway evoked population spikes similar in ampli-
The brain regions are described in Fig. 2. The data are expressed as the mean ± SD ng/mg tissue. The number of rats is shown in parentheses.

ACh, acetylcholine; MCA, middle cerebral artery; DH, dorsal hippocampus; VH, ventral hippocampus.

### Immunostaining of MAP2 and GFAP

Figure 4 shows the immunohistochemical staining of MAP2 and GFAP in the dorsal hippocampus ipsilateral and contralateral to the infarct from cognitively impaired, MCA-occluded rats. The shape of the ipsilateral hippocampus was markedly changed, whereas the contralateral hippocampus showed rather normal shape. However, intense MAP2-immunostaining was observed in the neuronal cell bodies and their dendrites of the hippocampus from each hemisphere. The immunostaining pattern of GFAP appeared to be normal, judging from the immunostaining intensity and morphology. The mean number of neither the MAP2-positive proximal dendrites nor the GFAP-positive cell soma in the CA1 region per section was significantly different between ipsilateral and contralateral to the infarct (Table 3). Hypertrophy of astrocytes, however, was occasionally observed in the white matter ipsilateral to the infarct (not shown).

### Table 1. The ACh levels in the hippocampus

<table>
<thead>
<tr>
<th>MCA occlusion</th>
<th>Cognitively impaired</th>
<th>Cognitively unimpaired</th>
<th>Sham</th>
<th>Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH</td>
<td>Ipsi (right)</td>
<td>20.41 ± 8.58 (11)</td>
<td>27.03 ± 6.60 (10)</td>
<td>23.54 ± 5.08 (10)</td>
</tr>
<tr>
<td></td>
<td>Contra (left)</td>
<td>23.22 ± 11.88 (11)</td>
<td>24.57 ± 9.45 (10)</td>
<td>20.25 ± 5.65 (10)</td>
</tr>
<tr>
<td>VH</td>
<td>Ipsi (right)</td>
<td>23.03 ± 9.12 (11)</td>
<td>32.96 ± 10.83 (10)</td>
<td>31.13 ± 13.91 (8)</td>
</tr>
<tr>
<td></td>
<td>Contra (left)</td>
<td>23.76 ± 11.93 (11)</td>
<td>27.54 ± 18.14 (10)</td>
<td>16.92 ± 5.16 (8)</td>
</tr>
</tbody>
</table>

- The relative population spike amplitude was measured at 60 min after tetanic stimulation. The data are expressed as the mean ± SD. The number of slices is shown in parentheses.
- LTP, long-term potentiation; MCA, middle cerebral artery.
FIG. 4. Immunohistochemical staining of microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) in the hippocampus ipsilateral (A, B) and contralateral (C, D) to infarct from the cognitively impaired rat (C, D). An enlargement of the CA1 subfield in the right column (E-H). A, C: Immunostaining of MAP2. B, D: Immunostaining of GFAP. No significant differences in either MAP2 or GFAP immunoreactivity could be observed between the ipsilateral and contralateral hippocampus. Bar = 500 μm (A), 50 μm (E); DG, dentate gyrus.
It has been proposed that a normal performance on the radial eight-arm maze task requires an intact hippocampal circuitry. Damage to the hippocampus and its connections impairs spatial memory in the radial-arm maze (Olton et al., 1978; Jarrard, 1993). In our study, about two thirds of the MCA-occluded rats showed a long-term spatial cognitive impairment in the radial eight-arm maze task. However, the hippocampus is not in the territory of the MCA. Permanent unilateral MCA occlusion in rats did not cause infarction in the hippocampus (Tamura et al., 1981b; Tyson et al., 1984). Moreover, in the acute or subacute phase after MCA occlusion, any histopathological damage of the hippocampus could not be observed (Tamura et al., 1981a; Tamura et al., 1986; Duverger and MacKenzie, 1988; Wahl et al., 1992). Other parameters such as induction of heat-shock protein, uptake of 2-deoxy glucose, or accumulation of calcium did not indicate any damage of the hippocampus (Kataoka et al., 1989; Nagasawa and Kogure, 1990; Kinouchi et al., 1993).

On the other hand, it remains possible that dysfunction of the hippocampal neurons might occur without any detectable histological alteration. Thus we studied the hippocampal function associated with spatial learning. Central cholinergic neurotransmission has been shown to play an important role in the regulation of learning behavior. It is widely accepted that two major cholinergic systems exist in the basal forebrain. One derives from the nucleus basalis magnocellularis and projects primarily to the neocortex, whereas the other is from both the medial septum and the diagonal band of Broca and projects primarily into the hippocampus via fimbria-fornix (Wenk et al., 1980; Mesulam et al., 1983). The septohippocampal cholinergic system is generally considered to be the main center of the memory process (Olton et al., 1978; Crutcher et al., 1983; Ikegami et al., 1989; Jarrard, 1993), and lesions located therein can produce a deficit of spatial cognition in the radial eight-arm maze task (Crutcher et al., 1983; Ikegami et al., 1989). In our study, the levels of ACh did not change significantly either in the dorsal or in the ventral hippocampus in the chronic phase of MCA occlusion. We cannot rule out the possibility that some other aspects of ACh function, such as ACh receptor populations, are affected after MCA occlusion. However, if severe and long-lasting spatial cognitive deficit after MCA occlusion is mainly due to the hippocampal damage, the levels of ACh in the hippocampus should be affected. Crutcher et al. (1983) suggested that an impairment of the radial-arm maze performance after medial septal lesions usually disappears within 3 weeks if the cholinergic denervation is not perfect (<90%). In addition, a 50% loss of hippocampal choline acetyltransferase (ChAT) activity caused a spatial cognitive deficit only when a delay interval was imposed on the radial maze task (Chrobak et al., 1988). Our results thus suggest that the septohippocampal cholinergic neurons may not be so greatly affected as a result of MCA occlusion.

Moreover, LTP is a persistent enhancement of synaptic efficacy induced by high frequent stimulation of afferent pathways (Anderson et al., 1980). It has been reported that the impairment of LTP in the CA1 region of the hippocampus is correlated with spatial learning deficit in rats (Morris et al., 1986; Deupree et al., 1991; Ramaker et al., 1993), and it is well known that the hippocampal CA1 region is selectively vulnerable to forebrain ischemia (Volpe et al., 1990; Gionet et al., 1991; Yamamoto et al., 1993; Akai et al., 1993). In our study, no significant difference in the degree of LTP formation could be observed between that in the ipsilateral and contralateral hippocampal CA1 region of the cognitively impaired rats. The magnitude of the LTP of slices from the cognitively impaired rats was slightly lower than that from intact 2-month rats but corresponded well with that from intact age-matched animals (12 month). These observations indicate that
the Schaffer collateral–CA1 pyramidal cell synapses in the hippocampus from the cognitively impaired MCA-occluded rats preserved the ability to induce LTP. The slight reduction of LTP magnitude in MCA-occluded rats as compared with intact 2-month rats is considered to be caused by the aging process rather than MCA occlusion. Because the fixed parameter of tetanus (100 Hz for 1 s) was used in our study, the possible impairment of LTP formation with lower stimulation frequency in the hippocampus from the cognitively impaired rats cannot be totally ruled out. Our study also showed that the same parameter of tetanic stimulation failed to induce LTP in the CA1 region of the hippocampal slices from intact 27-month rats. Similar age-related deterioration of LTP has been previously reported in the Senescence Accelerated Mouse, a murine model of accelerated senescence (Katsuki et al., 1990) and Fischer 344 rats (Hori et al., 1992). Kirino et al. (1992) reported that a majority of CA1 neurons lost the capacity for LTP at 48 h after 5 min of carotid artery occlusion of gerbils. They also demonstrated that loss of LTP was associated with abnormal Ca\textsuperscript{2+} homeostasis in ischemia-damaged CA1 neurons. The loss of LTP in the CA1 hippocampal neurons of aged rats may also be related to altered Ca\textsuperscript{2+} homeostasis because age-dependent alteration of Ca\textsuperscript{2+}-mediated potentials and currents have been found in these neurons (Landfield et al., 1992).

In addition, the hypertrophy of astrocytes, as reflected by an increase in the GFAP immunoreactivity, and the degradation of MAP2 have been considered to be sensitive and reliable markers of subtle and morphologically undetectable neuronal damage during the early stages of cerebral ischemia (Kuwai et al., 1989; Inuzuka et al., 1990; Schmidt-Kastner et al., 1990; Rischke and Kriegstein, 1991). In our study, neither the degradation of MAP2 nor the increase in the GFAP immunoreactivity could be observed in the ipsilateral hippocampal CA1 field from the cognitively impaired rats.

It has been suggested that damage to the cerebral cortex or to the striatum might thus also involve a deficit of learning and memory after MCA occlusion in the passive-avoidance task (Yamamoto et al., 1988), the Y-maze task (Wahl et al., 1992), or the water-maze task (Markgraf et al., 1992). In the radial-maze task, the rats with single lesions of the caudate nucleus or neocortex showed cognitive deficits, but this deficit later gradually recovered (Cook and Kesner, 1988; Colombo et al., 1989; Kesner et al., 1989; Packard et al., 1989). If a spatial memory system, which is generally used to solve the standard radial eight-arm maze, is destroyed in some way, there may be different compensatory or complementary systems on which the rats can rely to solve this maze. The lack of such helpful systems may produce the irreversible cognitive deficit in the radial-maze task. Damage to the cerebral cortex or to the striatum may also contribute to the long-term cognitive deficit observed after MCA occlusion in the radial-maze task. However, the long-term cognitive deficit may be the result of a disturbance in the several neuronal network systems at the same time rather than the lesions in a specific brain region. Further investigations are now being performed to elucidate this speculation.

In conclusion, MCA occlusion was capable of producing long-term spatial cognitive deficit in rats without significant damage of the hippocampus. It is most likely that the long-term spatial cognitive deficit of MCA-occluded rats is due to damage of the striatum, cortex, or circuits involving these structures.

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