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Effects of nefiracetam on spatial memory function and acetylcholine and GABA metabolism in microsphere-embolized rats

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Abstract

The present study aimed to determine whether nefiracetam, *N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, a cognition enhancer, has an effect on learning and memory function in sustained cerebral ischemia, and whether the effect, if any, may accompany modification of the cholinergic or γ-aminobutyric acid (GABA)ergic system, which are conceived to be involved in the learning and memory function, in the ischemic brain. Sustained cerebral ischemia was induced by the injection of 700 microspheres into the right hemisphere of the rat. The animals were treated once daily with 10 mg/kg nefiracetam p.o. from 15 h after the operation to either 10 days for the water maze study, or 3 or 5 days after the operation for neurochemical examination. Microsphere-embolized rats showed stroke-like symptoms 15 h after the operation and lengthened the escape latency in the water maze task on days 7–10, suggesting a spatial learning dysfunction. The delayed treatment did not reduce the stroke-like symptoms, but effectively shortened the escape latency. The animals at days 3 and 5 after the operation showed decreases in acetylcholine content and choline acetyltransferase activity, which were not prevented by nefiracetam. The microsphere-embolized rats showed decreases in GABA content and glutamic acid decarboxylase activity. The delayed treatment appreciably restored GABA content in the hippocampus on day 5 and reversed glutamic acid decarboxylase activity in both brain regions on day 5. These results suggest that the GABAergic activity rather than the cholinergic activity may be, at least in part, involved in the pharmacological effects of nefiracetam in the ischemic brain.

Keywords: Embolism, microsphere; GABA (γ-aminobutyric acid); Glutamic acid decarboxylase; Spatial memory function

1. Introduction

Nefiracetam, *N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, is a pyrrolidone derivative having a cognition-enhancing effect on amnesia of animals induced by scopolamine (Sakurai et al., 1989), γ-aminobutyric acid $(GABA)_A$ receptor inhibitor (Nabeshima et al., 1990b; Doyle et al., 1993), alcohol (Nabeshima et al., 1990a), benzodiazepine (Nabeshima et al., 1990a) and cyclohexamide (Nabeshima et al., 1991b). Since repeated administration of nefiracetam increased the choline acetyltransferase activity and glutamic acid decarboxylase activity in the cerebral cortex and hippocampus of normal rats (Kawajiri et al., 1990; Watabe et al., 1993), the pharmacological mechanisms underlying the cognition-enhancing effect are considered to be, at least in part, due to enhancement of the cholinergic activity and/or modification of the GABAergic activity. However, the effects of this agent on the cognition-enhancing effect and on the cholinergic and GABAergic neurotransmitter system in the ischemic brain have not been fully understood.

In the present study, we aimed to determine whether nefiracetam may have cognition-enhancing effects on sustained ischemia-induced experimental amnesia and whether such effects, if any, may accompany modification of the cholinergic and/or GABAergic activities in the ischemic model. The sustained cerebral ischemia was induced by microsphere embolism in rats (Miyake et al., 1993). This model induces widespread, small embolic infarcts, especially in the cerebral cortex, striatum and hippocampus (Miyake et al., 1993), and induces a failure in spatial

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memory function in the water maze task (Takagi et al., 1997; Nagakura et al., 2002). Thus, this model is conceived to mimic focal ischemia-induced human stroke (Lyden et al., 1992) or multi-infarct dementia (Naritomi, 1991). Using this model, the ability of this agent to restore spatial memory function and to recover changes in the cholinergic and/or GABAergic activities in the cerebral cortex and hippocampus was examined. The cerebral cortex and hippocampus are known to be vulnerable to ischemia (Smith et al., 1984; Kirino, 1982), and may be involved in learning and memory function (DiMattia and Kesner, 1988; Save et al., 1992).

2. Materials and methods

2.1. Experimental protocol

Two series of experiments were designed in the present study. In the first series of experiments, animals with microsphere embolism or sham operation were subjected to the water maze test from day 7 to day 10 after the operation. In the second series of experiments, the operated animals were subjected to determination of neurochemical variables including acetylcholine, choline and GABA contents, choline acetyltransferase and glutamate decarboxylase activities on days 3 and 5, and, finally, levels of glutamic acid decarboxylase proteins on day 5 after microsphere embolism.

2.2. Microsphere embolism

Male Wistar rats (Charles River Japan, Atsugi, Japan), weighing 180–220 g, were maintained in an animal room having a 12-h light/12-h dark cycle at a temperature of 23 ± 1 °C and a humidity of $55 \pm 5\%$ throughout the experiment. The animals had free access to food and water according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guidelines for Experimental Animal Care issued by the Prime Minister's Office of Japan. All efforts were made to minimize the suffering of animals, to reduce the number of animals used and to utilize alternatives to in vivo techniques, if available. The study protocol was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Science.

Microsphere-induced cerebral embolism was performed by the methods described previously (Miyake et al., 1993) with some modification. In brief, rats were anesthetized i.p. with 35 mg/kg of sodium pentobarbital. The right external carotid and pterygopalatine arteries were temporarily occluded with strings. A needle connected to a polyethylene catheter (3 French, Atom, Tokyo) was then inserted into the right common carotid artery. Seven hundred microspheres (47.5 ± 0.5 μm in diameter, NEN-005, New England Nuclear, Boston), suspended in 20% dextran solution (150 μl), were injected into the right internal carotid artery through the cannula. After the needle had been removed, and the puncture was repaired with surgical glue (Aron Alpha A, Sankyo, Tokyo), the strings occluding the right external carotid and pterygopalatine arteries were released. Following the occlusion of the right external carotid and pterygopalatine arteries, it took 2–3 min to restart the blood flow to the areas supplied by the arteries. The rats that underwent a sham operation were injected with the same volume of vehicle without microspheres. Fifteen hours after the operation, the behavior of the operated rats was scored on the basis of paucity of movement, truncal curvature and forced circling during locomotion, which were considered to be typical symptoms of stroke in rodents (Furlow and Bass, 1976, McGraw, 1977). The score of each item was rated from 3 to 0 (3 very severe, 2 severe, 1 moderate, 0 little or none). The rats with a total score of 7–9 points were used in the present study.

2.3. Treatment with nefiracetam

After examination of stroke-like symptoms of microsphere-injected rats at 15 h after the operation, the animals were randomly divided into the two groups, nefiracetam-treated and nefiracetam-untreated groups. Nefiracetam at a dose of 10 mg/kg, suspended with 0.5% carboxyl methyl cellulose, was administered into the stomach by gavage at 10:00 in the morning, and the administration was continued up to either 10 days after the operation for the water maze test, or 3 or 5 days for the neurochemical examination. Vehicle was administered to the untreated group. The dose employed in the present study was based on the findings described by others (Nabeshima et al., 1991b) and our dose–response measurements assessed in a preliminary study: treatment with 3 mg/kg/day p.o. of nefiracetam revealed less attenuation of the lengthening of the escape latency in the water maze task than that with 10 mg/kg/day nefiracetam. The water maze test and the examination of neurochemical variables were conducted at least 2 h after the daily administration of the agent.

2.4. Water maze test

The water maze test was performed according to the methods described previously (Morris, 1981; Takagi et al., 1997). The test was performed on days 7–10 after the operation. We examined the animal activity in the open field before the water maze test in a preliminary study. No significant difference in the open field activity was seen in the microsphere-embolized animals (373 ± 35 cm/min for the microsphere-embolized animals and 435 ± 36 cm/min for the sham-operated animals, $n = 6$ each). Microsphere-embolized and sham-operated animals were tested in the water maze using a 3 trials/day regimen. To eliminate rats that could not swim due to injury following microsphere

embolism, we performed the habituation study with the rats in the pool with a diameter of 100 cm on day 6 after the microsphere embolism. In the present study, no microsphere-embolized rats were eliminated due to a failure in swimming. The water maze apparatus (model TARGET/2, Neuroscience, Tokyo) consisted of a circular pool with a diameter of 170 cm, which contained water with 30-cm depth and a temperature of 23 ± 1 °C. A clear acrylic platform circle with a diameter of 12 cm was placed 1.5 cm below the surface of the water and kept in a constant position in the center of one of the four quadrants of the pool. The pool was surrounded by several cues such as circles, triangles and squares drawn on the shield fence. When the rat mounted the platform, it was kept there for 30 s. If the rat did not reach the platform, it was transferred onto the platform by hand. Measurements were automated by an on-line video-tracking device designed to track the object in the field, that is, a white rat moving above the black bottom of the pool. Tracking was achieved by the system consisting of a monochrome video camera with a 4.8-mm wide-angle lens mounted approximately 170 cm directly over the center of the pool. The tracker digitized coordinate values were sampled in turn using a PC-9801 computer. Escape latency, i.e., the time to climb onto the platform, was recorded for each trial with a behavioral tracing analyzer (BAT-2, Neuroscience). The cut-off time for each trial was set at 180 s. The mean latency of each of the three trials on each day for finding the hidden platform was recorded. After the third trial of day 10, the visible platform test was performed to examine the ability of the operated animals to navigate spatially.

2.5. Acetylcholine, choline and GABA content

In the second series of experiments, the acetylcholine and choline contents of the brain regions were determined according to the method described previously (Taguchi et al., 1993). The animals were sacrificed with focal microwave irradiation to the head for 0.85 s using a microwave applicator at 5.0 kW (model TMW-6402, Muromachi Kikai, Tokyo). After decapitation, the head of the animal was immersed into liquid nitrogen and left for 10 s (near-freezing). The cerebral cortex and hippocampus were isolated in the cold room at 4 °C. Each region was homogenized in 0.2 N $HClO_4$ and 0.01% of disodium ethylenediaminetetraacetate (EDTA-2Na) with a Polytron homogenizer (model PT-10, Kinematica, Lucerne, Switzerland). The extracts contained 1 μM of ethylhomocholine as an internal standard for detection of acetylcholine and choline. After being left for 10 min at 0 °C, the homogenate was centrifuged at $10,000 \times g$ for 15 min at 4 °C. A part of the supernatant fluid was separated as a sample for determination of GABA. The resultant supernatant fluid was neutralized with 2.5 M K_2CO_3. Then the resultant solution was centrifuged at $10,000 \times g$ for 15 min at 4 °C. The supernatant fluid was filtered through a membrane filter (0.45 μm), and the filtrate was sampled for determination of acetylcholine and choline.

Tissue acetylcholine and choline contents were determined by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD) (model ECD-100, Eicom, Kyoto). The extract was applied to a column for acetylcholine determination (Eicompak AC-gel + AC-enzymepak, Eicom). The eluate was detected at 450 mV by the electrochemical detector.

GABA content was determined by the HPLC-ECD method. The pH of the sample was adjusted to pH 9.5. The sample was reacted with 4 mM *o*-phthalaldehyde in 0.1 M sodium carbonate buffer (pH 9.5) for 2.5 min at 25 °C. The resulting derivatives were applied to the HPLC-ECD at 30 °C through a column (MA-5 ODS, Eicom) with an elution buffer of 0.0005% EDTA-2Na/0.05 M sodium phosphate buffer, pH 3.5, and methanol (1:1).

2.6. Choline acetyltransferase activity

In another set of experiments, the choline acetyltransferase and glutamic acid decarboxylase activities and glutamic acid decarboxylase proteins were determined. Rats were decapitated and their heads were near-frozen by liquid nitrogen for 10 s. The cerebral cortex and hippocampus were isolated from the hemispheres. The samples were stored at − 84 °C until assayed. The frozen tissues were homogenized with 150 mM potassium phosphate buffer, pH 6.8. Then the resulting homogenates were centrifuged at $10,000 \times g$ for 60 min. The supernatant solution was employed for determination of choline acetyltransferase activity according to a modified method of Kaneda and Nagatsu (1985). The samples were incubated at 37 °C for 20 min in 0.1 mM potassium phosphate buffer containing 10 mM choline chloride, 0.4 mM acetyl-CoA, 0.2 mM eserine sulfate, 300 mM NaCl and 20 mM EDTA-2Na. The reaction was stopped by the addition of 1 M perchloric acid. The sample containing 25 μl of 60 μM isopropylhomocholine was applied to the HPLC-ECD method as described above for determination of acetylcholine.

2.7. Glutamic acid decarboxylase activity

Glutamic acid decarboxylase activity was determined according to a modified method of Romero et al. (1998). The sample that had been obtained as above was incubated at 37 °C for 30 min in 150 mM buffer containing 32 mM L-glutamate and 1.5 mM pyridoxal-5′-phosphate. The reaction was stopped by the addition of 0.4 N perchloric acid containing 0.4 mM sodium disulfide and 0.90 mM EDTA-2Na. After centrifugation at $15,000 \times g$ for 3 min at 4 °C, the pH of the supernatant fluid was adjusted to 9.5 using 0.1 M sodium carbonate. The sample was reacted for 2.5 min at 25 °C with 4 mM *o*-phthalaldehyde in 0.1 M sodium carbonate buffer (pH 9.5). The resulting

derivatives were applied to the HPLC at 30 °C through a column (MA-5 ODS, Eicom) with an elution buffer of 0.01% EDTA-2Na/0.05 M sodium phosphate buffer, pH 3.5, and methanol (1:1).

2.8. Glutamic acid decarboxylase proteins

Glutamic acid decarboxylase protein was determined by using samples of day 5 for glutamic acid decarboxylase activity. The homogenate that had been prepared as above was solubilized by boiling for 5 min with 62.5 mM Tris–HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon PVDF, Millipore, Bedford, MA). The membrane was incubated for 2 h with the primary antibody, rabbit anti-glutamate decarboxylase 65/67 (Chemicon Int., USA) (1:4000), and then for 1 h with the secondary antibody, goat peroxidase-labeled rabbit anti-antibody (1:3000). Quantification of the immunoreactive bands was performed by densitometric scanning of autoradiograms using an enhanced chemiluminescence analysis system (Amersham Buckinghamshire, UK). For minimization of between-blot variability, an aliquot of pooled "control" membranes was run on one lane of every gel, and the immunolabeling was calculated relative to this standard.

2.9. Statistical analysis

The results were presented as means ± S.E.M. Statistical analysis for the water maze task was carried out using two-way repeated analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). Differences in neurochemical variables among the microsphere-embolized, nefiracetam-treated microsphere-embolized, sham-operated and nefiracetam-treated sham-operated groups were evaluated using two-way ANOVA followed by Fisher's PLSD. Differences with a probability of 5% or less were considered to be significant ($P<0.05$).

3. Results

3.1. Operation

In the present study, we used 169 rats (97 microsphere-embolized animals and 72 sham-operated animals). Among the microsphere-embolized rats, 11 animals (11%) died before all experimental protocols were completed. Seventy-one of the surviving rats (73%) showed stroke-like symptoms with a total score of 7–9 points and 15 animals (16%) showed stroke-like symptoms at less than 7 points as described in Materials and methods. Three rats with the stroke-like symptoms (two microsphere-embolized rats and one nefiracetam-treated microsphere-embolized rat) were eliminated from the study because of a failure of the animals to reach onto the platform within 60 s in the visible platform test. There were no stroke-like symptoms or mortality in the 72 sham-operated animals. Thus, 16 microsphere-embolized and 16 sham-operated animals were used in the first series of experiments (water maze test), whereas 52 microsphere-embolized and 56 sham-operated animals were used in the second series of experiments (neurochemical examination).

3.2. Neurological deficits

Fig. 1 shows changes in neurological deficits of the microsphere-embolized and sham-operated animals with or without nefiracetam treatment up to 10 days after the operation. The initial values for the neurological deficits of the nefiracetam-treated and nefiracetam-untreated microsphere-embolized rats were 8.0 ± 0.3 and 8.1 ± 0.3, respectively ($n=8$ each). The values were gradually decreased with time after the operation. The neurological deficits almost disappeared 7 days after the operation. There were no significant differences in the neurological deficits between the nefiracetam-treated and nefiracetam-untreated microsphere-embolized rats [$F(1,14)=1.118$, $P=0.308$]. No neurological deficits were seen in the sham-operated animals regardless of treatment with or without nefiracetam. Changes in neurological deficits of the nefiracetam-treated and nefiracetam-untreated microsphere-embolized animals after the operation in the second series of experiments up to 3 and 5 days after the operation were similar to those in the first series of experiments (data not shown).

3.3. The first series of experiments

3.3.1. Water maze task

The water maze test was performed on days 7–10 after the operation (Fig. 2). The escape latency of the microsphere-embolized rat was markedly prolonged compared

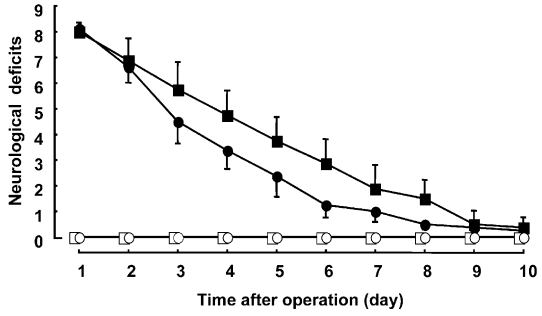

Fig. 1. The time course of changes in neurological deficits of the nefiracetam-treated (■) and nefiracetam-untreated (●) microsphere-embolized rats, and the nefiracetam-treated (□) and nefiracetam-untreated (○) sham-operated rats. Each value represents the mean ± S.E.M. of eight animals. The neurological deficits were scored by the criteria described in Materials and methods. No significant differences in the neurological deficits between the nefiracetam-treated and nefiracetam-untreated microsphere-embolized animals were seen ($P>0.05$).

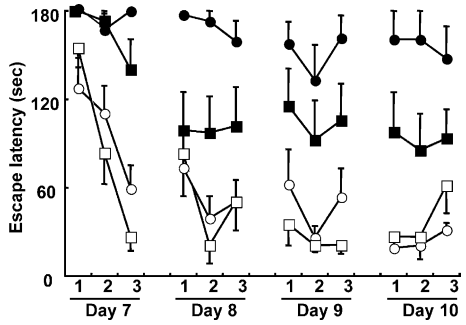

Fig. 2. Changes in the escape latency of the nefiracetam-treated (□) and nefiracetam-untreated sham-operated rats (○), and nefiracetam-treated (■) and nefiracetam-untreated microsphere-embolized rats (●). Each value represents the mean ± S.E.M. of eight animals. The water maze test was performed on days 7–10 after the operation.

with the sham-operated rat [$F(3,28)=24.4$, $P<0.0001$]. There were significant differences in the escape latency from the first trial of day 7 to the third trial of day 10 between sham-operated and microsphere-embolized animals [post hoc Fisher's PLSD $P<0.05$] and also those at the first to third trials of day 8, the third trial of day 9, and all trials of day 10 between the nefiracetam-treated and nefiracetam-untreated microsphere-embolized rats [post hoc Fisher's PLSD $P<0.05$]. No significant difference in the escape latency between the nefiracetam-treated and nefiracetam-untreated sham-operated animals was seen [post hoc Fisher's PLSD $P=0.7394$]. There were no significant differences in the swimming speed among these four groups (Table 1).

3.4. The second series of experiments

We examined acetylcholine and GABA metabolism after microsphere embolism. Changes in the neurochemical variables in the left cerebral cortex and hippocampus are not described in the text, because most of the neurochemical variables in the left hemisphere examined in the present study were unaltered by microsphere embolism or their changes, if any, were negligible.

Table 1
Swimming speed in the water maze task of the sham-operated and microsphere-embolized animals with and without nefiracetam treatment

Day	S	SN	ME	MN
7	22.3 ± 0.9	22.7 ± 2.0	28.8 ± 1.1	24.8 ± 1.1
8	24.5 ± 1.2	25.5 ± 0.7	31.7 ± 0.1	25.8 ± 0.3
9	25.8 ± 1.5	24.7 ± 1.6	30.1 ± 0.2	27.4 ± 0.4
10	28.0 ± 2.5	28.2 ± 2.3	29.4 ± 0.4	28.9 ± 0.4

Each value (cm/s) represents the mean ± S.E.M. of nefiracetam-untreated (S) and -treated (SN) sham-operated animals and of the nefiracetam-untreated (ME) and -treated (MN) microsphere-embolized animals ($n=8$ each). Swimming speed was recorded for each trial at 7–10 days after the operation. There were no significant differences in the swimming speed in the water maze task among these groups.

Table 2
Changes in the acetylcholine and choline contents of the right cerebral cortex and hippocampus of the sham-operated and microsphere-embolized rats with and without nefiracetam treatment on days 3 and 5 after the operation

	S	SN	ME	MN
Acetylcholine				
Day 3				
Cortex	15.1 ± 1.9	15.0 ± 1.1	7.4 ± 0.9[a]	8.7 ± 0.8
Hippocampus	25.0 ± 2.8	24.5 ± 1.9	10.1 ± 1.4[a]	11.8 ± 1.5
Day 5				
Cortex	15.0 ± 0.3	16.7 ± 1.5	10.0 ± 1.1[a]	8.5 ± 1.3
Hippocampus	27.2 ± 0.9	26.1 ± 1.2	11.6 ± 1.8[a]	15.4 ± 2.8
Choline				
Day 3				
Cortex	25.5 ± 3.4	22.1 ± 3.5	36.7 ± 5.6[a]	46.3 ± 5.8
Hippocampus	46.8 ± 4.4	39.9 ± 5.9	44.4 ± 3.6	44.8 ± 4.4
Day 5				
Cortex	29.2 ± 3.8	28.4 ± 4.1	36.1 ± 6.1	44.2 ± 6.6
Hippocampus	50.0 ± 3.8	51.6 ± 5.0	49.6 ± 4.7	50.0 ± 4.6

Each value (nmol/g frozen tissue) represents the mean ± S.E.M. of nefiracetam-untreated sham-operated (S, $n=5$ for day 3 and $n=7$ for day 5), nefiracetam-treated sham-operated (SN, $n=5$ for day 3 and $n=7$ for day 5), nefiracetam-untreated microsphere-embolized (ME, n = 6) and nefiracetam-treated microsphere-embolized (MN, $n=6$) groups. No appreciable improvement was seen by the delayed treatment with nefiracetam.

[a] Significantly different from the corresponding sham-operated groups ($P<0.05$).

3.4.1. Acetylcholine and choline content

Table 2 shows the changes in the acetylcholine and choline contents in the cerebral cortex and hippocampus of the right hemispheres of the microsphere-embolized and sham-operated animals. The acetylcholine content in the right cerebral cortex and hippocampus of the microsphere-embolized rats significantly decreased on day 3 [cortex: $F(3,18)=14.2$, $P<0.0001$, post hoc Fisher's PLSD

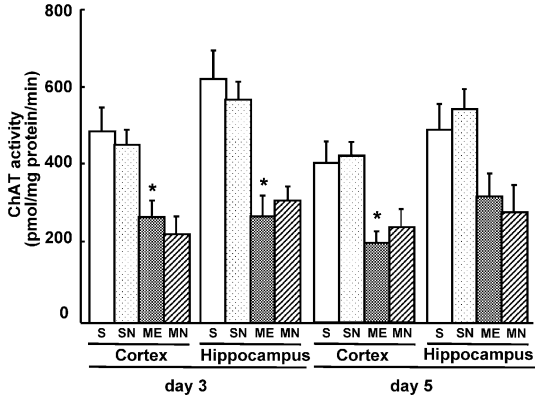

Fig. 3. The choline acetyltransferase activity in the right cerebral cortex and hippocampus of the nefiracetam-untreated (S) and nefiracetam-treated (SN) sham-operated groups, and the nefiracetam-untreated (ME) and nefiracetam-treated (MN) microsphere-embolized groups on days 3 and 5. Each value represents the mean ± S.E.M. of six experiments. *Significantly different from the nefiracetam-untreated sham-operated group ($P<0.05$).

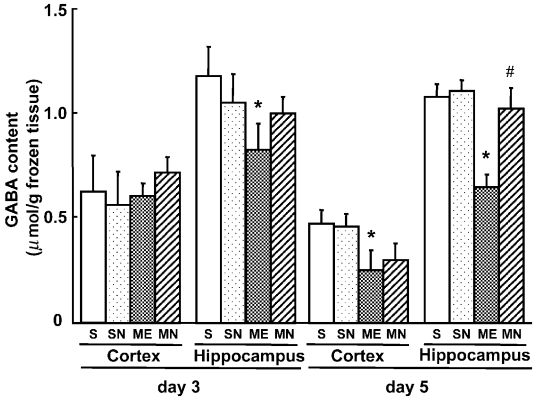

Fig. 4. The GABA content in the right cerebral cortex and hippocampus of the nefiracetam-untreated (S) and nefiracetam-treated (SN) sham-operated groups, and the nefiracetam-untreated (ME) and nefiracetam-treated (MN) microsphere-embolized groups on days 3 and 5. Each value represents the mean ± S.E.M. of seven (sham-operated group) and six (microsphere-embolilzed group) experiments. *Significantly different from the nefiracetam-untreated sham-operated group and #significantly different from the nefiracetam-untreated microsphere-embolized group ($P<0.05$).

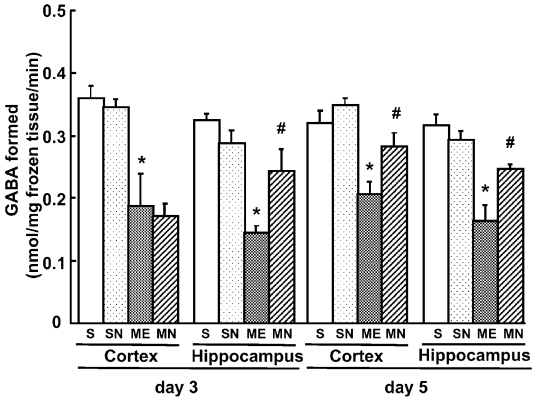

Fig. 5. The glutamic acid decarboxylase activity in the right cerebral cortex and hippocampus of the nefiracetam-untreated (S) and nefiracetam-treated (SN) sham-operated groups, and the nefiracetam-untreated (ME) and nefiracetam-treated (MN) microsphere-embolized groups on days 3 and 5. Each value represents the mean ± S.E.M. of seven experiments. *Significantly different from the nefiracetam-untreated sham-operated group and #significantly different from the nefiracetam-untreated microsphere-embolized group ($P<0.05$).

$P<0.0001$; hippocampus: $F(3,18)=22.1$, $P<0.0001$, post hoc Fisher's PLSD $P<0.0001$] and day 5 [cortex: $F(3,22)=14.0$, $P<0.0001$, post hoc Fisher's PLSD $P<0.0001$; hippocampus: $F(3,22)=24.2$, $P<0.0001$, post hoc Fisher's PLSD $P<0.0001$] as compared with that of the sham-operated animal. The delayed treatment with nefiracetam did not modify these changes in acetylcholine content.

Microsphere embolism induced a significant increase in the choline content in the ipsilateral cerebral cortex on day 3 [$F(3,18)=6.0$, $P<0.01$].

3.4.2. Choline acetyltransferase activity

Fig. 3 shows changes in choline acetyltransferase activity in the right cerebral cortex and hippocampus of microsphere-embolized and sham-operated rats. The choline acetyltransferase activity in the cerebral cortex and hippocampus was decreased on day 3 [cortex: $F(3,20)=7.3$, $P<0.01$, post hoc Fisher's PLSD $P<0.01$; hippocampus: $F(3,20)=10.7$, $P<0.001$, post hoc Fisher's PLSD $P<0.001$] and day 5 [cortex: $F(3,20)=7.2$, $P<0.01$, post hoc Fisher's PLSD $P<0.01$; hippocampus: $F(3,20)=4.8$, $P<0.05$, post hoc Fisher's PLSD $P=0.055$]. The delayed treatment with nefiracetam did not modify the decrease in choline acetyltransferase activity.

3.4.3. GABA content

GABA content of the microsphere-embolized and sham-operated animals is shown in Fig. 4. On day 3 after the operation, GABA content in the right hippocampus of the

Table 3
Changes in GABA content of the cerebral cortex, striatum and hippocampus of the right hemisphere of the sham-operated and microsphere-embolized rats with and without nefiracetam-treatment on days 3 and 5 after the operation

	S	SN	ME	MN
Day 3				
Cortex	0.63 ± 0.17	0.56 ± 0.16	0.61 ± 0.06	0.72 ± 0.07
Striatum	1.46 ± 0.23	1.35 ± 0.24	1.06 ± 0.22	1.17 ± 0.20
Hippocampus	1.19 ± 0.14	1.06 ± 0.14	0.83 ± 0.13[a]	1.00 ± 0.08
Day 5				
Cortex	0.48 ± 0.06	0.46 ± 0.06	0.25 ± 0.17[a]	0.30 ± 0.08
Striatum	1.46 ± 0.12	1.39 ± 0.13	1.14 ± 0.17	1.23 ± 0.17
Hippocampus	1.08 ± 0.06	1.11 ± 0.05	0.65 ± 0.07[a]	1.03 ± 0.10[b]

Each value (μmol/g frozen tissue) represents the mean ± S.E.M. of nefiracetam-untreated sham-operated (S, $n=7$), nefiracetam-treated sham-operated (SN, $n=7$), nefiracetam-untreated microsphere-embolized (ME, $n=6$) and nefiracetam-treated microsphere-embolized (MN, $n=6$) groups.
[a] Significantly different from sham-operated group ($P<0.05$).
[b] Significantly different from microsphere-embolized group ($P<0.05$).

Table 4
Changes in glutamic acid decarboxylase activity of the right cerebral cortex, striatum and hippocampus of the sham-operated and microsphere-embolized rats with and without nefiracetam treatment on days 3 and 5 after the operation

	S	SN	ME	MN
Day 3				
Cortex	0.36 ± 0.02	0.35 ± 0.01	0.19 ± 0.05[a]	0.17 ± 0.02
Striatum	0.35 ± 0.02	0.33 ± 0.02	0.21 ± 0.02[a]	0.15 ± 0.03
Hippocampus	0.32 ± 0.01	0.29 ± 0.02	0.15 ± 0.01[a]	0.24 ± 0.03[b]
Day 5				
Cortex	0.32 ± 0.02	0.35 ± 0.01	0.20 ± 0.02[a]	0.28 ± 0.02[b]
Striatum	0.31 ± 0.01	0.34 ± 0.01	0.18 ± 0.03[a]	0.29 ± 0.03[b]
Hippocampus	0.32 ± 0.02	0.29 ± 0.01	0.16 ± 0.02[a]	0.25 ± 0.01[b]

Each value (nmol/mg frozen tissue/min) represents the mean ± S.E.M. of nefiracetam-untreated sham-operated (S), nefiracetam-treated sham-operated (SN), nefiracetam-untreated microsphere-embolized (ME) and nefiracetam-treated microsphere-embolized (MN) groups ($n=7$ each).
[a] Significantly different from sham-operated group ($P<0.05$).
[b] Significantly different from microsphere-embolized group ($P<0.05$).

microsphere-embolized rats was decreased. On day 5, GABA content in the right cerebral cortex and hippocampus of the microsphere-embolized animal was further decreased [cortex: $F(3,22)=3.4$, $P<0.05$, post hoc Fisher's PLSD $P<0.05$; hippocampus: $F(3,22)=8.9$, $P<0.001$, post hoc Fisher's PLSD $P<0.001$]. The delayed treatment with nefiracetam tended to restore the GABA content in the right hippocampus of the microsphere-embolized animal on day 3 and significantly reversed it on day 5 [post hoc Fisher's PLSD $P<0.001$] (Table 3).

3.4.4. Glutamic acid decarboxylase activity

Fig. 5 shows changes in glutamic acid decarboxylase activity of the microsphere-embolized rat. The glutamic acid decarboxylase activity in the right cerebral cortex and hippocampus were markedly decreased on day 3 [cortex: $F(3,23)=15.1$, $P<0.0001$, post hoc Fisher's PLSD $P<0.001$; hippocampus: $F(3,23)=12.4$, $P<0.0001$, post hoc Fisher's PLSD $P<0.0001$] and day 5 [cortex: $F(3,24)=12.0$, $P<0.0001$, post hoc Fisher's PLSD $P<0.001$; hippocampus: $F(3,24)=20.8$, $P<0.0001$, post hoc Fisher's PLSD $P<0.0001$]. The delayed treatment with nefiracetam partially reversed the decrease in the activity in the hippocampus on day 3 [post hoc Fisher's PLSD $P<0.005$] and appreciably reversed the decrease in the cerebral cortex [post hoc Fisher's PLSD $P<0.005$] and hippocampus [post hoc Fisher's PLSD $P<0.001$] on day 5 after the operation (Table 4)

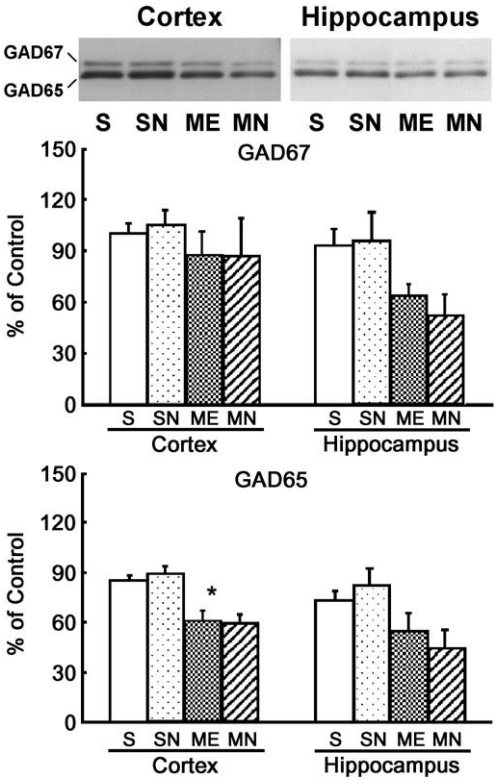

Fig. 6. The glutamic acid decarboxylase proteins, glutamic acid decarboxylase 67 (GAD67) and 65 (GAD65), in the right cerebral cortex and hippocampus of the microsphere-embolized and sham-operated rats on day 5. Representative Western immunoblots of the glutamic acid decarboxylase 67 (GAD67) and 65 (GAD65) in the right cerebral cortex and hippocampus from the nefiracetam-untreated (S) and nefiracetam-treated (SN) sham-operated groups, and the nefiracetam-untreated (ME) and nefiracetam-treated (MN) microsphere-embolized groups. Each value represents the mean ± S.E.M. of seven experiments. *Significantly different from the nefiracetam-untreated sham-operated group on day 5 after the operation ($P<0.05$).

Table 5
Changes in glutamic acid decarboxylase proteins of the cerebral cortex, striatum and hippocampus of the right hemisphere of the sham-operated and microsphere-embolized rats with and without nefiracetam treatment on day 5 after the operation

	S	SN	ME	MN
Glutamic acid decarboxylase 65				
Cortex	84.9 ± 3.0	90.0 ± 4.3	61.4 ± 5.6[a]	59.3 ± 6.0
Striatum	92.7 ± 8.4	100.7 ± 13.4	57.5 ± 17.1[a]	81.8 ± 12.2
Hippocampus	72.7 ± 6.2	82.7 ± 9.9	55.3 ± 10.1	45.2 ± 10.4
Glutamic acid decarboxylase 67				
Cortex	100.4 ± 5.8	105.5 ± 7.8	88.2 ± 13.2	87.4 ± 21.4
Striatum	94.9 ± 12.6	126.6 ± 13.9	88.9 ± 30.7	103.9 ± 18.2
Hippocampus	92.9 ± 10.0	95.0 ± 16.1	64.5 ± 6.8	53.2 ± 11.9

Each value (% of control) represents the mean ± S.E.M. of nefiracetam-untreated sham-operated (S), nefiracetam-treated sham-operated (SN), nefiracetam-untreated microsphere-embolized (ME) and nefiracetam-treated microsphere-embolized (MN) groups ($n=7$ each).
[a] Significantly different from sham-operated group ($P<0.05$).

3.4.5. Glutamic acid decarboxylase protein

Glutamic acid decarboxylase protein levels in the hippocampus were determined on day 5 after the operation. Fig. 6 shows the results of Western blot analysis of glutamic acid decarboxylase proteins, glutamic acid decarboxylase 65 and 67, of the microsphere-embolized rat. Glutamic acid decarboxylase 65 and 67 proteins in the right hippocampus of the microsphere-embolized rat were decreased [glutamic acid decarboxylase 65: $F(3,24)=3.8$, $P=0.02$; glutamic acid decarboxylase 67: $F(3,24)=3.7$, $P=0.03$]. In the right cerebral cortex, glutamic acid decarboxylase 65 of the microsphere-embolized rat on day 5 significantly decreased [$F(3,24)=12.4$, $P<0.0001$, post hoc Fisher's PLSD $P<0.005$]. No appreciable restoration of the glutamic acid decarboxylase protein content by treatment with nefiracetam was detected in the cerebral cortex or hippocampus (Table 5).

4. Discussion

Microsphere embolism employed in the present study showed long-lasting prolongation of the escape latency in the water maze task, which was consistent with the results described previously (Takagi et al., 1997; Nagakura et al., 2002). The prolongation of the escape latency appears to be specific in terms of the period and severity in the water maze task compared with four-vessel ligation/reperfusion- or

middle cerebral artery occlusion/reperfusion-induced failure in the spatial memory function (Imanishi et al., 1997; Yonemori et al., 1999). Delayed treatment with nefiracetam markedly ameliorated this prolongation of the escape latency, suggesting that this agent may have a potential to improve learning and memory function impaired by sustained cerebral ischemia. Since no improvement in the escape latency was seen in nefiracetam-treated sham-operated animals, this agent may be ineffective in the learning and memory function under normal conditions. As shown in Introduction, Nabeshima et al. (1990a,b, 1991a,b) extensively studied the improved effect of nefiracetam on drug- or agent-induced learning and memory dysfunction in the passive avoidance task of rodents. The present study demonstrated a possible therapeutic effect of this agent on memory function in ischemic animals.

Several studies have provided evidence for the possible involvement of the cholinergic system in learning and memory function (Beninger et al., 1989; Newhouse, 1990). Clinical studies also supported this idea in terms of the correlation of the acetylcholine levels, and choline acetyltransferase and cholinesterase activities with dementia of Alzheimer's disease (Bartus et al., 1982; Coyle et al., 1983) and of cerebrovascular disease (Danielsson et al., 1988). We observed a decrease in acetylcholine in the cerebral cortex and hippocampus of the ipsilateral hemisphere on days 3 and 5 after the operation. In contrast, the changes in choline content on days 3 and 5 were small. This relatively small change in choline content may be explained by the suggestion that the ischemia-induced release in choline occurs for a short period and is not due to accelerated hydrolysis of acetylcholine but due to a breakdown of the cell membrane phospholipids (Tucek, 1984). Choline acetyltransferase activity of the cerebral cortex and hippocampus of the ipsilateral hemisphere decreased after microsphere embolism. The delayed treatment with nefiracetam did not elicit any restoration of the acetylcholine content or any improvement of choline acetyltransferase activity of the microsphere-embolized animal, suggesting that this agent may not ameliorate the ischemia-induced changes in the cholinergic activity. It was reported that pretreatment with nefiracetam improved the acetylcholine content that had been impaired by scopolamine (Abe, 1991) and basal forebrain lesions (Nabeshima et al., 1991a). The apparent discrepancy in the observations on the cholinergic activity between the present and previous studies may be due to the differences in models, conditions for induction of the pathogenesis, doses of the agent, and timing of treatment. In particular, it is likely that the effects of drugs on the pathologic animal may be clearly different from those on normal animals or on drug-induced amnesic animals.

As described earlier, Nabeshima et al. (1988, 1990b, 1991b) strongly postulated the possible involvement of the GABAergic system in drug-induced amnesia in rodents. In patients with Alzheimer's disease or senile dementia, the GABA levels and glutamic acid decarboxylase activity were decreased (Davies, 1979). These observations suggest that the GABAergic system may be more or less involved in the learning and memory function. Watabe et al. (1993) reported that the repeated administration of nefiracetam to normal animals increased glutamic acid decarboxylase activity in the presence of a GABA transaminase inhibitor. This agent also enhanced the release of GABA and induced enhancement of synaptosomal uptake of GABA in the cerebrocortical slices (Watabe et al., 1993). It is also shown that nefiracetam increased the affinity to GABA receptors and increased the permeability of chloride channels (Huang et al., 1996). Considering these findings occurred under normal conditions, nefiracetam is likely to exert such effects on the GABAergic system even under the ischemic conditions. Thus, we examined changes in the GABAergic parameters of the microsphere-embolized animal. The GABA content of the hippocampus and/or cerebral cortex appreciably decreased on days 3 and 5 after microsphere embolism. The delayed treatment with nefiracetam partially reversed a change in GABA content in the hippocampus of the microsphere-embolized animal. These observations motivated us to further examine the effect of nefiracetam on the glutamic acid decarboxylase activity of the microsphere-embolized animal.

We found appreciable improvement of the glutamic acid decarboxylase activity in the cerebral cortex and hippocampus by the delayed treatment with nefiracetam, supporting that this agent may play some roles in the regulation of the GABAergic system in the ischemic brain. There are several factors regulating the glutamic acid decarboxylase activity under control conditions, including the amount of this enzyme, ATP, substrates and products. Glutamic acid decarboxylase is a sole enzyme to produce GABA, and is composed of two isoforms, glutamic acid decarboxylase 67 and 65 (Asada et al., 1997; Ji et al., 1999). We failed to demonstrate an appreciable reversal of the microsphere embolism-induced decrease in glutamic acid decarboxylase 67 and 65 proteins by the delayed treatment with nefiracetam. This suggests that the partial reversal of glutamic acid decarboxylase activity by nefiracetam appears not to be attributed to the reproduction of this enzyme. In the microsphere-embolized animal, a substantial decrease in the ATP synthesis occurred (Miyake et al., 1989). Furthermore, substances relating to the synthesis of GABA would be apparently decreased by the sustained ischemia because several amino acids and glycolytic metabolites were decreased in the ipsilateral hemisphere (Takeo et al., 1991; Taguchi et al., 1993). Determination of such substances in the microsphere embolism-induced ischemic brain would be necessary to elucidate the exact cause for changes in glutamic acid decarboxylase activity in the sustained cerebral ischemia in future studies.

In conclusion, we determined in the present study whether nefiracetam might affect the spatial learning function and the cholinergic and/or GABAergic activities in the ischemic brain. Although the agent has been shown to affect both activities under normal conditions, the GABAergic

activity may be preferentially affected by the delayed treatment with nefiracetam in the microsphere embolism-induced sustained cerebral ischemia. Reactivation of the GABAergic system may play a role in the improvement of spatial learning function in the ischemic brain.

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