



Improvement by nefiracetam of β -amyloid-(1-42)-induced learning and memory impairments in rats

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1 We have previously demonstrated that continuous i.c.v. infusion of amyloid β -peptide ($A\beta$), the major constituent of senile plaques in the brains of patients with Alzheimer's disease, results in learning and memory deficits in rats.

2 In the present study, we investigated the effects of nefiracetam [N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, DM-9384] on $A\beta$ -(1-42)-induced learning and memory deficits in rats.

3 In the $A\beta$ -(1-42)-infused rats, spontaneous alternation behaviour in a Y-maze task, spatial reference and working memory in a water maze task, and retention of passive avoidance learning were significantly impaired as compared with $A\beta$ -(40-1)-infused control rats.

4 Nefiracetam, at a dose range of 1–10 mg kg⁻¹, improved learning and memory deficits in the $A\beta$ -(1-42)-infused rats when it was administered p.o. 1 h before the behavioural tests.

5 Nefiracetam at a dose of 3 mg kg⁻¹ p.o. increased the activity of choline acetyltransferase in the hippocampus of $A\beta$ -(1-42)-infused rats.

6 Nefiracetam increased dopamine turnover in the cerebral cortex and striatum of $A\beta$ -(1-42)-infused rats, but failed to affect the noradrenaline, serotonin and 5-hydroxyindoleacetic acid content.

7 These results suggest that nefiracetam may be useful for the treatment of patients with Alzheimer's disease.

Keywords: Alzheimer's disease; amyloid β -peptide; nefiracetam (DM-9384); reference memory; voltage-sensitive calcium channels; working memory

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; $A\beta$, amyloid β -peptide; ACh, acetylcholine; AD, Alzheimer's disease; ANOVA, one-way analysis of variance; APP, β -amyloid precursor protein; ChAT, choline acetyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ -aminobutyric acid; HVA, homovanillic acid; VSCC, voltage-sensitive calcium channels

Introduction

Alzheimer's disease (AD) is the most common cause of progressive decline of cognitive function in aged humans. The disease is characterized neuropathologically by the presence of numerous senile plaques and neurofibrillary tangles accompanied by neuronal loss. The senile plaques are composed of amyloid β -peptide ($A\beta$), a 40–42 amino acid peptide fragment of the β -amyloid precursor protein (APP) (Kang *et al.*, 1987). Transgenic mice, which overexpress human APP containing the mutations associated with familial AD, develop many of the pathological characterizations associated with AD (Games *et al.*, 1995; Johnson-Wood *et al.*, 1997; Sturchler-Pierrat *et al.*, 1997). Furthermore, $A\beta$ is cytotoxic to neurons (Yankner *et al.*, 1990) and renders neurons vulnerable to various insults including excitotoxicity (Koh *et al.*, 1990; Mattson *et al.*, 1992). These previous findings suggest that $A\beta$ has a central role in the pathogenesis of AD.

We have previously demonstrated that continuous infusion of $A\beta$ -(1-40) into the cerebral ventricle of rats results in learning and memory deficits, suggesting that accumulation of $A\beta$ in the brain is related to cognitive impairments in AD (Nitta *et al.*, 1994; Nabeshima & Nitta, 1994). Continuous $A\beta$ -(1-40) infusion causes a decrease in choline acetyltransferase

(ChAT) activity in the cerebral cortex and hippocampus (Nitta *et al.*, 1994), changes in ciliary neurotrophic factor levels (Yamada *et al.*, 1995a), activation of glial cells as shown by an increase in glial fibrillary acidic protein immunoreactivity (Nitta *et al.*, 1997), and a deficiency of long-term potentiation in the CA1 field of the hippocampus (Nabeshima & Itoh, 1997). Repeated administration of propentofylline, which stimulates nerve growth factor synthesis/secretion in quiescent astroglial cells (Shinoda *et al.*, 1990), improves $A\beta$ -(1-40)-induced learning and memory deficits (Yamada *et al.*, 1998). We have also demonstrated that NC-1900, an active fragment analogue of arginine vasopressin, improves cognitive dysfunction in this non-transgenic animal model of AD (Tanaka *et al.*, 1998). Although the mechanisms of the brain dysfunction caused by accumulation of $A\beta$ in the brain are obscure, dysfunction of cholinergic and dopaminergic neuronal systems may be responsible, since *in vivo* brain microdialysis revealed a marked reduction of nicotine- and/or KCl-induced stimulation of acetylcholine (ACh) release in the hippocampus/cerebral cortex and 3,4-dihydroxyphenylethylamine (dopamine) release occurred in the striatum without affecting the basal levels of these neurotransmitters (Itoh *et al.*, 1996).

Nefiracetam, a pyrrolidone derivative, is a novel compound developed for drug therapy against cognitive disorders in aged humans (for review see Nabeshima, 1994; Yamada & Nabeshima, 1996). This compound shows remarkable effects

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in animals on cognitive impairments caused by various drug treatments (Nabeshima *et al.*, 1990; 1991b; 1994), basal forebrain lesion (Nabeshima *et al.*, 1991a), cerebral ischemia (Tanaka *et al.*, 1992), and carbon monoxide exposure (Hiramatsu *et al.*, 1992). Electrophysiological studies have demonstrated that nefiracetam modulates the currents through L-type voltage-sensitive calcium channels (VSCC) (Yoshii & Watabe, 1994), γ -aminobutyric acid (GABA)_A receptors (Huang *et al.*, 1996), and nicotinic ACh receptors (Nishizaki *et al.*, 1998), by interacting with a cyclic AMP-dependent protein kinase and/or a Ca²⁺-dependent protein kinase C pathway. Since the ameliorating effect of nefiracetam on scopolamine-induced memory impairments is antagonized by treatment of VSCC antagonists, it is suggested that activation of VSCC is involved in the improving effect of nefiracetam on cognitive deficits (Yamada *et al.*, 1994).

In the present study, we investigated the effects of nefiracetam on A β -induced impairments of spontaneous alternation behaviour in a Y-maze task, spatial reference and working memory in a water maze task, and retention of passive avoidance learning. Furthermore, the effect of this compound on ChAT activity and monoamine metabolism were examined in the A β -treated rats. Since recent evidence suggests that A β -(1-42) plays a more important role than A β -(1-40) in the pathology of AD (Jarrett & Lansbury, 1993; Iwatsubo *et al.*, 1994), rats were continuously infused with A β -(1-42) into the cerebral ventricle. Control rats were infused with non-toxic reverse fragment A β -(40-1). We confirmed that the vehicle itself failed to induce any behavioural and neurochemical changes at this flow rate (Nitta *et al.*, 1994).

Methods

Animals

The rats used in the present study were males of the Wistar strain (7 weeks old; Charles River Japan Inc., Yokohama, Japan) weighing 250 \pm 20 g at the beginning of experiments. They were housed in groups of two or three in a temperature- and light-controlled room (23°C; 12-h light cycle starting at 09 h 00 min) and had free access to food and water, except

during the behavioural experiments. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

Rats were anaesthetized with pentobarbital (50 mg kg⁻¹) i.p., and the infusion cannula for A β was implanted into the right ventricle (A: -0.3, L: 1.2, V: 4.5), according to the atlas of Paxinos & Watson (1986). Continuous infusion of A β -(1-42) (300 pmol day⁻¹) was maintained for at least 2 weeks by attaching an infusion cannula to a mini-osmotic pump (Alzet 2002; Alza, Palo Alto, CA, U.S.A.) (Nitta *et al.*, 1994). The control rats were infused with A β -(40-1). We have confirmed that the vehicle by itself has no effect on learning behaviour at this flow rate (Nitta *et al.*, 1994; 1997).

Drug administration and experimental design

The experimental schedule is shown in Figure 1. The behavioural tests and the administration of nefiracetam were started on day 7 after the start of A β -(1-42) infusion. The behavioural tests were carried out sequentially. Nefiracetam at doses of 1, 3 and 10 mg kg⁻¹ or saline was administered orally to A β -(1-42)-infused rats for 14 consecutive days, 1 h before the behavioural test throughout the experimental period. A β -(40-1)-infused control rats were administered with saline. Each group consisted of eight to nine rats. On day 20 after the start of A β infusion, rats were killed 1 h after the last administration of nefiracetam for the measurement of ChAT activity and the contents of monoamines and their metabolites in the brain.

Measurement of locomotor activity

Locomotor activity was measured on day 7 after the start of A β infusion. The experimental apparatus consisted of a locomotor cage (25 \times 42 \times 20 cm), with photobeams placed 2 cm above the floor at 1-inch intervals along two sides of the

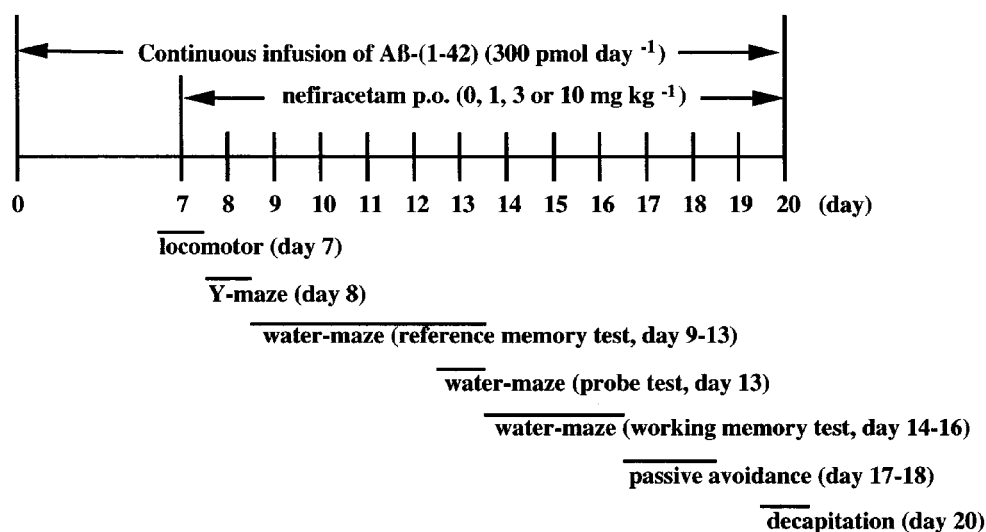


Figure 1 Experimental schedule.

cage (Colombus Instruments, U.S.A.). Locomotor activity was measured during a 10-min period (Fuji *et al.*, 1993).

Y-maze task

The Y-maze task in rats was carried out on day 8 after the start of $A\beta$ infusion, as described previously (Maurice *et al.*, 1994). The experimental apparatus consisted of a black-painted Y-maze made of plywood. Each arm of the Y-maze was 35 cm long, 25 cm high and 10 cm wide and positioned at an equal angle. Each rat was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of arm entries was recorded manually. A spontaneous alternation behaviour, which is regarded as a measure of spatial memory (Maurice *et al.*, 1994; Yamada *et al.*, 1996), was defined as entry into all three arms on consecutive choices in overlapping triplet sets. The per cent spontaneous alternation behaviour was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries $- 2$) $\times 100$.

Water maze task

The water maze task (Morris, 1984), with some modification (Tanaka *et al.*, 1998), was carried out from day 9 to 16 after the start of $A\beta$ infusion. The experimental apparatus consisted of a circular water tank (140 cm in diameter and 45 cm high). A transparent platform (10 cm in diameter and 25 cm high) was set inside the tank, which was filled, to a height of 27 cm, with water of temperature approximately 23°C; the surface of the platform was 2 cm below the surface of the water. The pool was located in a large test room, in which there were many cues external to the maze (e.g. pictures, lamps, etc.). The position of the cues remained unchanged throughout the water maze task.

Reference memory test: For each training trial, the rat was put into the pool at one of five starting positions, the sequence of the positions being selected randomly. The platform was located in a constant position throughout the test period in the middle of one quadrant, equidistant from the centre and edge of the pool. In each training session, the latency to escape onto the hidden platform was recorded. If the rat found the platform, it was allowed to remain there for 15 s and was then returned to its home cage. If the rat was unable to find the platform within 90 s, the training was terminated and a maximum score of 90 s was assigned. Training was conducted for 5 consecutive days, twice a day, from day 9 to day 13 after the start of $A\beta$ infusion.

Probe test: Immediately after the tenth training trial on day 13 after the start of $A\beta$ infusion, the platform was removed from the pool and animals were tested on a 30 s spatial probe trial. The time spent in the platform-quadrant where the platform had been located during training was measured.

Working memory (repeated acquisition) test: Working memory test was conducted for three consecutive days from day 14 to day 16 after the start of $A\beta$ infusion, and consisted of five trials (one session) per day. The working memory test was procedurally similar to standard water maze test training, except that the platform location was changed for each session. Since the platform position was changed daily, this task evaluated working memory (Frick *et al.*, 1995). For each trial, the rat was put into the pool at one of five starting positions, the sequence of the positions being selected randomly. The first trial of each session was an informative sample trial in which

the rat was allowed to swim to the platform in its new location and to remain there for 15 s. The rat was then placed in a home cage for an intertrial interval of 1 min. The platform remained in the same location throughout the remaining four trials of the day. Spatial working memory was regarded as the mean escape latency of the second to fifth trials. The ability of working memory in each rat was assessed by the mean performance for three consecutive days from day 14 to day 16.

Multiple-trial passive avoidance task

The multiple-trial passive avoidance task was carried out on day 17 and 18 after the start of $A\beta$ infusion, as described previously (Yamada *et al.*, 1996). The experimental apparatus consisted of two compartments (25 \times 15 \times 15 cm high), one illuminated and one dark, both equipped with a grid floor. The two compartments were separated by a guillotine door. In the acquisition trial, each rat was placed in the illuminated compartment; when the animal entered the dark compartment, the door was closed and an inescapable footshock (0.3 mA, 5 s) was delivered through the grid floor. The rat was removed after receiving the footshock and was placed back into the light compartment by the experimenter. The door was again opened 30 s later to start the next trial. Training continued in this manner until the rat stayed in the light compartment for 120 s on a single trial. In the retention trial, given 24 h after the acquisition test, the rat was again placed in the illuminated compartment and the time taken to enter the dark compartment was measured as step-through latency. When the rat did not enter for at least 300 s, a score of 300 s was assigned.

Measurement of ChAT activity and monoamine and metabolite content

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of $A\beta$ -(1-42) infusion. The brains were dissected into four regions, the frontal cortex, parietal cortex, striatum and the hippocampus, after which they were rapidly frozen and stored in a deep freezer at -80°C until assayed. Measurement of ChAT activity was carried out as reported previously (Kaneda & Nagatsu, 1985). The content of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and noradrenaline was determined by a high-performance liquid chromatography system with an electrochemical detector (Eicom, Kyoto, Japan), as described by Yamada *et al.* (1995b).

Drugs

$A\beta$ -(1-42) and $A\beta$ -(40-1) were obtained from Bachem (Torrance, CA, U.S.A.), and dissolved in 35% acetonitrile containing 0.1% trifluoroacetic acid at a concentration of 25 μM . Nefiracetam [N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidiny) acetamide, DM-9384] was kindly provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan), and dissolved in saline.

Statistical analysis

Results were expressed as means \pm s.e.mean. Statistical significance between $A\beta$ -(40-1)-treated control and $A\beta$ -(1-42)-treated groups was determined by Student's *t*-test for locomotor activity, number of arm entries in the Y-maze task, number of training trials in the passive avoidance task, and the

neurochemical data. Mann-Whitney *U*-test was also used for the two group comparison in spontaneous alternation behaviour in Y-maze, performance in water maze task and step-through latency in the passive avoidance task. Statistical significance in the behavioural and neurochemical effects of nefiracetam was determined by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test, followed by Bonferroni's test. Two-way ANOVA was also conducted for analysing data of the training trials of the water maze.

Results

Effects of nefiracetam on locomotor activity in the β -(1-42)-treated rats

There was no difference in locomotor activity counts on day 7 after the start of $A\beta$ infusion between $A\beta$ -(40-1)-infused control (1734 ± 21 counts/10 min, $n=8$) and the $A\beta$ -(1-42)-infused group (1708 ± 38 counts/10 min, $n=9$) ($P=0.9112$). The activity counts in the $A\beta$ -(1-42)-infused rats that received an oral administration of nefiracetam at doses of 1, 3 and 10 mg kg^{-1} prior to the measurement of locomotor activity, were 1842 ± 21 , 2089 ± 19 , and 1817 ± 37 counts/10 min, respectively. One-way ANOVA revealed that there was no statistical difference in locomotor activity counts between vehicle-treated and nefiracetam-treated $A\beta$ -(1-42)-infused rats [$F(3,30)=0.835$, $P=0.4853$].

Effects of nefiracetam on spontaneous alternation behaviour in the β -(1-42)-treated rats

As shown in Figure 2A, the spontaneous alternation behaviour in the $A\beta$ -(1-42)-infused group was significantly lower than that in the $A\beta$ -(40-1)-infused control group ($P<0.01$). There was a statistical difference in spontaneous alternation behaviour between vehicle-treated and nefiracetam-treated $A\beta$ -(1-42)-infused rats [$KW=8.395$, $P=0.0385$]. *Post-hoc* analysis revealed that nefiracetam at a dose of 10 mg kg^{-1} significantly attenuated the impairment of this behaviour induced by $A\beta$ -(1-42) ($P<0.05$). The number of arm entries did not differ between the five different treatment groups of rats (Figure 2B), indicating that changes in alternation behaviour were not due to generalized exploratory, locomotor or motivational effects.

Effects of nefiracetam on performance of the water maze task in the β -(1-42)-treated rats

Changes in escape latency time taken to find the hidden platform produced by training trials in each group of rats are shown in Figure 3A. Two-way ANOVA with all treatment groups revealed significant main effects of group [$F(4,370)=3.437$, $P=0.0089$] and training [$F(9,370)=86.354$, $P<0.0001$], but not group by trial interactions [$F(36,370)=0.9808$, $P>0.05$]. *Post hoc* analysis indicated that performance in the $A\beta$ -(1-42)-infused group was significantly impaired, compared to that in the $A\beta$ -(1-40)-infused control group ($P<0.0188$). Repeated daily administration of nefiracetam at a dose of 3 mg kg^{-1} significantly ameliorated the impairment of performance caused by $A\beta$ -(1-42) ($P<0.001$) (Figure 3A). Nefiracetam at 10 mg kg^{-1} also improved performance in the $A\beta$ -(1-42)-treated rats, but the effect was not significant ($P=0.0514$).

A 30-s spatial probe trial was carried out on day 13 after the start of $A\beta$ -(1-42) infusion, following the tenth training trial

(Figure 3B). The $A\beta$ -(1-42)-infused group, compared with the $A\beta$ -(40-1)-treated control group, showed a significant decrease in the time spent in the quadrant in which the platform had been located (platform-quadrant) during training (control group: $53.8 \pm 3.9\%$, $A\beta$ -(1-42)-infused group: $33.0 \pm 2.4\%$) (Figure 3B). There was a significant group effect on the time spent in the platform-quadrant between the vehicle- and nefiracetam-treated $A\beta$ -(1-42)-infused groups [$KW=11.098$, $P<0.0112$]. *Post-hoc* analysis revealed that all doses of nefiracetam examined in the present study significantly increased the time spent in the platform-quadrant ($P<0.01$), relative to the $A\beta$ -(40-1)-treated control group.

The escape latencies in the first trial (sample trial) and in the second to fifth trials (test trials) of the working memory test are shown in Figure 4A and B, respectively. Although there was no difference in the escape latency in the sample trial (Figure 4A), a marked difference in the test trials was observed between $A\beta$ -(40-1)-infused control and $A\beta$ -(1-42)-treated groups

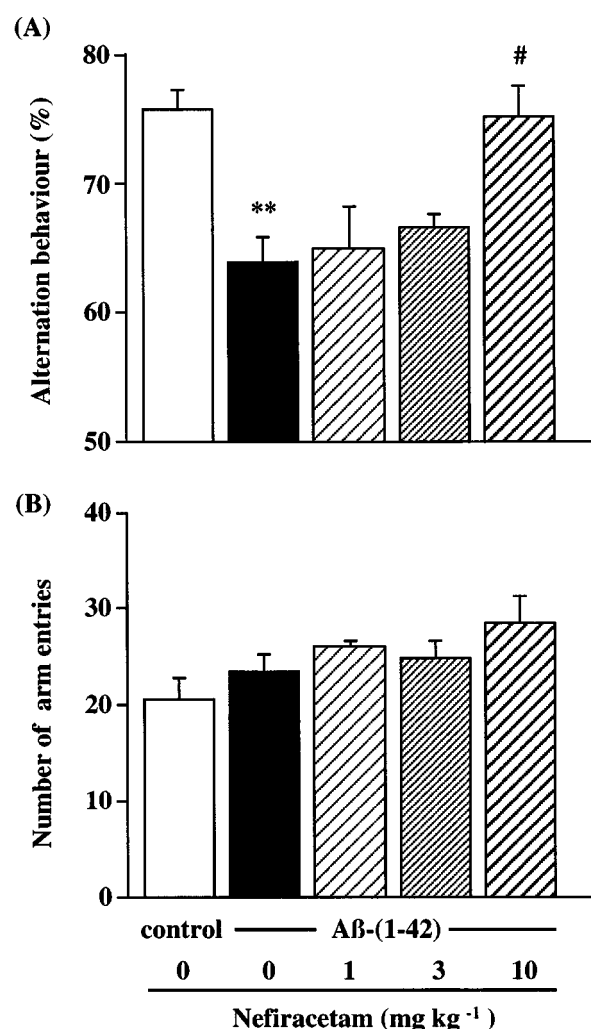


Figure 2 Effects of nefiracetam on spontaneous alternation behaviour in the $A\beta$ -(1-42)-treated rats. Spontaneous alternation behaviour (A) and the number of arm entries (B) during an 8-min session in the Y-maze task were measured on day 8 after the start of $A\beta$ infusion. Daily oral administration of nefiracetam was started on day 7, and spontaneous alternation behaviour was measured 1 h after the administration. Columns indicate means \pm s.e.mean ($n=8-9$). ** $P<0.01$ vs $A\beta$ -(40-1)-treated control rats. # $P<0.05$ vs $A\beta$ -(1-42)-treated rats.

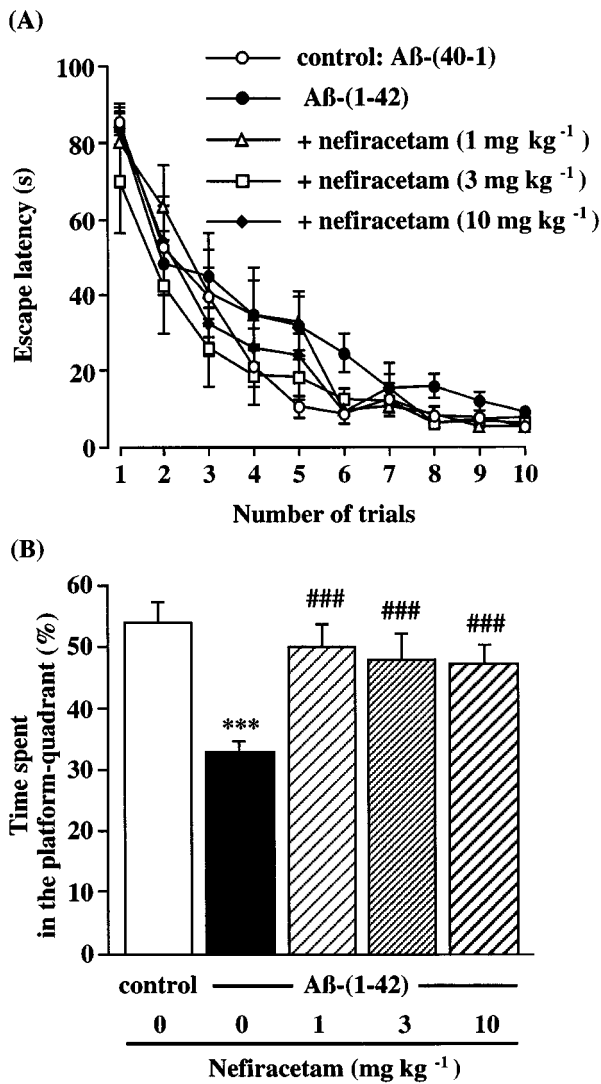


Figure 3 Effects of nefiracetam on performance in the training trials (A) and in the probe trial (B) of the water maze task in the A β -(1-42)-treated rats. The training trials were carried out on days 9–13 after the start of A β infusion. The probe trial was carried out on day 13 after the start of A β infusion, immediately after the tenth training trial. Daily oral administration of nefiracetam was started on day 7, and the training was carried out 1 h after the administration. Symbols indicate the means \pm s.e.mean ($n = 8-9$). *** $P < 0.001$ vs A β -(40-1)-treated control rats. ### $P < 0.001$ vs A β -(1-42)-treated rats.

($P < 0.001$) (Figure 4B). Nefiracetam significantly and dose-dependently ameliorated the impairment of performance in the test trials in the A β -(1-42)-infused rats ($P < 0.001$) (Figure 4B), without affecting the escape latency in the sample trial (Figure 4A).

Effects of nefiracetam on performance of the multi-trial passive avoidance task in the A β -(1-42)-treated rats

In the acquisition trial, there was no difference in the step-through latency (Figure 5A) or the number of training trials (Figure 5B) among the five treatment groups. Furthermore, there was no apparent difference in vocalization of rats in each treatment group when they received a footshock.

In the retention trial, however, a significant reduction of the step-through latency was observed in the A β -(1-42)-treated

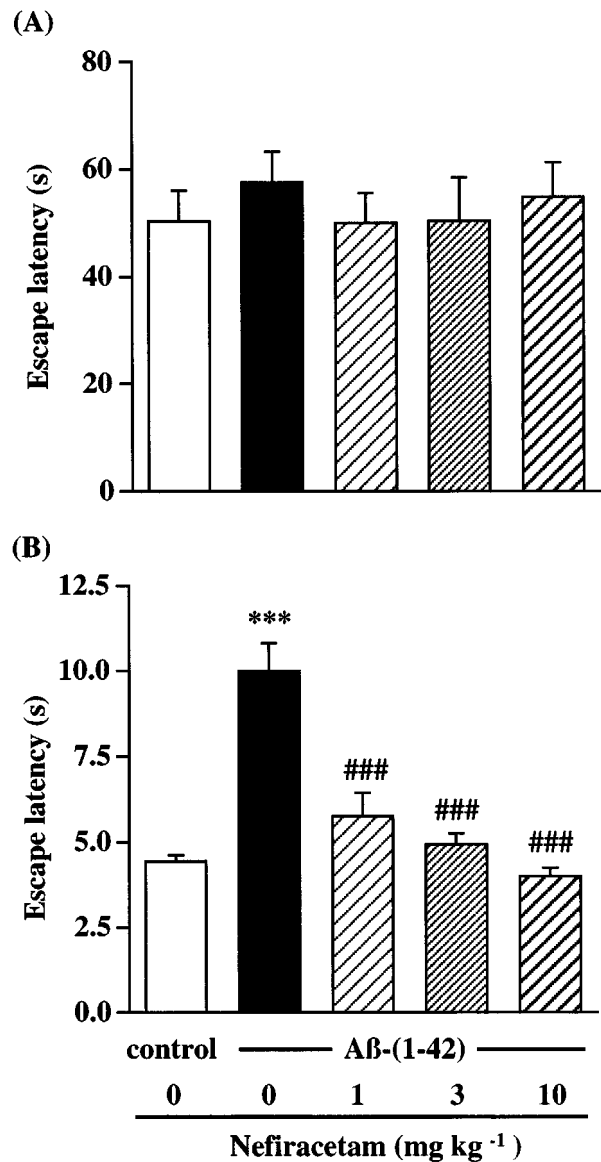


Figure 4 Effects of nefiracetam on performance in the sample trial (A) and in the test trials (B) of the working memory test of the water maze task in the A β -(1-42)-treated rats. The working memory test (five trials per day) was carried out on days 14–16 after the start of A β infusion. Daily oral administration of nefiracetam was started on day 7, and the test was carried out 1 h after the administration. Values are the means \pm s.e.mean ($n = 8-9$). *** $P < 0.001$ vs A β -(40-1)-treated control rats. ### $P < 0.001$ vs A β -(1-42)-treated rats.

groups compared with the A β -(40-1)-treated control group ($P < 0.01$). The deficits of retention of the passive avoidance learning in the A β -(1-42)-treated rats were improved by the treatment with nefiracetam at doses of 1, 3, and 10 mg kg⁻¹ [KW = 10.110, $P = 0.0177$].

Effects of nefiracetam on choline acetyltransferase activity in the A β -(1-42)-treated rats

Table 1 shows that continuous infusion of A β -(1-42) had no significant effect on ChAT activity in the hippocampus, frontal cortex or the striatum. Repeated oral administration of nefiracetam at a dose of 3 mg kg⁻¹ for 14 days caused a significant increase (25%) in ChAT activity in the hippocampus, but not in other areas of the brain such as the frontal

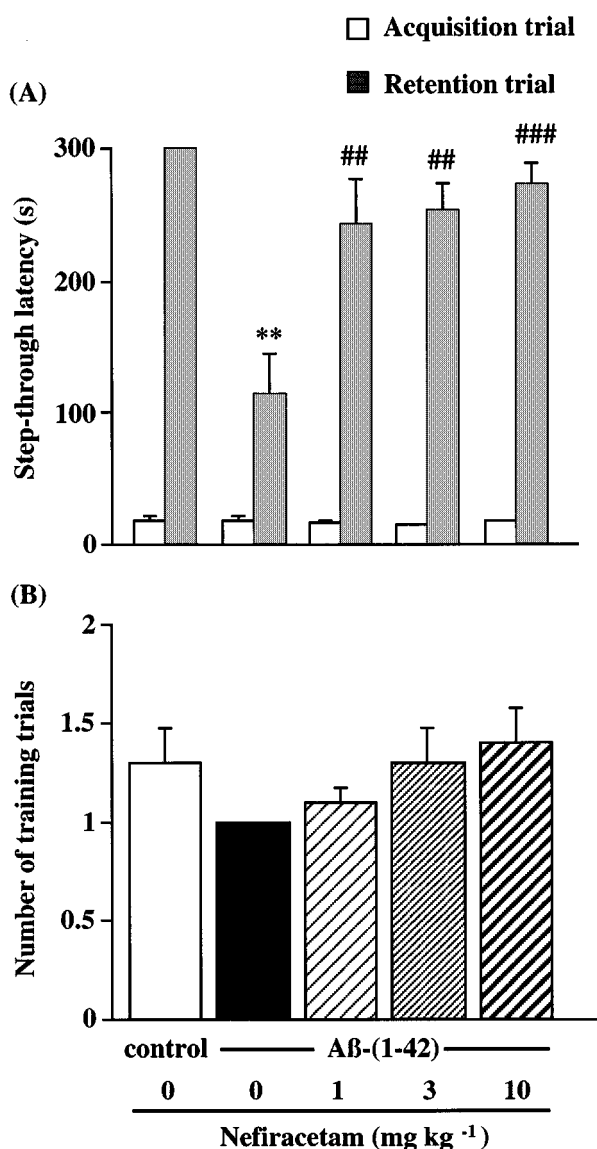


Figure 5 Effects of nefiracetam on step-through latency (A) and the number of training trials (B) in the multiple-trial passive avoidance task in the $A\beta$ -(1-42)-treated rats. The task was carried out on days 17–18 after the start of $A\beta$ infusion. Daily oral administration of nefiracetam was started on day 7, and the passive avoidance task was carried out 1 h after the administration. Values are the means \pm s.e.mean ($n=8-9$). ** $P<0.01$ vs $A\beta$ -(40-1)-treated control rats. ### $P<0.01$, #### $P<0.001$ vs $A\beta$ -(1-42)-treated rats.

cortex and striatum, of the $A\beta$ -(1-42)-treated rats. Nefiracetam, at doses of 1 and 10 mg kg⁻¹, failed to affect ChAT activity in any of the brain areas examined.

Effects of nefiracetam on monoamine and metabolite content in the $A\beta$ -(1-42)-treated rats

Table 2 shows the effects of nefiracetam on dopamine metabolism in the brains of rats continuously infused with $A\beta$ -(1-42) into the cerebral ventricle. It is evident that continuous infusion of $A\beta$ -(1-42) had no significant effect on dopamine, DOPAC and HVA levels in the frontal and parietal cortex, striatum and hippocampus (Table 2). There were also no differences in 5-HT, 5-HIAA and noradrenaline contents in

Table 1 Effects of nefiracetam on the ChAT activity in the brains of rats continuously infused with $A\beta$ -(1-42) into the cerebral ventricle

Treatment	Dose (mg kg ⁻¹)	ChAT activity (pmol min ⁻¹ mg ⁻¹ protein)		
		Hippocampus	Frontal cortex	Striatum
Control	0	1617 \pm 145	1299 \pm 96	4649 \pm 280
$A\beta$ -(1-42)	0	1571 \pm 88	1137 \pm 62	4556 \pm 254
+Nefiracetam	1	1556 \pm 123	1175 \pm 48	4955 \pm 200
	3	1978 \pm 97**	1150 \pm 36	4755 \pm 177
	10	1517 \pm 98	1229 \pm 43	4726 \pm 162

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of the $A\beta$ -(1-42) infusion. Control rats were infused with $A\beta$ -(40-1). Each value represents the means \pm s.e.mean ($n=8-9$). ** $P<0.01$ vs $A\beta$ -(1-42)-treated rats.

any of these areas of the brain between $A\beta$ -(40-1)-infused control and $A\beta$ -(1-42)-treated animals (data not shown).

Nefiracetam significantly increased dopamine content in the frontal and parietal cortex, and striatum, but not hippocampus, in the $A\beta$ -(1-42)-treated rats. The effect was most significant at the lowest dose (1 mg kg⁻¹). Nefiracetam also increased DOPAC levels in the parietal cortex, and DOPAC and HVA levels in the striatum at doses of 1 and 3 mg kg⁻¹. Furthermore, this compound increased the DOPAC:dopamine ratio in the parietal cortex, by more than 2 fold, at the dose range of 1–10 mg kg⁻¹. Nefiracetam did not affect the content of 5-HT, 5-HIAA or noradrenaline in any areas of the brain examined in the $A\beta$ -(1-42)-treated animals (data not shown).

Discussion

Previous studies, including those from our laboratory, have demonstrated that intracerebral injection of $A\beta$ causes brain dysfunction as evidenced by neurodegeneration and an impairment of learning and memory (Kowall *et al.*, 1991; Flood *et al.*, 1994; Nitta *et al.*, 1994; 1997; Giovannelli *et al.*, 1995; Maurice *et al.*, 1996; Yamada *et al.*, 1998) although neurotoxic effects of $A\beta$ *in vivo* have been somewhat controversial (Clemens & Stephenson, 1992; Games *et al.*, 1992; Winkler *et al.*, 1994). In the present study, therefore, we extensively investigated behavioural changes in $A\beta$ -(1-42)-treated rats.

We observed that continuous infusion of $A\beta$ -(1-42) into the cerebral ventricle caused an impairment of spontaneous alternation behaviour in a Y-maze and performance in a water maze task, both of which are considered to involve spatial memory. Retention of long-term passive avoidance learning was also impaired by the continuous infusion of $A\beta$ -(1-42). The feature of learning and memory impairments in rats induced by continuous infusion of $A\beta$ -(1-42) is, in general, similar to that found in rats infused with $A\beta$ -(1-40) (Nitta *et al.*, 1994; 1997; Yamada *et al.*, 1998; Tanaka *et al.*, 1998). However, ChAT activities in the brains of $A\beta$ -(1-42)-infused rats did not differ from those in the $A\beta$ -(40-1)-infused control rats whereas continuous infusion of $A\beta$ -(1-40) decreased the hippocampal ChAT activity (Nitta *et al.*, 1994; 1997; Yamada *et al.*, 1998). Therefore, there may be some difference in the neurotoxicity *in vivo* caused by $A\beta$ -(1-40) and $A\beta$ -(1-42).

It is unlikely that the impairment of performance of the $A\beta$ -(1-42)-treated rats in learning and memory tasks is due to changes in motivation or sensorimotor function since different

Table 2 Effects of nefiracetam on dopamine metabolism in the brains of rats continuously infused with A β -(1-42) into the cerebral ventricle

Treatment	Dose (mg kg ⁻¹)	Dopamine	DOPAC (ng g ⁻¹ tissue)	HVA	DOPAC/Dopamine	HVA/Dopamine
<i>Frontal cortex</i>						
Control	0	28.6 ± 6.7	20.7 ± 4.6	7.3 ± 2.0	0.741 ± 0.062	0.247 ± 0.020
A β -(1-42)	0	22.2 ± 1.4	15.2 ± 1.3	5.5 ± 0.5	0.704 ± 0.077	0.252 ± 0.023
+ Nefiracetam	1	29.7 ± 2.5*	18.1 ± 1.5	7.9 ± 1.4	0.617 ± 0.038	0.267 ± 0.040
	3	29.4 ± 1.3*	19.6 ± 1.9	5.6 ± 0.6	0.690 ± 0.091	0.195 ± 0.019
	10	23.8 ± 1.4	16.0 ± 1.1	5.1 ± 0.5	0.674 ± 0.036	0.213 ± 0.017
<i>Parietal cortex</i>						
Control	0	12.8 ± 1.1	9.4 ± 0.8	2.3 ± 0.1	0.747 ± 0.059	0.183 ± 0.011
A β -(1-42)	0	10.0 ± 0.9	9.3 ± 2.0	1.9 ± 0.1	0.915 ± 0.156	0.193 ± 0.008
+ Nefiracetam	1	15.9 ± 1.4**	8.6 ± 0.5	7.9 ± 1.7**	0.563 ± 0.044	0.474 ± 0.071**
	3	13.0 ± 1.1	10.7 ± 1.0	5.8 ± 1.0*	0.906 ± 0.153	0.435 ± 0.077*
	10	10.1 ± 0.6	9.5 ± 1.3	4.7 ± 0.3	0.998 ± 0.185	0.476 ± 0.042**
<i>Striatum</i>						
Control	0	19189 ± 1435	2035 ± 112	2508 ± 130	0.109 ± 0.006	0.135 ± 0.009
A β -(1-42)	0	18794 ± 905	1952 ± 112	2605 ± 163	0.104 ± 0.004	0.140 ± 0.008
+ Nefiracetam	1	24791 ± 941**	2571 ± 166*	3186 ± 196	0.104 ± 0.007	0.130 ± 0.009
	3	22875 ± 2010	2949 ± 192**	3432 ± 218**	0.140 ± 0.023	0.164 ± 0.029
	10	19901 ± 758	1891 ± 82	2606 ± 71	0.095 ± 0.003	0.132 ± 0.005
<i>Hippocampus</i>						
Control	0	10.2 ± 0.5	13.9 ± 1.0	4.4 ± 0.4	1.386 ± 0.120	0.428 ± 0.033
A β -(1-42)	0	9.6 ± 0.8	15.4 ± 1.5	4.3 ± 0.4	1.586 ± 0.145	0.468 ± 0.051
+ Nefiracetam	1	9.7 ± 0.6	12.6 ± 1.5	3.7 ± 0.2	1.277 ± 0.132	0.387 ± 0.028
	3	9.0 ± 1.5	12.4 ± 3.0	3.7 ± 0.5	1.330 ± 0.208	0.420 ± 0.021
	10	9.2 ± 0.7	13.2 ± 1.5	4.1 ± 0.3	1.410 ± 0.090	0.458 ± 0.036

Rats were killed 1 h after the last administration of nefiracetam on day 20 after the start of the A β -(1-42) infusion. Control rats were infused with A β -(40-1). Each value represents the mean \pm s.e.mean ($n = 8-9$). * $P < 0.05$, ** $P < 0.01$ vs A β -(1-42)-treated rats.

motivation is involved in these behavioural tasks, and different skill is required for better performance in each task. Actually, the locomotor activity and the number of total arm entries in the Y-maze task in the A β -(1-42)-treated rats did not differ from those in the A β -(40-1)-treated control rats, indicating no changes in motor function and exploratory activity. There were also no differences between the A β -(40-1)-treated control and A β -(1-42)-treated rats in the sensitivity to electric footshock in the acquisition trial of the passive avoidance task and the escape latency onto the submerged platform in the first training trial and the sample trial of working memory test in the water maze task. These results indicate no changes in shock sensitivity and swimming ability. Therefore, it is likely that impairment of performance in the A β -(1-42)-treated rats is due to learning and memory deficits.

Nefiracetam, at a dose range of 1–10 mg kg⁻¹, significantly ameliorated impairment of spontaneous alternation behaviour in the Y-maze task, performance in the water maze task, and shortened step-through latency in the retention trial of passive avoidance task in the A β -(1-42)-treated rats. This compound did not affect locomotor activity, sensitivity to electric footshock in the acquisition trial of the passive avoidance task or the escape latency onto the submerged platform in the first training trial and the sample trial of working memory test in the water maze task in the A β -(1-42)-treated rats. Therefore, it is unlikely that the observed improvement by nefiracetam of performance in various cognitive tasks is due to changes in sensorimotor function and/or motivation in the A β -(1-42)-treated rats. Rather, it is plausible that nefiracetam ameliorates learning and memory deficits caused by the continuous infusion of A β -(1-42) into the cerebral ventricle in rats.

The mechanism of learning and memory dysfunction in the A β -(1-42)-treated rats is unknown at present. We have previously demonstrated, by using *in vivo* brain dialysis, that high potassium- and nicotine-induced increase in ACh and dopamine release in the hippocampus/cerebral cortex and the striatum, respectively, is markedly impaired by the continuous infusion of A β -(1-40) although the basal levels of these neurotransmitters in the A β -(1-40)-infused rats did not differ from those in vehicle-infused control animals (Itoh *et al.*, 1996). Harkany *et al.* (1998) showed that bilateral injection of β -amyloid(Phe(SO₃H)²⁴)₂₅₋₃₅ peptide, a metabolically stable analogue of A β -(25-35), into the rat nucleus basalis magnocellularis caused a reduction of cortical acetylcholinesterase-positive projections. Maurice *et al.* (1996) demonstrated that tacrine, a cholinesterase inhibitor, and (-)-nicotine improved the A β -(25-35)-induced impairment of spontaneous alternation behaviour in a Y-maze, passive avoidance learning, and water maze learning in mice. An *in vitro* experiment has shown that A β -(25-35) activates tau protein kinase 1/glycogen synthase kinase 3 β , leading to an inactivation of pyruvate dehydrogenase, and causes an impairment of ACh synthesis without affecting ChAT activity (Hoshi *et al.*, 1996). In light of these previous findings, we consider that dysfunctions of cholinergic and dopaminergic neuronal systems are responsible, at least in part for the A β -induced learning and memory deficits.

Nefiracetam activates VSCC *in vitro* (Yoshii & Watabe, 1994) and the effect is involved in the amelioration by nefiracetam of scopolamine-induced memory impairment (Yamada *et al.*, 1994). Accordingly, it is plausible that nefiracetam ameliorates the A β -(1-42)-induced learning and

memory deficits by activating VSCC, and thereby stimulates release of ACh and dopamine in the brain. Indeed, it has been shown that nefiracetam stimulates the turnover of ACh (Abe et al., 1994; Kawajiri et al., 1994), dopamine (Abe et al., 1992; 1994) and GABA (Watabe et al., 1993), as well as ACh release in the frontal cortex (Sakurai et al., 1998). In the present study, we have demonstrated that nefiracetam can increase dopamine turnover in the cerebral cortex and striatum in the $A\beta$ -treated rats.

One might argue against our hypothesis that the amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory deficits is due to the activation of VSCC, since accumulating evidence suggests that high concentration of $A\beta$ destabilizes intracellular calcium mobilization (Fraser et al., 1997). Some studies have suggested that calcium influx through VSCC contributes to $A\beta$ -induced neurotoxicity *in vitro* and that VSCC antagonists, by reducing such influx, are able to attenuate $A\beta$ -induced neurotoxicity (Weiss et al., 1994; Abe & Kimura, 1996).

We do not argue against the calcium hypothesis of the $A\beta$ -induced neurotoxicity. However, ChAT activities in the brains of $A\beta$ -(1-42)-treated rats did not differ from those in $A\beta$ -(40-1)-treated control rats, suggesting that memory deficits in the $A\beta$ -(1-42)-treated rats are not directly associated with cholinergic neurotoxicity. Furthermore, nefiracetam did not improve nor potentiate the $A\beta$ -(1-42)-induced learning and memory impairments if daily administration of this compound began 3 days before the start of $A\beta$ -(1-42) infusion (unpublished observation). In such experiments, nefiracetam was administered p.o. 1 h after the behavioural test. Therefore, it is unlikely that the ameliorating effects of nefiracetam observed in the present study are due to its neuroprotective effect against $A\beta$ -(1-42). Rather it is possible that amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory impairments is associated with its acute memory-enhancing effect (Nabeshima, 1994).

It has been suggested that low concentrations of soluble $A\beta$ can potentially induce cholinergic and dopaminergic hypoactivity (Itoh et al., 1996) that is not dependent on concurrent neurotoxicity (for review see Auld et al., 1998). Accordingly, we speculate that learning and memory deficits observed in the $A\beta$ -(1-42)-treated rats are accompanied by hypoactivity of cholinergic and dopaminergic neurons, which is not directly related to the neurodegeneration and that nefiracetam can ameliorate learning and memory impairments by stimulating ACh (Sakurai et al., 1998) and dopamine release through the activation of VSCC.

In addition, nefiracetam modulates GABA_A (Huang et al., 1996) or nicotinic ACh receptor currents (Nishizaki et al., 1998), stimulates ChAT and glutamate decarboxylase activities (Shiotani et al., 1992; Tanaka et al., 1992; Watabe et al., 1993), and muscarinic ACh receptor binding (Nabeshima et al., 1991b). Therefore, these effects may also contribute to the amelioration by nefiracetam of $A\beta$ -(1-42)-induced learning and memory deficits. In fact, we observed an increase in the hippocampal ChAT activity in the $A\beta$ -(1-42)-treated rats which had received repeated oral administration of nefiracetam at a dose of 3 mg kg⁻¹ for 14 days although the effect was not dose-related.

In conclusion, we have demonstrated in the present study that a novel cognition enhancing drug, nefiracetam, ameliorates learning and memory deficits in rats which had previously received a continuous infusion of $A\beta$ -(1-42) into the cerebral ventricle. Our findings suggest that nefiracetam may be useful for the treatment of patients with AD and that clinical trials of nefiracetam for the treatment of AD are warranted.

We are grateful to Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) for the generous donation of nefiracetam. This study was supported, in part, by Grants-in-Aid for Science Research from the Ministry of Education, Science, and Culture of Japan (No. 07557009, 08457027, 10897005 and 97450) and by an SRF Grant for Biomedical Research.

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(Received June 22, 1998
Revised October 5, 1998
Accepted October 20, 1998)