Continuous Infusion of β-Amyloid Protein into the Rat Cerebral Ventricle Induces Learning Impairment and Neuronal and Morphological Degeneration

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ABSTRACT—To investigate the toxicity of β-amyloid protein, a component of the senile plaques in Alzheimer's disease, it was infused into the cerebral ventricle of rats for 14 days by a mini-osmotic pump. Performances in the water maze and passive avoidance tasks in β-amyloid protein-treated rats were impaired. Choline acetyltransferase activity significantly decreased in the hippocampus both immediately and 2 weeks after the cessation of the infusion. However, the learning impairment was recoverable 2 weeks after cessation of the infusion. Both immediately and 2 weeks after the cessation of the infusion, glial fibrillary acidic protein immunoreactivity increased. Furthermore, β-amyloid protein altered the staining in the nuclei of hippocampal cells for only 2 weeks after the cessation. These results suggest that β-amyloid protein produces some damage in the central nervous system in vivo.

Keywords: Amyloid protein, Alzheimer's disease, Learning and memory, Cholinergic neuron, Glial fibrillary acidic protein

Alzheimer's disease (AD) is characterized by the presence of senile plaques and neurofibrillary tangles accompanied by synaptic and neuronal loss. The core of the plaque consists of β-amyloid protein (1) and other proteins (2, 3). The extent of β-amyloid protein deposition correlates with the degree of neuronal damage, cognitive impairment and memory loss (4). Although this protein has been well characterized biochemically, its primary biological function and role in the pathogenesis of AD are unknown (5). In AD patients, learning and memory are impaired with a concomitant loss of the cholinergic marker enzyme, choline acetyltransferase (ChAT), in the cerebral cortex (6). However, direct evidence that β-amyloid protein is related to the impairment of learning and memory has not been demonstrated. We have preliminarily reported that β-amyloid protein impairs learning and memory and confirmed the accumulation of β-amyloid protein after continuous infusion into the cerebral ventricles in adult rats (7, 8). Furthermore, nicotine- and K^+-stimulated acetylcholine and/or dopamine release from the frontal cortex/hippocampus and striatum, respectively, was impaired in the β-amyloid protein-infused rats (9). In this study, we investigated whether memory impairment and neuronal dysfunction of cholinergic neurons occurred and whether glial fibrillary acidic protein (GFAP), which is one of parameters of changes in the expression of intermediate filament protein, increases after continuous infusion of β-amyloid protein into the cerebral ventricles of adult rats. In addition, we also investigated whether the animals could recover from the toxicities of β-amyloid protein on their neuronal function.

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MATERIALS AND METHODS

Animals

Male Kbl Wistar rats (Oriental Bio Service, Co., Kyoto) weighing 280-320 g at the beginning of the experiments were used. They were housed in groups of two or three in a temperature- and light-controlled room (23℃, 12-hr light cycle starting at 9:00 a.m.). The animals were treated in accordance with the institutional guidelines of Nagoya University. The rats had free access to food and water, except during the task performance.

Surgery and experimental design

The synthesized human β-amyloid protein(1−40) was obtained from Bachem (Torrance, CA, USA). The β-amyloid protein was dissolved in 35% acetonitrile/0.1% trifluoacetic acid (TFA). Continuous infusion of the β-amyloid protein (0, 3, 30, 100, 300 and 500 pmol/day) was maintained for two weeks by attaching a cannula to a modified mini-osmotic pump (Alzet 2002; Alza, Palo Alto, CA, USA) (10). The control rats were only infused with the vehicle (35% acetonitrile/0.1% TFA). We did not use reverse amyloid protein(40−1), since we had confirmed that reverse β-amyloid protein(40−1) has no effects on learning ability and ChAT activity in our preliminary experiments. Each group consisted of seven rats. The cannula was implanted into the right ventricle (A −0.3, L 1.1, V 3.6) on day1. In one group, the water maze and the passive avoidance tasks were carried out on day 9 to day 13 and on day 14 to day 15, respectively, after the start of infusion. In the other group, they were carried out on day 23 to day 27 and on day 28 to day 29, respectively. After the behavioral experiments, four rats of each group were decapitated for the ChAT assay. Three rats of each group were used for GFAP immunochemical and Nissl's stain.

Water maze task

Water maze task was carried out as reported previously (11). The animal was trained for 90 sec. When it failed to get to the hidden platform, the training was terminated and a maximum score of 90 sec was assigned. One session consisting of two training periods was given daily. Training was conducted on 4 consecutive days (four sessions of two trials each).

Step-through passive avoidance task

The step-through passive avoidance task was carried out as reported previously (12). The criterion was whether the animals remained in the light compartment for at least 300 sec. The results are expressed as the percent of animals per group that showed a step-through latency of 300 sec or more (retention %).

Measurement of ChAT activity

Measurements for ChAT activity were carried out as reported previously (12, 13).

Histochemical study

Rats were anesthetized and killed immediately after the cessation of the β-amyloid protein infusion by transaortic perfusion-fixation with cold saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed and postfixed for 12 hr in the same fixative. The brains were left in 10% sucrose in phosphate-buffered saline (PBS) for 4 hr, 15% sucrose in PBS for 4 hr, and 20% sucrose in PBS for 12 hr at 4℃. Frozen brains were cut at 20 μm with a cryostat, and the areas surrounding the ventricles were collected and stained with Nissl's stain.

To identify GFAP, the tissue samples were stained with rabbit anti-GFAP monoclonal antibody (Dako Japan, Kyoto) (1 : 40). The tissues were incubated overnight at 4℃ with the primary antibody. Immunolabeled sections were processed and developed with diaminobenzidine using a Vectastain kit (Vector Laboratories, Burlingame, CA, USA).

Statistical analyses

The data from the maze task were analyzed by a repeated-measure analysis of variance and Tukey's test. ChAT activity data were analyzed by one-way analysis of variance and Tukey's test. In the passive avoidance task, the two-tailed Fisher's Exact Probability Test was used for the statistical analysis of the differences (retention %) between the β-amyloid protein- and vehicle-treated group.

RESULTS

The accumulation of β-amyloid protein in the brain was confirmed by the results of immunohistochemical staining (7).

Characteristics of the impairment of water maze task performance induced by β-amyloid protein

The mean latencies (the time taken to escape onto the hidden platform) in each training session of the water maze task for the six groups are shown in Fig. 1A. The latency of the vehicle-treated group in the first session of training was not different from those of the β-amyloid protein-treated rats. Although the latencies of the β-amyloid protein-treated groups were only slightly shortened by repeated training, those in the vehicle-treated group were shortened rapidly [$F(5,3)=1.213$, $P<0.002$]. In the third and fourth training sessions, the latencies of the β-amyloid protein (300 pmol/day)-treated rats were significantly longer than those of the vehicle-treated group (Tukey's test, $P<0.05$). In Fig. 1B, the latencies of the β-amyloid protein-treated groups described as percent of the vehicle-treated group are shown. The latencies of β-amyloid protein (300 pmol/day)-treated rats were longer than those of vehicle-treated rats at the third and fourth training sessions, when the water maze task was performed under the infusion of β-amyloid protein. However, the degree of prolongation was less at 2 weeks after cessation of infusion compared to that under the infusion of β-amyloid protein [$F(1,3)=1.3577$, $P<0.05$]. In

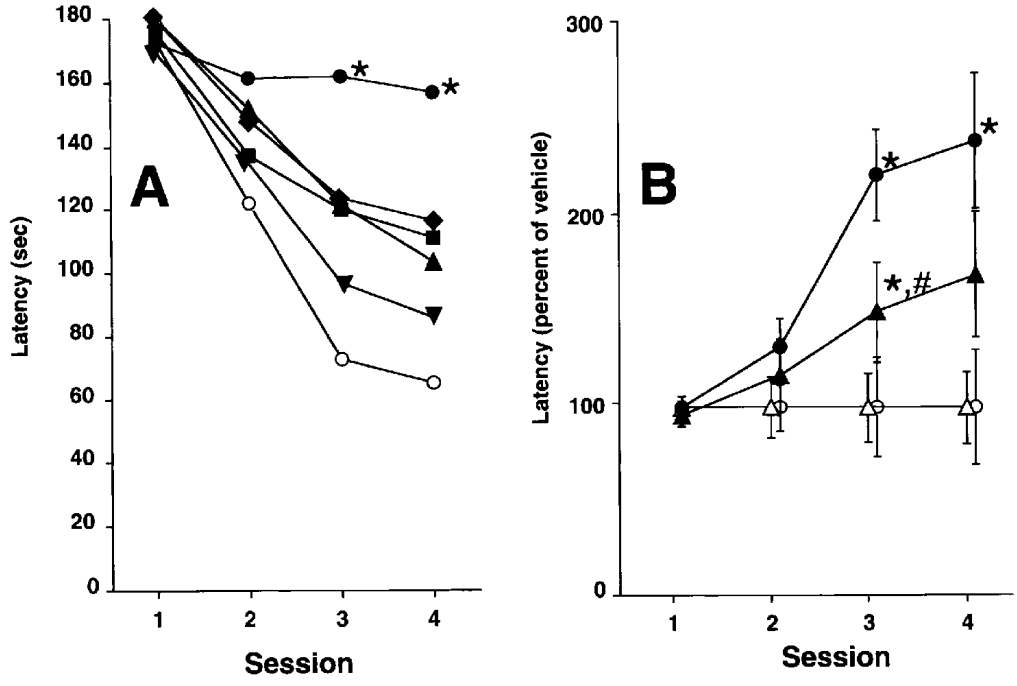

Fig. 1. Characteristics of impairment of the performance in the water maze task induced by β-amyloid protein. The task was carried out on days 9–13 (under the infusion) and days 23–27 (2 weeks after the cessation of the infusion) after the start of the infusion of β-amyloid protein. Each group consisted of seven rats. A (latency: the value expresses the mean): ○, 0 pmol/day; ■, 3 pmol/day; ▲, 30 pmol/day; ▼, 100 pmol/day; ●, 300 pmol/day; ◆, 500 pmol/day. B (percent of vehicle: the value expresses the mean±S.E.M.): ○, 0 pmol/day (under the infusion); △, 0 pmol/day (2 weeks after the cessation); ●, 300 pmol/day (under the infusion); ▲, 300 pmol/day (2 weeks after the cessation). *$P<0.05$ vs vehicle (β-amyloid protein: 0 pmol/day)-treated rats (under the infusion) (Tukey's test). #$P<0.05$ vs 300 pmol/day under the infusion group (Tukey's test).

the third training session, the latencies were significantly different (Tukey's test, $P<0.05$).

Characteristics of the impairment of passive avoidance task performance induced by β-amyloid protein

Each value in Fig. 2 represents the percent of animals that reached the criterion of 300-sec step-through latency (retention %). As shown in Fig. 2A, the retention percent of the β-amyloid protein-treated (300 pmol/day) rats was smaller than that of the vehicle-treated rats under the infusion of the protein. However, this effect of the β-amyloid protein was not observed two weeks after the cessation of the infusion (Fig. 2B).

Effects of continuous infusion of β-amyloid protein on ChAT activity in the frontal cortex, parietal cortex, striatum and hippocampus in rats

ChAT activities in the frontal cortex, parietal cortex, striatum and hippocampus in the vehicle-treated rats were 1169.4±24.4, 718.9±45.3, 4041±691.6 and 899.7±56.9 pmol/min/mg protein, respectively. ChAT activities in the frontal cortex (3, 30 pmol/day) and hippocampus (300 pmol/day) were decreased by β-amyloid protein immediately after the cessation of the infusion (Table 1). The decreased ChAT activities in the hippocampus were also observed 14 days after the cessation of the β-amyloid protein infusion (Table 1).

Effects of continuous infusion of β-amyloid protein on the morphology of the nuclei of hippocampal cells

Figure 3 shows the results of Nissl's stain in the hippocampal area 2 weeks after the cessation of the infusion of β-amyloid protein. The nuclear morphological changes were observed in CA2 and the dentate gyrus. However, in the hippocampal area immediately after the cessation of the infusion of β-amyloid protein, cell death, neuronal tangle and/or atrophy could not be observed in any hippocampal areas such as the CA1, CA2 and dentate gyrus (data not shown).

Effects of continuous infusion of β-amyloid protein on the GFAP immunoreactivity

As shown in Fig. 4, GFAP immunoreactivity increased both immediately (4B) and 2 weeks (4D) after the β-amyloid protein infusion in the hippocampal CA1 area. However, in the CA2 area, it did not increase (data not shown).

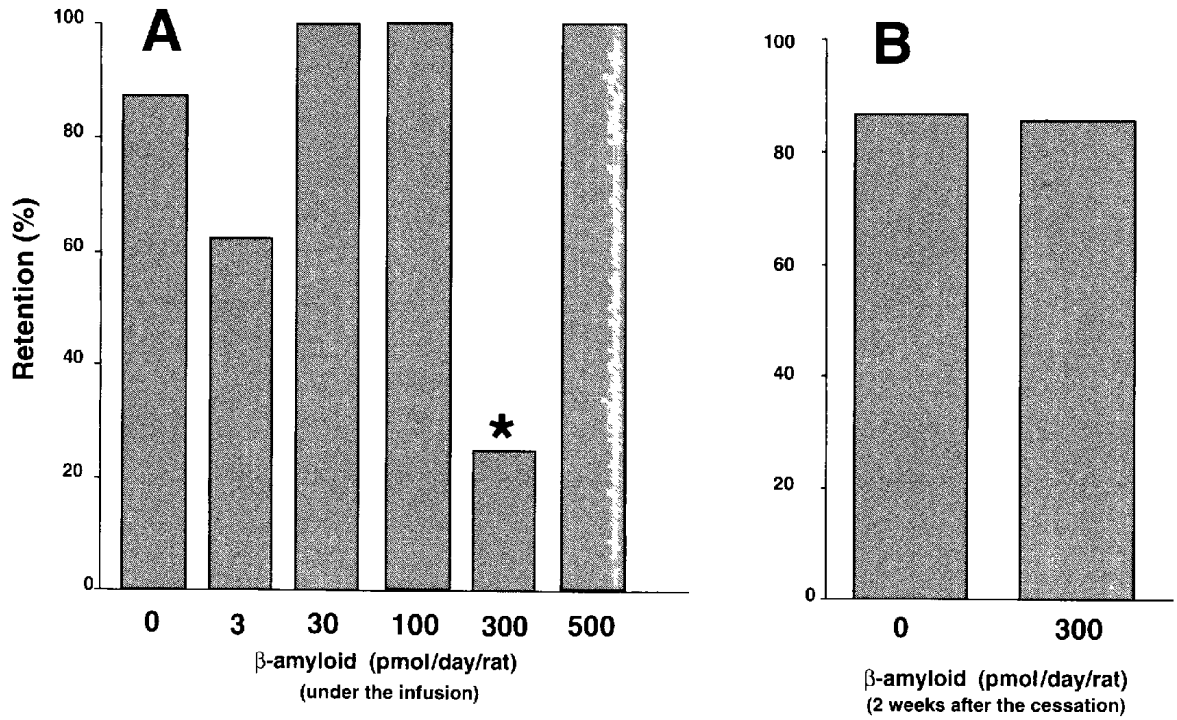

Fig. 2. Characteristics of impairment of the performance in the passive avoidance task induced by β-amyloid protein. The task was carried out on days 14–15 (under the infusion: A) and days 28–29 (2 weeks after the cessation of the infusion: B) after the start of the infusion of β-amyloid protein. Each group consisted of seven rats. The value of column expresses the percent of animals per group that showed as step-through latency of 300 sec or more. *$P<0.05$ vs vehicle (β-amyloid protein: 0 pmol/day)-treated rats (Fisher's Exact Probability Test).

Table 1. Effects of continuous infusion of β-amyloid protein on ChAT activities in the frontal cortex, parietal cortex, striatum and hippocampus in rats

Brain region	β-Amyloid protein (pmol/day)					
	0	3	30	100	300	500
ChAT activity (% of control), immediately after cessation of the infusion						
Frontal cortex	100±2.1	85.5±5.4*	81.3±7.1*	86.4±3.6	91.5±6.6	80.8±7.6
Parietal cortex	100±6.3	90.1±6.3	91.7±6.1	89.9±5.9	99.4±9.4	85.8±1.3
Striatum	100±17.1	106.6±6.3	105.6±5.1	117.1±5.5	106.8±8.5	111.4±8.0
Hippocampus	100±6.3	91.8±7.5	90.6±14.4	91.3±8.5	82.1±5.1*	91.6±7.9
ChAT activity (% of control), 2 weeks after cessation of the infusion						
Frontal cortex	100±4.6	—	—	—	97±8.8	—
Parietal cortex	100±4.7	—	—	—	110.5±5.8	—
Striatum	100±16.7	—	—	—	76.6±7.6	—
Hippocampus	100±3.6	—	—	—	83.8±11.6*	—

Each value shows the mean±S.E.M. of four animals. —: not determined. *$P<0.05$ vs vehicle (β-amyloid protein: 0 pmol/day)-treated rats (Tukey's test).

DISCUSSION

Our experimental conditions of continuous infusion of β-amyloid protein(1–40) using a mini-osmotic pump are very similar to the deposition process of β-amyloid protein in AD patients. The important findings in this study are that as in AD patients, the deposition of β-amyloid protein in the brain is related to learning disability, morphological changes and cholinergic neuronal degeneration.

In agreement with our previous results (7–9) in the β-amyloid protein-treated rats, the performance on the

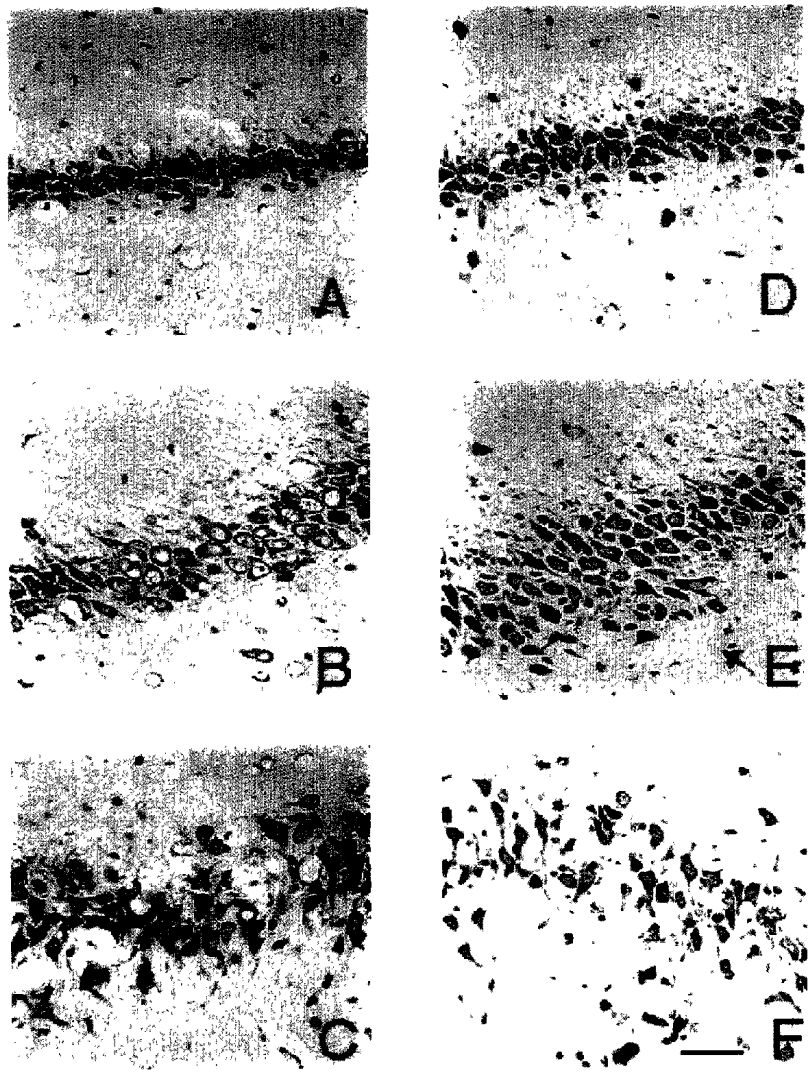

Fig. 3. The effects of continuous infusion of β-amyloid protein on the morphology of nuclei of cells in the hippocampal CA1 (A, D), CA2 (B, E) and dentate gyrus (C, F) 2 weeks after the cessation of the infusion. A, B, C: β-amyloid protein (0 pmol/day)-treated rats; D, E, F: β-amyloid protein (300 pmol/day)-treated rats. The rats were killed 2 weeks after the cessation of the β-amyloid protein infusion. Bars: 20 μm.

water maze and passive avoidance tasks was impaired and reduction of ChAT activity in the hippocampus was demonstrated. The β-amyloid protein-induced impairment of learning and memory was confirmed by the electrophysiological experiment using the hippocampal slices: Long-term potentiation in the hippocampal CA1 area of β-amyloid-infused rats was significantly inhibited (14).

These toxicities could be observed only at the dose of 300 pmol/day of β-amyloid protein but not at other doses. The phenomenon may be due to the amount of the aggregated form and the degree of penetration into neuronal cells after aggregation of β-amyloid protein, because following incubation under physiological conditions, β-amyloid protein adopts an aggregated form and shows a change in its biological effects on the hippocampal neurons in vitro from neurite-promoting to neurotoxic effects (15). These results suggest that the aged peptide has an altered, aggregated structure evidenced by the stability of the high molecular weight species and that the aggregated forms may be related to the observed toxicity. In the present study, the neuronal degeneration may be also related to the aggregated protein, because the β-amyloid protein was incubated in the cerebrospinal fluid (CSF) during the 14-day infusion. High doses of β-amyloid protein might produce a large amount of the aggregated form, but low degree of penetration. However, it is difficult to confirm this hypothesis, because mass spectroscopy and amino acid sequence analysis are essential to determine whether the incubated and non-incubated β-amyloid proteins are the same structure in the

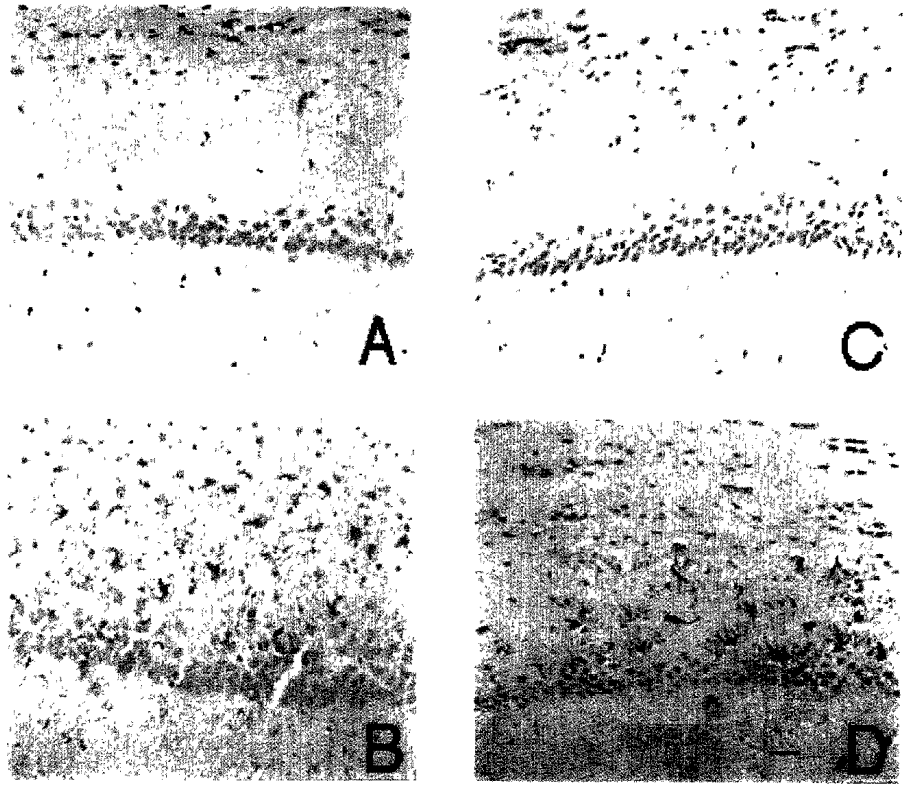

Fig. 4. The effects of continuous infusion of β-amyloid protein on the GFAP immunoreactivity immediately (A, B) and 2 weeks (C, D) after the cessation of the infusion. A, C: β-amyloid protein (0 pmol/day)-treated rats; B, D: β-amyloid protein (300 pmol/day)-treated rats. Bars: 50 μm.

CSF or deposited sites (16).

We dissolved β-amyloid protein in acetonitrile/TFA in this study. From previous solubility studies, β-amyloid protein solution at 15 mg/ml in acetonitrile/TFA (80 times more concentrated than the highest dose in the present study) does not precipitate after 24 hr at 22℃ or 37℃ (16). When β-amyloid protein is dissolved in water or PBS, β-amyloid protein is precipitated following incubation under physiological conditions as described above (16). If β-amyloid protein is precipitated in the osmotic pump, it cannot be delivered from the pump to the brain. Acetonitrile toxicity in vivo has been reported to be linked to its conversion to cyanide by microsomal cytochrome P450 (17). Cyanide toxicity, like hypoxia, is known to involve a compromised calcium homeostasis caused by a deficiency in energy metabolism (18). It is also plausible that the 35% acetonitrile may extract some components of the membranes and allow toxic calcium influx. Calcium-mediated neurotoxicity has been shown to be potentiated by β-amyloid protein (19). Acute injection of acetonitrile into the rat hippocampus produces very serious damage to the neuronal cells (16). However, neuronal damage was not observed under our experimental conditions in which the solvent containing β-amyloid protein was infused continuously (2 weeks) and slowly (0.5 μl/hr) by a mini-osmotic pump into the cerebral ventricles. In the preliminary experiments, no differences in learning ability and memory and activities of cholinergic neuronal marker enzymes such as ChAT between the acetonitrile-treated and untreated rats were demonstrated. Based upon these observations, we used acetonitrile as the solvent for β-amyloid protein. Taken together, neurotoxicities found in the present experiments may be induced by β-amyloid protein, but not by the used solvent.

GFAP immunoreactivity increased both immediately and 2 weeks after stopping the continuous infusion of β-amyloid protein. Changes in nuclear morphology in CA2 and dentate gyrus were observed 2 weeks after the cessation of the infusion. Furthermore, the decrease of ChAT activity was also observed 2 weeks after the cessation. Cholinergic neuronal dysfunction did not recover even 2 weeks after the cessation of β-amyloid protein infusion. The morphological changes were not immediately observed but could be seen 2 weeks after the cessation. Small morphological changes that we could not see by microscopy might have occurred before 2 weeks. An electron microscopy study should be done to clarify the result

of the cholinergic dysfunction immediately after the cessation of infusion.

However, the impairment of learning and memory observed during the infusion of β-amyloid protein tended to recover 2 weeks after the cessation of infusion. The reversible mechanism of β-amyloid protein toxicities is yet unknown. One possible explanation is that some other neuronal systems may compensate for the neuronal dysfunction induced by β-amyloid protein. Further study should be done on this point.

The present results suggest that the deposition of β-amyloid protein in the brain is related to learning and memory impairment, morphological and cholinergic neuronal degeneration.

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