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Effects of Intracerebroventricular Injection of AF64A on Learning Behaviors in Rats

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Abstract—The effects of intracerebroventricular (ICV) injection of ethylcholine aziridinium ion (AF64A) (3 nmole/2 μl, each lateral ventricule), a putative selective cholinotoxin, on learning behaviors and choline acetyltransferase (ChAT) activity were studied in rats. AF64A-treated rats (AF64A-rat) exhibited deficient performance in a passive avoidance task and a delayed alternation task in the T-maze, but demonstrated superior avoidance response in a two-way shuttle avoidance task. These changes in learning behaviors were associated with the selective decrease of hippocampal ChAT activity. Physostigmine (0.1 mg/kg, i.p.) significantly improved the retention latency of AF64A-rats in the passive avoidance task. AF64A-rats receiving physostigmine (0.2 mg/kg, i.p.) exhibited a slight but not significant improvement of performance in the delayed alternation task in the T-maze. These findings suggested that ICV injection of AF64A may be useful for producing an experimental amnesia model with hippocampal cholinergic hypofunction like Senile dementia of the Alzheimer type (SDAT), if appropriate learning tests are selected.

In recent years, a number of choline analogues have been suggested as potential tools in developing selective animal models with central cholinergic hypofunction (1–3). The Na^+-dependent, high affinity choline transport system (HAChT) at cholinergic nerve terminals is considered to be the rate-limiting step for the synthesis of acetylcholine (ACh) (4, 5), and interference to such a process should provide a useful means for experimental reduction of cholinergic activity (1). Reversible inhibitors of HAChT, e.g., hemicholinium-3, are expected to transiently impair cholinergic function in vivo. Several studies have shown memory retention defects in animals treated with these inhibitors (6–8). Certain mustard analogues of choline, which are irreversible inhibitors of HAChT, have been shown to cause a specific reduction of presynaptic cholinergic parameters in vivo, suggesting that cholinergic terminals degenerate as the results of treatment with these compounds (3). One of these compounds, ethylcholine aziridinium ion (AF-64A), has been shown to impair the retention of a passive avoidance task and disrupt acquisition of a radial-arm maze task in rats after ICV injection (9, 10).

In the present paper, we have investigated whether ICV injection of AF64A to rats is suitable for making an amnesia model in animals. The behavioral studies consisted of a one-trial passive avoidance task, a delayed alternation task in a T-maze and a two-way shuttle active avoidance task. Choline acetyltransferase (ChAT) activity was measured in different brain regions as an indicator of cholinergic integrity. Furthermore, effects of physostigmine, a cholinomimetic agent which has been reported to have beneficial effects on learning and memory task in amnesia-model animals (11–14) and patients of Alzheimer's disease (15), were also examined in AF64A-treated rats in a passive avoidance task and a delayed alternation task in a T-maze.

Materials and Methods

1. Materials

Acetyl-AF64 was kindly given to us by Dr. A. Fisher, Israel Institute for Biological Research, Ness-Ziona. Physostigmine sulfate was purchased from Wako Pure Chemical Industries. Anesthetic was prepared by dissolving chloral hydrate (6 g) and $MgSO_4$ (2 g) in distilled water (72 ml), and then by further addition of propylene glycol (40 ml), pentobarbital sodium solution (2.5 g/50 ml) (Nembutal, Abbott Laboratories) and 99.5% ethylalcohol (8 ml).

2. Animals and surgical procedures

Male Sprague-Dawley rats (200–250 g, Charles River Japan) were housed individually in a temperature and light controlled room, and they were allowed free access to food and water. For the surgical procedures, rats were anesthesized with the above anesthetic (0.3 ml/100 g, i.p.) and placed into a stereotaxic apparatus. Following a midline incision, holes were drilled in the skull overlaying the lateral ventricles. Coordinates for the injection were 0.8 mm posterior to the bregma, ±1.5 mm lateral to the midline and 4.8 mm ventral to the skull surface. The incisor bar of the stereotaxic instrument was set at 4.0 mm below the interaural line. Injections of 2 μl of AF64A (3 nmole) were given at each lateral ventricle over an 8-min period, and the needle (30 guage, stainless steel) was left in place for 4 min to allow diffusion of AF64A. For sham-operated rats, 2 μl of physiological saline solution was injected in the same way as for AF64A treatment.

3. AF64A preparation

AF64A was freshly prepared from acetyl-AF64 by the method of Mantione et al. (2). Briefly, acetyl-AF64 (16 mg) was rapidly dissolved in 6.4 ml distilled water under constant stirring. The pH was then adjusted and maintained between 10.5 and 11.0 by addition of NaOH solution as needed. The reaction was allowed to proceed for approximately 25 min, until no rapid drop of pH was observed. After termination of the reaction, HCl solution was added to reduce the pH once to 5.5–7.0 and then the final pH was adjusted to 7.4 by NaOH solution. Distilled water was added to bring the final concentration of AF64A to 10.0 mM. This stock solution was diluted with physiological saline solution to 1.5 mM to prepare the injection solution. The injection solution was kept on ice until used, usually within 7 hr.

4. Behavioral experiments

General behavior: The electric shock sensitivity in rats was evaluated by the same apparatus as used for the passive avoidance task 3 weeks after surgery. The shocks in duration of 3 sec were delivered at 10 different intensity levels between 12.5 and 125 volts. Flinch, vocalization and jumping responses were recorded at each shock level. Spontaneous motor activity before and after the surgery was measured by an animal activity monitoring apparatus (MK-ANIMEX, Muromachi Kikai Co.). Each rat was placed into an individual plastic cage, which was located directly over the activity monitor, and the activity was automatically measured during 23 hr (12 hr in light and 11 hr in dark period) every 3 days.

One-trial passive avoidance task: Memory retention deficit was evaluated by a step-through passive avoidance apparatus (SFK-1; O'hara & Co., Ltd.) 19–21 days after surgery. On the acquisition trial, each rat was placed in the light chamber. After a 60-sec habituation period, the guillotine door separating the light and the dark chambers was opened, and the initial latency time to enter the dark chamber was recorded. Immediately after the rat entered the dark chamber, the guillotine door was closed and electric foot shock (75 V, 0.2 mA, 50 HzAC) was delivered to the floor grids for 3 sec. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. Rats that had an initial latency time of more than 60 sec were excluded from the further experiments. Twenty-four hours later, retention latency time was measured in the same way as in the acquisition trial, but foot shock was not delivered, and the latency time was recorded to a maximum of 600 sec.

When the effects of physostigmine were examined, physostigmine solution (0.1 mg/kg) or physiological saline solution (vehicle) was intraperitoneally administered to rats immediately after the acquisition trial. The

retention trial was performed 24 hr later.

Delayed alternation task in the T-maze: The T-maze was made of white plastic board. The start arm was 30 cm long, 12 cm wide and 20 cm high, and both of the two goal arms were 60 cm long, 12 cm wide and 20 cm high. A guillotine door was located 55 cm from the edge in each of goal arm, and a 4-cm diameter plastic food cup was put at the edge of each goal arm. Rats were subjected to this task 5 weeks after surgery. Prior to the behavioral training, 1 week was allowed for stabilization of the rat's body weight at approximately 85% the free-feeding level and habituation for the maze. On the first training week, a guillotine door was introduced, which could block off either side arm at the choice point. The rats received 6 trainings daily for 5 days and were forced to alternate between two arms on the successive trials, so as to ensure that all rats entered both arms of the maze. On the second training week, the rats received 6 trials daily in an alternation task with 5-min intertrial interval for 5 days. Each trial consisted of 2 runnings (the first is information running and the second is choice running). On the information running, one side arm was blocked at the choice point by a guillotine door, so that the rats were forced to choose the single open arm, which was baited with 2 pellets. Immediately after consumption of the pellets, the guillotine door was removed and the rats were replaced at the start arm for the choice running. At this time, the rats were permitted to choose between the two arms. However, the just-forced arm was empty, whereas the other side arm was baited with 2 pellets, such that the rats had to alternate the just-forced response for the correct choice. The rats which achieved the criterion, i.e., more than 4 correct choices out of 6 trials in 5 training days, were used for a delayed alternation task in which a delay time (10, 30, 90 and 270 sec) was introduced between the information running and the choice running. Each rat received 2 trials daily on left and right information running at each delay time for 4 days.

To examine the effects of physostigmine, each rat received 6 trials of testing on the delayed alternation task in the T-maze with 5-min intertrial interval. The delayed time (60 sec) was introduced between the information running and the choice running. Physostigmine (0.2 mg/kg, i.p.) or vehicle was administered to the rat 15 min before the first trial of the testing.

Active avoidance task: The active avoidance task was evaluated by a two-way shuttle avoidance apparatus (MSA-100; Muromachi Kikai Co.) 5 weeks after rats received surgery. The conditioned stimulus (CS) consisted of the illumination of a 6 W light and 50 dB tone, presented for 3 sec, in the compartment occupied by a rat. If the rat did not run to the other compartment within 3 sec after the onset of the CS, the unconditioned stimulus (UCS), 2.0 mA foot shock, was delivered to the floor grids for 3 sec. The intertrial interval (ITI) was 24 sec. A correct response was recorded if the rat ran to the other compartment during the onset of the CS. Each rat was subjected to 4 sessions with 120 trials per session every 2 days.

5. ChAT activity

Rats were sacrificed by decapitation 5 weeks after surgery. ChAT activity was assayed in tissue homogenates of the frontal cortex, hippocampus and striatum by the method of Fonnum (16).

Results

1. Behavioral experiments

General behaviors: Approximately 10% of the AF64A-treated rats (AF64A-rat) died within 5 days after surgery. On the contrary, all sham-operated rats (sham-rat) survived. Although some AF64A-rats showed hypersensitivity to external stimuli for the first week after surgery, this sign became gradually weaker and disappeared in the second week. In terms of electric shock sensitivity, there was no significant difference between AF64-rats and sham-rats in each response threshold (Table 1). With respect to the spontaneous motor activity, both AF64A-rats and sham-rats were more active during the dark period than during the light period. During the dark period, AF64A-rats showed slightly higher activity than sham-rats 6–10 days after surgery, but the differences were not statistically significant. Since apparent hyperactivity in AF64A-rats disappeared within 15

Table 1. Shock sensitivity in rats responding to foot-shock 3 weeks after ICV injection of AF64A

	Response threshold (Voltage)	
	sham-rats (N=13)	AF64A-rats (N=15)
Flinch	36.5±1.7	33.3±2.9
Vocalization	46.2±2.6	46.5±4.0
Jump	74.0±3.6	76.7±4.2

Values represent the mean±S.E.M. No significant differences were observed according to Student's *t*-test between sham-treated control-rats and AF64A-rats.

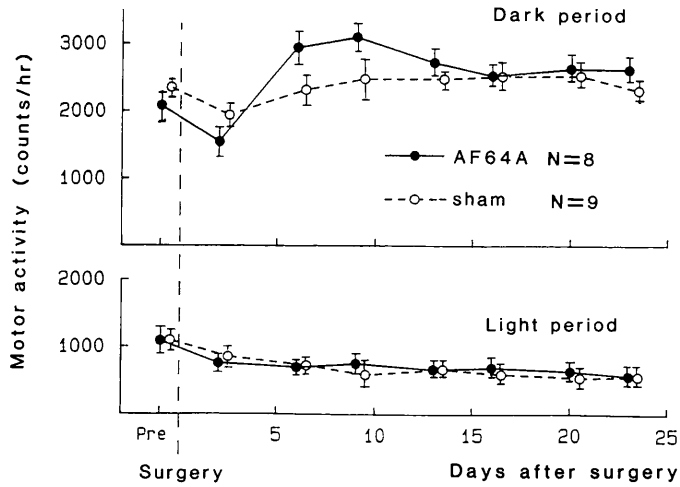

Fig. 1. Spontaneous motor activity of sham-rats and AF64A-rats during the dark period (top) and the light period (bottom) before and after surgery. Values represent the mean±S.E.M. No significant differences were observed in each point according to Student's *t*-test.

days after surgery (Fig. 1), learning studies were made later than day 16 after surgery.

One-trial passive avoidance task: In terms of the initial latency to enter the dark chamber on the acquisition trial, there was no significant difference between AF64A-rats and sham-rats. On the retention trial, the latency of AF64A-rats was significantly shorter than that of sham-rats (Fig. 2).

Delayed alternation task in T-maze: Both AF64A-rats and sham-rats quickly learned to consume the pellets in the food cup at the edge of the goal arm during the maze habituation. On the alternation training, AF64A-rats required a few more trials to achieve the criterion as compared with the sham-rats. Figure 3 shows the results of the delayed alternation task in the T-maze. Both AF64A-rats and sham-rats made correct choices more than 95% of the time when they had no delay time, and there was no significant difference between the two groups. When delay time was introduced, however, AF64A-rats made significantly more errors than sham-rats at 10, 30, 90 and 270 sec delay time.

Active avoidance task: In the two-way shuttle avoidance task, AF64A-rats tended to perform more active avoidance responses than sham-rats throughout the 4 sessions. There was a significant difference in the avoidance responses in the first session between the two groups (Fig. 4). With respect to the total response (ITI, US and UCS), there was no significant difference between the two groups at each session (Table 2).

2. ChAT activity

ICV injection of AF64A (3 nmoles/each laterral ventricle) resulted in significant decrease of ChAT activity in the hippocampus (56% of that in sham-rats), but not in the frontal cortex and the striatum (Table 3).

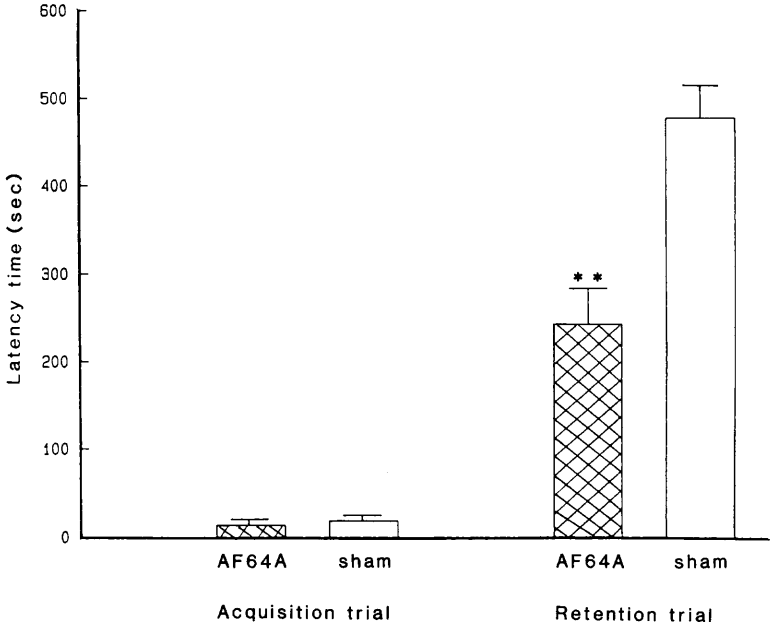

Fig. 2. Latency time to enter the dark chamber of a passive avoidance apparatus in the acquisition and retention trial for sham-rats and AF64A-rats. Values represent the mean±S.E.M. of 12 sham-rats and 13 AF64A-rats. **: Significant difference from sham-rats with $P<0.01$, Mann-Whitney U-test.

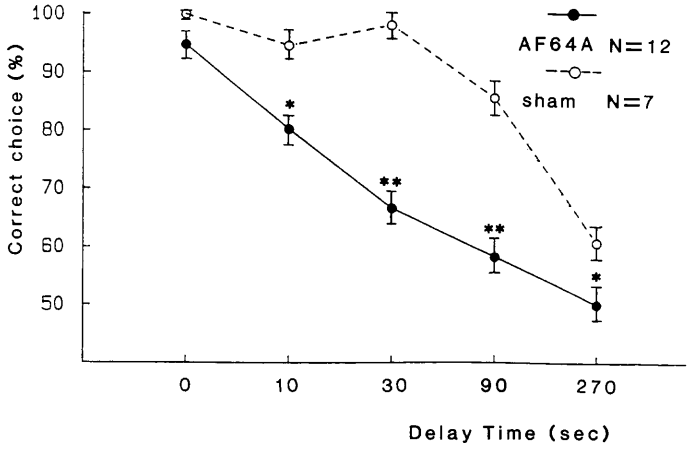

Fig. 3. Correct choice on the delayed alternation task in the T-maze. AF64A-rats and sham-rats were tested with 2 trials (left and light choice running) for each delay time for a successive 4 days. Values represent the mean±S.E.M. Significantly different from the sham-rats: *$P<0.05$, **$P<0.01$, Mann-Whitney U-test.

3. Effects of physostigmine

There was a significant difference in the retention latency between sham-rats receiving physiological saline solution (vehicle) and AF64A-rats receiving vehicle (Fig. 5), confirming our previous results (Fig. 2). When physostigmine (PHY) was intraperitoneally administered at the dose of 0.1 mg/kg, performance of AF64A-rats was improved in the retention trial. That is, there was no sig-

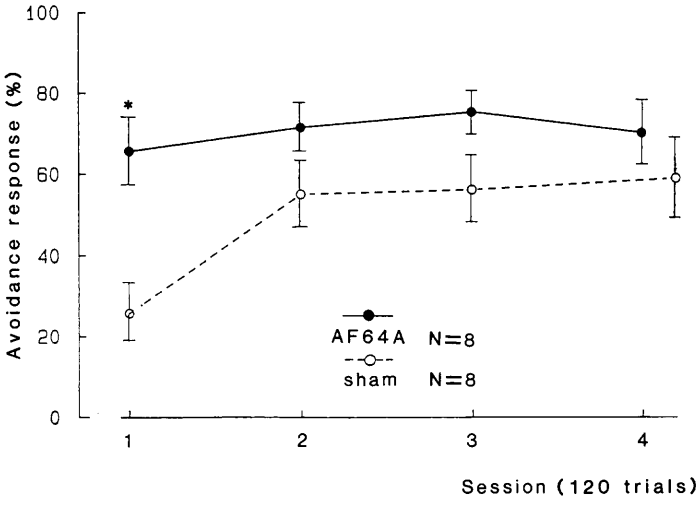

Fig. 4. Active avoidance responses in a two-way shuttle box. AF64A-rats and sham-rats were tested for 4 sessions, with 120 trials per session, every 2 days. Values represent the mean±S.E.M. *: Significant difference from the sham-treated control-rats with $P<0.05$, Student's t-test.

Table 2. Total responses in a two-way shuttle avoidance task

	1st session	2nd session	3rd session	4th session
Sham-rats (N=8)	132.1±11.6	146.9± 8.1	141.3±6.0	137.8±7.1
AF64A-rats (N=8)	131.2±13.2	153.1±10.7	135.9±7.0	125.0±4.9

Values represent the mean±S.E.M. No significant differences were observed according to Student's t-test between sham-treated control-rats and AF64A-rats at each session.

Table 3. Choline acetyltransferase activity in brain regions 5 weeks after ICV injection of AF64A

	ChAT activity (mole/hr/mg·protein)	
	sham-rats (N=14)	AF64A-rats (N=24)
Frontal cortex	87.9± 2.0	86.1± 2.0
Hippocampus	114.9± 5.7	64.3± 4.2**
Striatum	398.0±15.9	395.2±10.7

Values represent the mean±S.E.M. **Significant difference from the sham-treated control-rats, with $P<0.01$, Student's t-test.

nificant difference in the retention latency between sham-rats and AF64A-rats receiving PHY, but there was a significant difference between AF64A-rats receiving vehicle and AF64A-rats receiving PHY. No significant difference in latency to enter the dark chamber was observed in the acquisiotion trial (Fig. 5).

In the delayed alternation task in the T-maze, where the delay time (60 sec) was introduced, there was a significant difference in correct response between sham-rats receiving vehicle and AF64A-rats receiving vehicle. PHY (0.2 mg/kg, i.p.) slightly improved the performance of AF64A-rats, but there was no significant difference in correct respones between AF64A-rats receiving vehicle and AF64A-rats receiving PHY (Table 4).

Discussion

AF64A injected into the lateral ventricles (3 nmole in 2 μl, each side) produced significant reduction of ChAT activity in the hippocampus (56% of that in sham-rats),

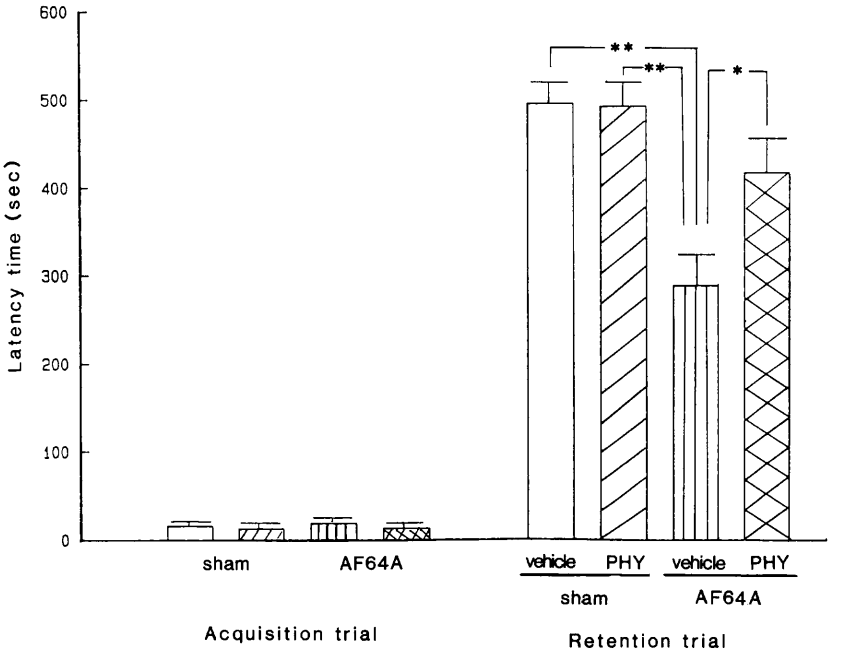

Fig. 5. Effects of physostigmine (0.1 mg/kg, i.p.) on a passive avoidance task in AF64A-rats and sham-rats. Values represent the mean±S.E.M. of 21, 22, 18 and 19 rats for sham-rats receiving vehicle, sham-rats receiving physostigmine (PHY), AF64A-rats receiving vehicle and AF64A-rats receiving PHY, respectively. Significantly different from the AF64A-rats receiving vehicle: *$P<0.05$, **$P<0.01$, Mann-Whitney *U*-test.

Table 4. Effects of physostigmine on the delayed alternation task in the T-maze

	Percent of correct responses
Sham-rats+Vehicle	97.6±2.4
AF64A-rats+Vehicle	59.7±5.2**
AF64A-rats+PHY (0.2 mg/kg)	66.7±6.2**

Values represent the mean±S.E.M. **Significant difference from the sham-treated control rats receiving vehicle, with $P<0.01$, Mann-Whitney *U*-test.

without concomitant reduction of the activity in the frontal cortex and striatum 5 weeks after treatment, suggesting that ICV injection of AF64A appears to produce selective hippocampal hypofunction in rats. The reduction of ChAT activity in AF64A-rats was accompanied by impairment of performance in the one-trial passive avoidance task and in the delayed alternation task in the T-maze. However, no significant difference was found between AF64A-rats and sham-rats in the spontaneous motor activity and the electric foot shock sensitivity, suggesting that the impairment of learning performance in AF64A-rats was not due to the possible abnormality in the general behaviors. There has been a considerable amount of evidence suggesting that cholinergic function has a vital role in memory processing in rats (13, 17–19). Our present results provide evidence that hippocampal cholinergic function is involved in memory function.

In marked contrast with the passive avoidance task and the delayed alternation task in the T-maze, the AF64A-rats could perform the active avoidance task in the

two-way shuttle box apparently better than the sham-rats. Myhrer (20) reported apparently better performance in a shuttle avoidance task in rats with complete fimbria lesions. Jarrard (21) reported that rats with extensive hippocampal lesions exhibit a facilitated acquisition in the two-way shuttle avoidance task, but a deficient performance in a passive avoidance task and in a spatial reversal learning task in a Y-maze. At the same time, he found that rats with extensive hippocampal lesions are more active than normal rats during the day as well as during the night. Furthermore, it has been reported that rats with extensive damage in the hippocampal region are more sensitive to electric shock (22). In AF64A-rats, we have observed slight damage in the fimbria-fornix and loss of CA2 and CA3 fields in the hippocampus by microscopic examination of tissues taken 30–40 days after AF64A treatment (N. Nakahara et al., unpublished observation). With respect to total responses in a shuttle avoidance task and general behaviors including shock sensitivity and spontaneous motor activity, however, there was no significant difference between AF64A-rats and sham-rats. These results demonstrated that in the AF64A-rats, septo-hippocampal damage may be not large enough to change shock sensitivity and motor activity.

In the delayed alternation task in the T-maze, two different aspects may be characterized as following: 1) a reference memory component which involves the invariant response of running down the arm of the T-maze and consumption of the pellets in the food cup and 2) a working memory component in which the animals must maintain some representation of the previous stimulus event to perform correctly (23, 24). Both AF64A-rats and sham-rats quickly learned to run down the arm and consume the pellets during the habituation period. At training for the alternation task in the T-maze, AF64A-rats required slightly more training than the sham-rats to achieve the criterion of more than 4 correct choices out 6 trials. AF64A-rats also showed poorer performance than sham-rats in the T-maze, when the delay time was introduced. In the T-maze without delay time, however, AF64A-rats performed as correctly as sham-rats. These findings suggest that the working memory of AF64A-rats is inferior to that of sham-rats, but their reference memory is unaffected; i.e., AF64A-rats are deficient in holding previous information that is valid only for a short period of time.

There is controversy about whether AF64A is a selective cholinergic neurotoxin or not. Walsh et al. (10) reported that at 120 days after ICV treatment with 7.5 or 15 nmole AF64A, bilaterally, significant reductions were observed in hippocampal ACh levels, as well as in cortical ACh levels after a higher dose of AF64A. Levels of choline (Ch), as well as concentrations of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites, homovanillic acid (HVA), 3,4-dihydroxyphenyl acetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), respectively, were not affected in the same tissues following AF64A treatment. Kasa et al. (25, 26) made morphological observations that ICV injection of 5 nmole of AF64A produced selective cholinotoxic effects in the brain and that, on the other hand, higher concentration of AF64A (>10 nmole) produced nonspecific degeneration. Potter et al. (27) demonstrated that: 1) ICV injection of AF64A produces only a transient decrease in hippocampal DA, NA and 5-HT contents, suggesting that with up to 10 nmoles the neurotoxin, hippocampal aminergic nervous systems are not destroyed, and 2) ICV injection of AF64A exerts a major and long-lasting inhibitory effect on the hippocampal cholinergic system. On the contrary, Jarrard et al. (9) claimed from histological examination that AF64A (1.5–3.0 nmole in 0.5 μl, for each lateral ventricle) caused non-specific and extensive damage to the fimbria-fornix. At the same time, they carried out neurochemical measurement and found great depletions of ACh in the hippocampus and striatum, but no depletion of NA and DA. Taken together with our findings of reduction of ChAT activity in the hippocampus but not in the frontal cortex and striatum, it seems most likely that in the hippocampus of the rats treated with a low dose of AF64A in the present study, the cholinergic system was selectively impaired, resulting in cognitive

dysfunction in the passive avoidance task and T-maze task. Defects of other neurotransmitter systems including monoaminergic systems might be, if any, minor in the hippocampus and other regions, and it seems less likely that they contribute to the present cognitive dysfunction.

Although AF64A-rats with hippocampal cholinergic hypofunction were impaired relative to sham-rats in regards to the learning performance in the passive avoidance task and the alternation task in the T-maze, they had a residual learning capacity. In the passive avoidance task, for example, the mean retention latency of AF64A-rats was significantly longer (243 sec) than the initial latency (13 sec), suggesting that they kept some memory of the shocks of the previous days. Physostigmine (0.1 mg/kg, i.p.), an acetylcholinesterase inhibitor and cholinomimetic agent, significantly improved the retention latency of AF64A-rats in the passive avoidance task. In addition, physostigmine (0.2 mg/kg, i.p.) had a tendency to improve the performance of AF64A-rats in the delayed alternation task in the T-maze. These finding suggest that a residual learning capacity in AF64A-rats might be enhanced by physostigmine.

Senile dementia of the Alzheimer type (SDAT) is characterized behaviorally by a general decline in cognitive function. Although a couple of neurotransmitter systems are reported to be impaired, one of the most profound neurochemical changes observed in this disease is a marked presynaptic cholinergic hypofunction in selective brain regions including the hippocampus (28–30). On the contrary, postsynaptic muscarinic receptors appear to be relatively unaffected (28, 31–33). In the experimental study by Mantione et al. (34) it was also demonstrated that intracerebral injection of AF64A (2 nmoles) into the dorsal hippocampal area of rats caused a significant decrease in the activity of HAChT and ChAT activity, while choline levels and muscarinic ACh receptors were unchanged in the same brain area. Thus, it seems that neurochemical and learning behavioral impairments in AF64A-rats mimic two important aspects of SDAT in humans: presynaptic cholinergic deficit and cognitive impairment.

In conclusion, ICV injection of AF64A can be considered as a useful means for producing an experimental amnesia model like SADT which can be used for evaluating new drugs for treating the disease.

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