Beneficial Effects of FKS-508 (AF102B), a Selective M₁ Agonist, on the Impaired Working Memory in AF64A-Treated Rats

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Abstract—The effects of FKS-508 [AF102B; cis-2-methylspiro(1,3-oxathiolane-5,3')quinuclidine], a selective M₁ muscarinic receptor agonist, were examined to predict the possible activity on memory disorders using a T-maze and radial-arm maze task in experimental amnesia models. The amnesia models were produced by bilateral intracerebroventricular injection of ethylcholine aziridinium ion (AF64A), a selective cholinotoxin, in rats. Repeated administrations of FKS-508 (5 mg/kg/day, i.p.) for 5 weeks significantly ameliorated impaired performance of AF64A-treated rats (AF64A-rats) in a delayed alternation task in the T-maze. Repeated administrations of FKS-508 (1 and 5 mg/kg/day, p.o.) for 5 weeks significantly ameliorated acquisition failures of AF64A-rats in a radial-arm maze task. Single administration of FKS-508 (1 and 5 mg/kg, p.o.) significantly reduced the incorrect choices of AF64A-rats in a radial-arm maze task with 6 hr-delay time. No abnormalities in general behaviors, such as loss of appetite and ataxia, were observed in rats treated with FKS-508 repeatedly during 5 weeks. Our present results showed that FKS-508 can ameliorate memory impairments in AF64A-rats with central cholinergic hypofunction without causing any behavioral abnormalities. FKS-508 may be considered as a candidate for the clinical examination of the cholinergic hypothesis of senile dementia of the Alzheimer type.
examinations on the displacement of $[^3]$H-QNB binding in the rat forebrain and cerebellum (13), (b) electrophysiological investigations on rabbit superior cervical ganglia (14, 15), and (c) neurochemical studies on presynaptic muscarinic autoreceptor and heteroreceptor regulating neurotransmitter release (16). Moreover, we confirmed that FKS-508 crosses the blood-brain barrier (our unpublished observation).

In the present paper, we examined the effects of repeated and single administrations of FKS-508 on the impaired working memory of AF64A-rats in a T-maze and a radial-arm maze. The preliminary account of part of this work has been given elsewhere (17, 18).

Materials and Methods

1. Materials: Acetyl-AF64 (HCl) was kindly supplied by Dr. A. Fisher (Israel Inst. Biol. Res., Ness-Ziona, Israel). Drugs used were FKS-508 [AF102B; cis-2-methylspiro(1,3-oxathiolane-5,3')quinucilne hydrochloride hemihydrate] and oxotremorine sesquifumarate (Sigma, St. Louis, U.S.A.).

2. Animals and AF64A treatment: Male Sprague-Dawley rats (200–250 g, Charles River Japan, Atugi) were individually housed in a stainless steel mesh cage in a temperature and light controlled room. Ethylcholine aziridinium ion (AF64A) was freshly prepared from acetyl-AF64 (HCl) by the method of Mantione et al. (19) as described elsewhere (11). Surgery was carried out as described in the previous paper (11). Injections of 2.0 $\mu$l of AF64A (3 nmole) or saline solution (for sham-operated rat) were made into each lateral ventricle over an 8-min period. Rats were subjected to behavioral experiments 5 weeks after surgery.

3. Effects of repeated administrations of FKS-508 on a delayed alternation task in a T-maze: The pre-testing for a delayed alternation task in the T-maze was performed as described in the previous paper (11). Briefly, 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 rat was forced to choose the single opened arm, which was baited with 2 pellets. Immediately after consumption of the pellets, the guillotine door was removed, and the rat was replaced at the start arm for the choice running. At this time, the rat was permitted to choose both arms. However, the just-forced arm was empty, whereas the other side arm was baited with 2 pellets, such that the rat had to alternate the just-forced response for the correct choice. All of the rats which received 6 trainings daily for 5 pre-testing days were randomly divided into the group subjected to FKS-508 and the group subjected to saline solution (vehicle).

Over the following 5 weeks, 5 days per week, each rat received 4 daily trials on a delayed alternation task with 5 min inter-trial interval, in which 60 sec of delay time was introduced between the information running and the choice running. FKS-508 (5 mg/kg, i.p.) or vehicle was daily administered to rats 15 min before the first trial.

Four days after the completion of testing, sham-operated rats (sham-rat) and AF64A-rats were sacrificed by decapitation to measure ChAT activities in the hippocampus and frontal cortex by the method of Fonnum (20).

4. Effects of repeated administrations of FKS-508 on a radial-arm maze task: The apparatus, made of white painted wood, was elevated 60 cm above the floor and was placed in a testing room with many visual extra-maze stimuli, such as a refrigerator, table, blackboard, locker and posters on the wall. Eight arms (60-cm long and 12-cm wide) octagonally extended from a central platform (38 cm in diameter), and the sides of the arms were 4-cm high. A 4-cm diameter plastic food cup was put at the end of each arm.

The pre-testing week (5 days) was allowed
for stabilization of rat’s body weight at approximately 85% free-feeding level and habituation for the maze. On the first day for habituation, 40 mg food pellets were put all over the maze and rats were placed on the central platform to explore the maze and consume the pellets in pairs for 15 min. On the second day, a single rat was placed on the central platform for 10 min with a few pellets in each arm, but no pellet in the central platform. For the remaining 3 days, one pellet was put into the food cup of each arm, and each rat was allowed to consume the pellets for 10 min.

Over the following 5 weeks, 5 days per week, each rat received 1 daily trial of testing as described below. All 8 arms were baited with 1 pellet each. The rat was placed into the opaque plastic cylinder (30 cm in diameter and 30 cm high) put on the central platform. Ten seconds later, the cylinder was removed, and the rat was permitted to run from arm to arm until 8 pellets were collected. In this task, a correct choice means to enter the arm with a pellet, and an incorrect choice is reentry into an arm that a pellet had been taken from already. FKS 508 (0.5, 1 and 5 mg/kg, p.o.) or distilled water (vehicle) was daily administered to rats 30 min before the trial.

5. Effects of single administration of FKS-508 and oxotremorine on a radial-arm maze task with 6 hr-delay time: Stabilization of rat’s body weight and habituation for the maze were carried out in the same manner as described above. After that, each rat received 1 trial a day for 8 training-days. Each one of the 8 arms were baited with a pellet. The rat was placed into the plastic cylinder. Ten seconds later, the cylinder was removed, and the rat was permitted to run from arm to arm until 8 pellets were taken. The criterion of acquisition performance was set up for a rat to make the correct choice more than 6 times in the first 8 choices. The rats that attained the criterion more than twice in the 8 training-days were used for further experiments.

A period of 6 hr-delay time was introduced to examine the maintenance of the working memory by the rats. That is, rats were allowed to collect 6 pellets, and then they were removed from the maze and returned to their home cage. After a period of 6 hr-delay time, the rats were placed again in the maze to collect the remaining 2 pellets, during which the incorrect choices were measured to examine the maintenance of working memory. FKS-508 (0.5, 1 and 5 mg/kg), oxotremorine (0.04, 0.2 and 1 mg/kg) or distilled water (vehicle) was perorally administered 30 min before the first trial collecting 6 pellets.

Results

1. Effects of repeated administrations of FKS-508 on a delayed alternation task in a T-maze: In the pre-testing trial, where the delay time was not introduced, AF64A-rats made significantly fewer correct choices than sham-rats* (Fig. 2). In the previous study, we also observed that AF64A-rats required more trials to learn the T-maze task as compared with the sham-rats during the 5 pre-testing days (11).

There was no significant difference of correct choices in the pretesting trial between AF64A-rats subjected to FKS-508 and AF64A-rats subjected to the vehicle (Fig. 2). In the testing trial, where the delay time (60 sec) was introduced, correct choices decreased in the first week in both sham- and AF64A-rats when compared with that in the pre-testing trial (Fig. 2). Sham-rats treated with vehicle exhibited a rapid increase of correct choices as the number of testing trials increased. On the other hand, AF64A-rats treated with vehicle did not exhibit the increase of correct choices at all throughout 5 testing weeks. AF64A-rats treated with FKS-508 (5 mg/kg/day, i.p.) gradually ameliorated their performance in the T-maze as the number of testing trials increased, and significant differences of correct choices were found between AF64A-rats treated with vehicle and AF64A-rats

* We previously reported that there was no significant difference of correct choices in the pre-testing trial between AF64A-rats and sham-rats, which were used for further experiments with delay time (in Fig. 3 of ref. 11). The apparent discrepancy is due to the facts that the rats which achieved the criterion, i.e., more than 4 correct choices out of 6 trials in 5 pre-testing days, were used for further experiments in the previous study, while all AF64A-rats were used without selection and randomly divided into 2 groups for further experiments in the present study.
Fig. 2. Effects of repeated administrations of FKS-508 on a delayed alternation task in a T-maze in AF64A-rats. Each rat was tested with 20 trials on each week, and the percentage of correct choices in each group was calculated for each week over the 5 weeks of testing. The percentage of correct choices in each group over 5 days of pre-testing (without delay time) are indicated on the left. Values represent the mean±S.E. of 14, 19 and 18 rats for sham-rats treated with vehicle (X), AF64A-rats treated with FKS-508 (5 mg/kg, i.p.: ●) and AF64A-rats treated with vehicle (○), respectively. One-way ANOVA (LSD method) was used for statistical analysis. Significantly different from AF64A-rats treated with vehicle: **P<0.01.

2. Effects of repeated administrations of FKS-508 on a radial-arm maze task: AF64A-rats failed significantly more times than sham-rats in this task. Compared to AF64A-rats treated with vehicle, AF64A-rats treated with FKS-508 showed a tendency to make more correct choices throughout 5 testing weeks (Table 1). AF64A-rats treated with FKS-508 (5 mg/kg/day, p.o.) made significantly more correct choices than AF64A-rats treated with vehicle during 5 testing weeks.

In terms of ChAT activity in the hippocampus and frontal cortex, no significant difference was observed between AF64A-rats treated with FKS-508 and AF64A-rats treated with vehicle. However, in confirmation of our previous results (11), hippocampal ChAT activity in AF64A-rats was significantly reduced by 37%, when compared with that in sham-rats (Fig. 3).

Fig. 3. Choline acetyltransferase (ChAT) activity in the hippocampus and frontal cortex 4 days after the completion of a T-maze task. Values represent the mean±S.E. with the number of animals in columns. Significantly different from AF64A-rats treated with vehicle: *P<0.05, **P<0.01 (LSD method).
Table 1. Effects of repeated administrations of FKS-508 on the acquisition performance of AF64A-rats in a radial-arm maze task

| Treatment                        | Dose (mg/kg, p.o.) | 1st week | 2nd week | 3rd week | 4th week | 5th week |
|----------------------------------|--------------------|----------|----------|----------|----------|----------|
| Sham-rat + Vehicle (n=23)        | —                  | 2.4±0.3  | 1.4±0.2  | 1.0±0.2  | 0.8±0.2  | 0.8±0.1  |
| Sham-rat + FKS-508 (n=18)        | 5                  | 1.8±0.2  | 1.0±0.2  | 0.8±0.2  | 0.5±0.1  | 0.4±0.1  |
| AF64A-rat + Vehicle (n=23)       | —                  | 8.1±0.8**| 7.3±0.9**| 6.4±1.0**| 6.7±1.0**| 4.6±0.8**|
| AF64A-rat + FKS-508 (n=22)       | 5                  | 6.2±0.8**| 5.5±1.0**| 3.6±0.8***| 3.4±0.7****| 3.1±0.9**|
| Sham-rat + Vehicle (n=33)        | —                  | 2.4±0.2  | 1.6±0.2  | 1.0±0.2  | 1.2±0.2  | 1.0±0.2  |
| AF64A-rat + Vehicle (n=30)       | —                  | 7.2±0.8**| 5.7±0.7**| 5.2±0.6**| 5.0±0.8**| 4.2±0.6**|
| AF64A-rat + FKS-508 (n=33)       | 1                  | 6.7±0.6**| 4.6±0.5**| 3.2±0.4***| 2.9±0.5***| 2.9±0.6**|
| Sham-rat + Vehicle (n=17)        | —                  | 2.0±0.3  | 0.9±0.1  | 0.8±0.1  | 0.7±0.2  | 0.5±0.1  |
| AF64A-rat + Vehicle (n=28)       | —                  | 6.7±0.8**| 6.1±0.9**| 5.6±0.9**| 4.2±0.7**| 3.9±0.7**|
| AF64A-rat + FKS-508 (n=26)       | 0.5                | 6.1±0.5**| 4.9±0.6**| 4.4±1.0**| 3.4±0.6**| 3.4±0.6**|

Mean number of incorrect choices per trial by each animal in each group was calculated for each week over 5 weeks of testing. Significant difference from sham-rats treated with vehicle (one-way ANOVA): *P<0.05, **P<0.01. Significant difference between AF64A-rats treated with vehicle and AF64A-rats treated with FKS-508: *P<0.05, **P<0.01.

Fig. 4. Effects of single administrations of FKS-508 and oxotremorine on a radial-arm maze task with 6 hr-delay time in AF64A-rats. Compounds were perorally administered 30 min before the first trial collecting 6 pellets. Values represent the mean±S.E. with the number of animals in columns. Significantly different from AF64A-rats treated with vehicle: *P<0.05, **P<0.01 (LSD method).

Even when the dose of FKS-508 was reduced to 1 mg/kg/day, p.o., there were still significant differences between AF64A-rats treated with FKS-508 and AF64A-rats treated with vehicle at the first, third and fourth week. However, at a lower dose of FKS-508 (0.5 mg/kg/day, p.o.), there was no significant difference between AF64A-rats treated with FKS-508 and AF64A-rats treated with vehicle. Repeated administrations of FKS-508 (5 mg/kg/day, p.o.) to sham-rats had no influence on the acquisition performance in this task, and it caused no behavioral abnormality.
3. Effects of single administration of FKS-508 and oxotremorine on a radial-arm maze task with 6 hr-delay time: AF64A-rats treated with vehicle made significantly more incorrect choices than sham-rats treated with vehicle for collection of the remaining 2 pellets (Fig. 4). FKS-508 (5 mg/kg, p.o.) significantly decreased the incorrect choices in AF64A-rats. Even when the dose of FKS-508 was reduced to 1 mg/kg, p.o., there was still a significant difference between AF64A-rats treated with vehicle and AF64A-rats treated with FKS-508 in performance of the radial-arm maze task. However, the improving effect of FKS-508 disappeared at lower dosage (0.5 mg/kg, p.o.). Oxotremorine (0.04, 0.2 and 1 mg/kg, p.o.) showed a tendency for improving the performance of AF64A-rats in this test, but the effect was not significant (Fig. 4).

Discussion

The functions of the septo-hippocampal system in rats are well-known to play an important role for spatial memory, which is classified into working memory and reference memory. The component of working memory involves flexible stimulus-responses which are useful for one trial of an experiment, but not for subsequent trials, whereas the reference memory component involves fixed stimulus-responses which are not altered over consecutive trials in the maze task (21). Lesions to the hippocampal formation or its major connections are reported to produce disruption of a maze learning task, because of selective impairment of working memory (1-5). On the other hand, Dunnett et al. (22) observed that septal grafts (rich in cholinergic neurons) ameliorate the performance of rats with fornix-fimbria lesions in a T-maze. These findings suggested that cholinergic deficits occurred in the hippocampus of AF64A-rats and that the effects of FKS-508 and vehicle were examined in a homogeneous group of rats with cholinergic deficits. The performance of AF64A-rats repeatedly treated with FKS-508 in the T-maze task was significantly ameliorated as compared to that of AF64A-rats treated with vehicle. In a radial-arm maze task, the acquisition performance of AF64A-rats was significantly inferior to that of sham-rats. Again, repeated administrations of FKS-508 significantly ameliorated the impaired performance of AF64A-rats. In the radial-arm maze task with 6 hr-delay time imposed between the sixth and seventh choices, AF64A-rats made significantly more incorrect choices than sham-rats, showing the impairment in the working memory component. The performance of AF64A-rats was significantly ameliorated by single administration of FKS-508. Fisher et al. (13) have also found that FKS-508 ameliorates the memory deficits in the radial-arm maze task with 2 hr-delay time imposed between the forth and fifth choices concluded in a review article that at low doses (≤5 nmole), the effects of AF64A appear to be limited to the cholinergic system, and that at higher doses, noncholinergic effects become prominent. Recent studies by other groups showed that the neurochemical deficit induced by AF64A is associated with a significant impairment of the working memory component in the radial-arm and T-maze task (8-10). We have also observed that ICV injection of AF64A (3 nmole per each side) into rats produce the working memory impairment in the T-maze task with a concomitant hypofunction in the hippocampal cholinergic system (11).
in AF64A-rats. These results suggested that ICV injection of AF64A into rats caused the learning and memory deficits in the maze task, more specifically the impairment in working memory, and that single or repeated administrations of FKS-508 ameliorated the impaired working memory in AF64A-rats with hippocampal cholinergic hypofunction.

Single administration of oxotremorine to AF64A-rats produced slight but not significant improvement in the radial-arm maze task with 6 hr-delay time. However, we previously found positive effects of oxotremorine on the performance in a passive avoidance task in AF64A-rats (12). The reason for these differential effects of oxotremorine on AF64A-rats is not known. It might be due to the different character of the task used, i.e., negative reinforcement (the passive avoidance task) and positive reinforcement (the T-maze and radial-arm maze task). It might be also due to central side-effects induced by oxotremorine, which might have disturbed the learning performance of the rats treated with oxotremorine in this task. We observed that oxotremorine (1 mg/kg, p.o.) produced significant hypothermia, a central muscarinic response, in rats (H. Arisawa et al., unpublished observation). In addition, our recent examination of FKS-508 and oxotremorine in central muscarinic responses in mice showed 1) that FKS-508 (1–5 mg/kg, p.o.) and oxotremorine (0.2–1 mg/kg, p.o.) significantly ameliorated the passive avoidance failure in scopolamine-induced amnesia mice; 2) that ED50 of FKS-508 for producing tremor was 95 mg/kg, p.o., and that of oxotremorine was 2.7 mg/kg, p.o.; and 3) that ED50 of FKS-508 for hypothermia was 33 mg/kg, p.o., and that of oxotremorine was 0.19 mg/kg, p.o. (N. Nakahara et al., manuscript in preparation). These results indicate that oxotremorine is slightly more potent than FKS-508 in amelioration of experimental amnesia induced by scopolamine, but far more potent in inducing central side-effects, such as tremor and hypothermia.

Recently, pharmacological evidence suggested that muscarinic receptors in the central nervous system may be divided into at least two subtypes, M1-subtype and M2-subtype, on the basis of their affinities for pirenzepine (24). One of the most intriguing findings is that M1 sites are rich in the neocortex and hippocampus in both rats and humans (25, 26). Such a distribution suggests some role for M1 receptors in cognitive processes. In fact, based on the effects of pirenzepine on the performance of rats in a Morris water maze, Hagan et al. (27) claimed that M1 receptors play an important role in spatial learning and memory. Caulfield et al. (28) suggested by ICV injection of pirenzepine in mice that M1 sites are involved in learning and memory processes, whereas M2 receptors are involved in other central muscarinic side-effects such as tremor, hypothermia and antinociception. Senile dementia of the Alzheimer type (SDAT) is characterized clinically by a general decline in cognitive function. The decrease of cognitive function is well-correlated with reduction of ChAc activity, the synthetic enzyme for the neurotransmitter acetylcholine, in selective brain regions including the hippocampus (29, 30). Based on observed neurochemical deficits (29–31), it is proposed that muscarinic agonists, more specifically, M1-selective agonists, are a rational therapeutic approach in SDAT (32). It has been well-documented that FKS-508 is a selective M1 agonist (13–16). Our present results showed that FKS-508 can ameliorate the impaired working memory in the T-maze and radial-arm maze task in AF64A-rats with hippocampal cholinergic hypofunction (see also ref. 13). Repeated administrations of FKS-508 ameliorated the impaired performance of AF64A-rats, suggesting that pharmacological tolerance was not brought out during 5-weeks treatment. Moreover, repeated administrations neither affected the acquisition performance nor induced any behavioral abnormalities in sham-rats. Taken together with earlier results reported by Fisher et al. (13) and those from our laboratory (12) that FKS-508 ameliorates passive avoidance failure in AF64A-rats and scopolamine-induced amnesia mice, FKS-508 deserves further examination for development as a possible therapeutic drug for SDAT.

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