Pharmacology Biochemistry and Behavior, Vol. 60, No. 2, pp. 377–386, 1998

0091-3057/98 $19.00 + .00

ELSEVIER

PII S0091-3057(97)00601-1

Comparative Studies of Huperzine A, E2020, and Tacrine on Behavior and Cholinesterase Activities

DONG HANG CHENG AND XI CAN TANG

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China

Received 1 August 1997; Revised 27 October 1997; Accepted 12 November 1997

CHENG, D. H. AND X. C. TANG. *Comparative studies of huperzine A, E2020, and tacrine on behavior and cholinesterase activities.* PHARMACOL BIOCHEM BEHAV **60**(2) 377–386, 1998.—Comparative effects of cholinesterase inhibitors (ChEI) huperzine A with E2020 and tacrine on the radial maze performance in ethylcholine mustard aziridinium ion (AF64A)-treated rat and inhibition of cholinesterase activity were studied. The intracerebroventricular (ICV) injection of AF64A (3 nmol/side) caused significant impairment in the rat's ability to fulfill the partially baited maze paradigm. Oral huperzine A (0.5–0.8 mg/kg), E2020 (1.0–2.0 mg/kg), and tacrine (8.0 mg/kg) effectively reversed AF64A-induced working memory deficit. The doses that improved AF64A-induced memory deficit were correlated to about 25–30% (huperzine A) and less than 10% (E2020, tacrine) inhibition of acetylcholinesterase (AChE) activity in the cortex and hippocampus. Huperzine A, E2020 and tacrine all produced dose-dependent inhibition of brain AChE following ICV and oral administration. Oral huperzine A exhibited higher efficacy on the inhibition of AChE in the cortex and hippocampus than those of E2020 and tacrine. Tacrine was more effective in inhibiting plasma butyrylcholinesterase (BuChE) than it was brain AChE. Conversely, the BuChE activity was less affected by huperzine A and E2020. The results showed that huperzine A had high bioavailability and more selective inhibition on AChE activity in cortex and hippocampus. Huperzine A fits more closely with the established criteria for an ideal AChE inhibitor to be used in clinical studies. © 1998 Elsevier Science Inc.

Alzheimer's disease Cholinesterase inhibitor Huperzine A E2020 Tacrine Rat radial maze Learning and memory Cholinesterase

ALZHEIMER'S disease (AD) is a slowly progressive neuropsychiatric illness, principally characterized by memory deficits, and is of unknown etiology. It has become the fourth leading cause of death in developed nations. Increasing pharmacological and neurochemical evidence has linked AD to the cholinergic hypothesis of memory dysfunction (1,5,7,8). Moreover, the degree of cholinergic deficit in senile dementia of Alzheimer's type (SDAT) has been correlated with the severity of cognitive impairment (29,36). Therefore, treatment strategies were focused on replacement therapy for deficits of central cholinergic neurotransmission (6). Recently, clinical trials have shown that cholinesterase inhibitors (ChEI) are the most promising drugs demonstrating efficacy in the treatment for AD (15). There are only two ChEIs, physostigmine and tacrine, that have been evaluated on a large scale in patients with AD. However, the short half-life and peripheral cholinergic side effects of physostigmine and the dose-dependent hepatotoxicity of tacrine limit their clinical value (35,37). An ideal ChEI suitable for the prevention or palliation of memory deficit in AD should produce a long-term acetylcholinesterase inhibition with minimal side effects at therapeutic doses. Such a requirement has not been satisfied by any ChEI so far. Therefore, the search for a potent, long-acting acetylcholinesterase (AChE) inhibitor that exerts minimal side effects for the treatment of AD is still ongoing. Huperzine A and E2020 are such promising compounds. Huperzine A, a new *lycopodium* alkaloid that was isolated from the Chinese herb *Huperzia serrata (*Thunb) Trev (22), is a potent and selective AChE inhibitor (4,24,34) with a better therapeutic index than that of physostigmine (32,33). E2020 is a novel central-acting AChE inhibitor under development by the Eisai company. The improved properties of both huperzine A and

Requests for reprints should be addressed to X. C. Tang, Chinese Academy of Sciences, Shanghai Institute of Materia Medica, State Key Lab. of Drug Research, Shanghai 200031, China.

E2020 overcome the problem of tacrine and physostigmine occurring in clinical treatment of AD (31,33). Huperzine A and E2020 deserve further study as promising candidates for therapy of cognitive impairment with AD. Ethylcholine mustard aziridinium ion (AF64A) is a neurotoxic derivative of choline that produces a long-lasting presynaptic central cholinergic deficit in rats similar to some characteristics observed in AD. Therefore, it should prove to be an important tool for elucidating the underlying mechanisms of this disease and also for evaluating potentially useful therapeutic drugs for its treatment (11,13). The aim of this study was to provide comparative data of huperzine A, E2020, and tacrine on the inhibition of cholinesterase *in vivo* and on amnesia produced by AF64A on a partially baited radial maze paradigm. The chemical structures of the three drugs are shown in Fig. 1.

METHOD

Animals

Male Sprague–Dawley rats weighing 280–350 g, were housed individually in a climatically controlled room in a 12 L: 12 D cycle. Five days prior to behavioral training, daily food was limited to reduce the rats to 85% of their free-feeding weight. Food deprivation was maintained throughout the whole experiment, except for 7 days prior to and following surgical procedure. Male and female rats of Sprague–Dawley strain were used for the biochemical experiments.

Behavioral Experiments

Apparatus. The plastic radial maze was elevated 70.5 cm above the floor and had an octagonal center platform with eight arms separated from it. The platform was 51.5 cm in diameter and each arm was 61 cm long and 12 cm wide. Plexiglas walls were 10 cm high extending along the length of each arm. Food wells were 1-cm deep and 2 cm in diameter located 3 cm from the distal end of each arm. Several obvious extramaze cues (e.g., wall, picture, light, curtain) were present in the room and remained in the same position with respect to the maze.

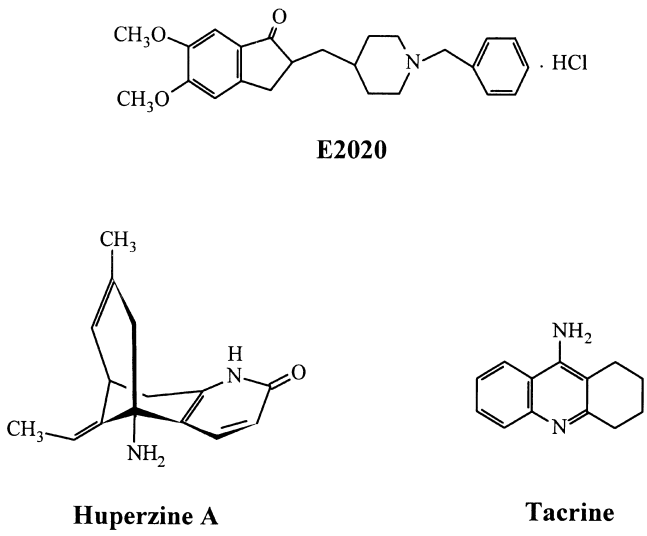

FIG. 1 Chemical structures of huperzine A, E2020, and tacrine.

Preparation and intraventricular injection of AF64A . AF64A was prepared as described previously by Fisher et al. (12). Briefly, an aqueous solution of 10 mM acetylethylcholine mustard HCl was adjusted to pH 11.3–11.5 with NaOH. After stirring for 20 min at room temperature, the pH was lowered to 7.3–7.5 using HCl. For intracerebroventricular (ICV) administration, the final concentration of AF64A was 1.0 mM. Solutions of AF64A were always prepared immediately prior to use and kept on ice at all times. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, IP) and positioned in a Narishige stereotaxic instrument and AF64A (3 nmol/3 ml/side) or saline (sham-operated control) was infused at the flow rate 1 μl/min into the lateral ventricle. The stereotaxic coordinates were from bregma: anterior −0.8 mm; lateral ±1.4 mm; and vertical −3.8 mm from the skull (27).

Radial maze training and testing. The experimental procedure reported here has been described in detail elsewhere (38). In brief, rats were placed individually on the center platform of the maze and allowed to explore the maze and to consume food pellets that were scattered on the floor of the maze for 10 min daily on 3 consecutive days to initially habituate them. Beginning with the fourth day, rats were given one training session daily, 6 days a week. At the start of each session, only four predetermined arms were used as the baited arms. The baiting pattern remained the same throughout the experiment but varied from rat to rat to limit the development of odor cues within the maze as well as controlling for directional preference with respect to the extramaze cues. Each rat was placed on the platform and left until all four baited arms were exhausted, 14 choices were made, or 10 min had elapsed, whichever occurred first. Rats were trained to a criterion of at most one error over four consecutive trials. Once a rat reached the criterion, training for this rat was reduced to two trials a week until all rats reached the criterion (range 35–50 days). The first entry into a baited arm was defined as a correct response. Three types of error were recorded as follows: first entry into the unbaited arm was regarded as an error in reference memory (RM); re-entry into the baited arm was considered as working memory (WM) error; re-entry into the unbaited arm was consider as working-reference memory (WRM) error.

Drug Testing

After all rats achieved the criterion, they received bilateral injections of AF64A or saline as described. One week following surgery, behavioral testing was resumed. Three weeks after surgery the rats were used to examine the effects of huperzine A (provided by the Department of Phytochemistry, this Institute), E2020 (provided by the Department of Synthetic Chemistry, this Institute), and tacrine (Sigma Chemical Co.) on ameliorating AF64A-induced memory deficits. Huperzine A, E2020, and tacrine were dissolved in saline and administered orally in a volume of 10 ml/kg body weight (b.wt.) 30 min prior to testing.

Activity of Choline Acetyltransferase

The choline acetyltransferase (ChAT) activity was measured as described by Leventer et al. (20). Samples were homogenized in 19 vol of ice-cold sodium phosphate buffer (75 mM, pH 7.4, 4°C). Ten microliters of aliquot of this homogenate was added to 10 μl of buffer substrate solution (sodium phosphate, 75 mM, pH 7.4; NaCl, 600 mM; $MgCl_2$, 40 mM; physostigmine, 2.0 mM; bovine serum albumin 0.05%; choline iodide, 10 mM; and [^{3}H]acetyl-coenzyme A, 0.87 mM). After

30-min incubation at 37°C, 150 μl of 3-heptanone containing 75 mg/ml sodium tetraphenylboron was added to extract radiolabelled ACh. The organic and aqueous phases were separated by centrifugation, and a 100 μl aliquot of the top (organic) layer was measured by liquid scintillation spectrometry (Beckman LS6000LL). ChAT activity was expressed as ACh formed/mg protein/h.

Biochemical Experiments

Administration of drugs. The huperzine A, E2020, and tacrine that the rats received in biochemical experiments were obtained from the same sources as they were in the behavioral experiments described above. For the studies using ICV injection, each rat was stereotaxically implanted with a permanent polyethylene cannula into the right lateral ventricle under anesthesia with pentobarbital (40 mg/kg, IP). The coordinates were 0.8 posterior to the bregma, 1.5 mm lateral to the middle, and −3.6 mm vertical to the surface of the animal's skull according to the atlas of Paxinos and Watson (27). The placement was tested by slowly draining cerebrospinal fluid. All drugs for the ICV injection were dissolved in a sterile physiological saline, and the appropriate dose was intracerebroventricularly administered in a volume of 8 μl over 1 min. For studying the effects of oral administration of each drug on cholinesterase, all drugs were dissolved in saline and administered orally in a volume of 10 ml/kg b.wt.

Preparation of tissue. For the analysis of AChE activity, rats were killed by decapitation 30 min after adminstration of the ChEI. Brains were extirpated rapidly and frontal cortex, hippocampus, striatum, and hypothalamus were dissected out on ice according to Glowinski and Iversen (16). The various brain regions of ICV-injected rats were dissected from the injected side of the brain. For the study of butyrylcholinesterase (BuChE) activity, blood was collected from the trunk of the rat immediately after decapitation. Serum was separated for determining the activity of BuChE.

Activity of cholinesterase (ChE). The ChE assay was performed using a colorimetric method by Ellman et al. (10) with slight modifications. Frontal cortex, hippocampus, hypothalamus, and striatum were homogenized in 49, 49, 49, and 249 vol of sodium phosphate buffer (75 mM, pH 7.4, 4°C), respectively. The homogenate was preincubated for 5 min at 37°C with 0.1 mM of tetraisopropyl pyrophosphoramide (iso-OMPA), a selective inhibitor of BuChE activity. For the assay of AChE or BuChE activity, a 4 ml reaction mixture that contained acetylthiocholine iodide (0.3 mM) or butyrylthiocholine iodide (0.4 mM), sodium phosphate buffer (0.1 mM, pH 7.4) 1 ml, and homogenate 0.1–0.2 ml was incubated at 37°C for 8 min. The reaction was terminated by adding 1 ml of 3% sodium lauryl sulphate, then 1 ml of 0.2% 5,5′-dithio-bis (2-nitrobenzoic) acid to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The color production was measured spectrophotometrically at 440 nm. All samples were assayed in duplicate. ChE activity was calculated as optical density (OD) value per mg protein for AChE and OD value per g protein for serum BuChE. Protein concentrations were determined with the coomassie blue protein-binding method (3) using bovine serum albumin as standard.

Statistics

Analysis of variance (ANOVA) followed by Duncan's multiple-range test was used for data obtained in the behavioral tests. The data from the biochemical studies were analyzed by Student's *t*-test.

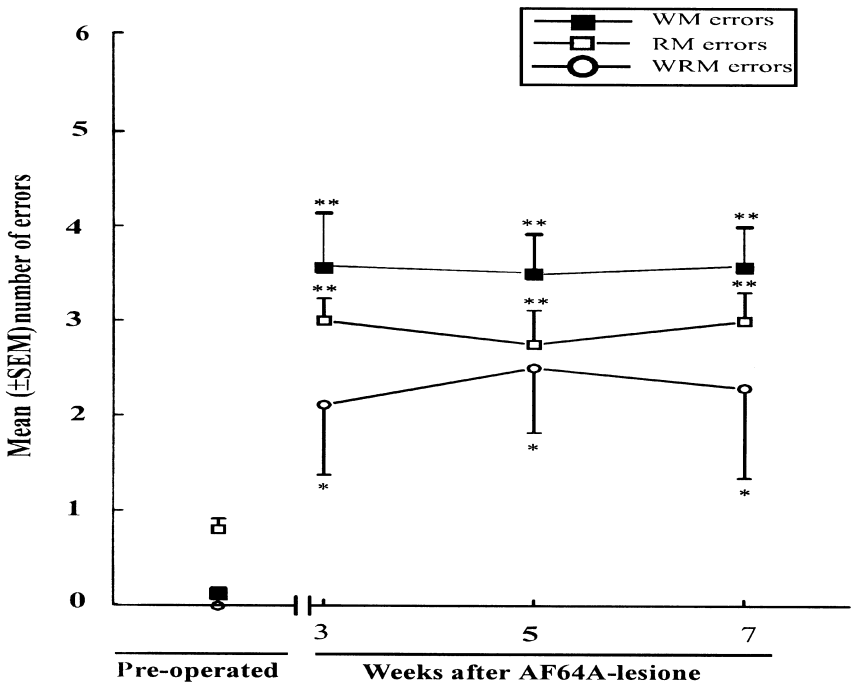

FIG. 2. Effect of ICV injection AF64A on partially baited radial maze performance in rat. Data represent mean ± SEM of eight animals in a group. $*p < 0.05$, $**p < 0.01$ vs. preoperated rats.

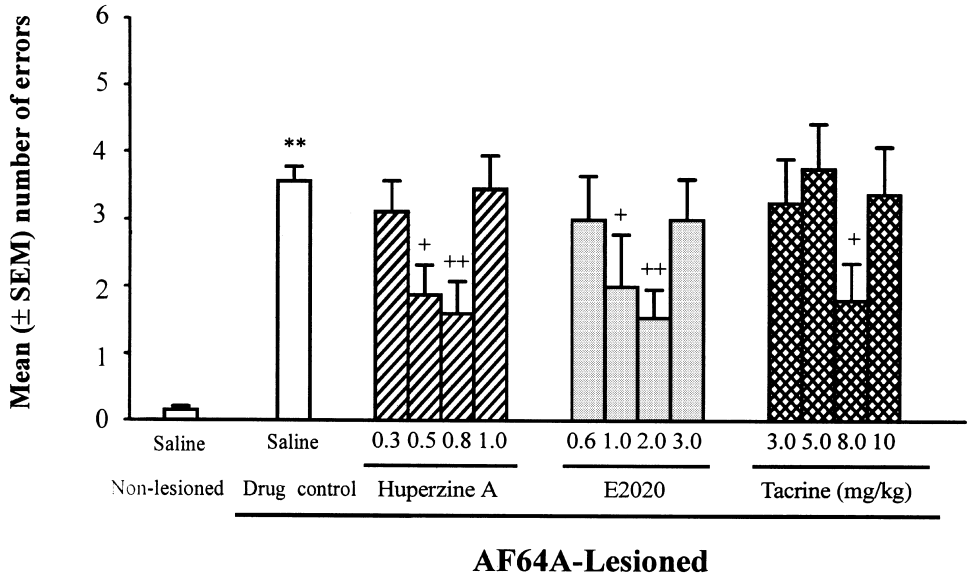

FIG. 3. Effects of oral huperzine A, E2020 and tacrine on AF64A-induced working memory deficit in a partially baited radial maze paradigm. Data expressed as mean ± SEM indicated by vertical bars ($n = 35$ for saline, non-lesioned group, $n = 43$ for AF64A-lesioned, drug control, $n = 8$–12 for each drug). $^{**}p < 0.01$ vs. the saline, non-lesioned group. $^{+}p < 0.05$, $^{++}p < 0.01$ vs. AF64A-lesioned, saline drug control group.

RESULTS

Behavioral Experiments

Stability of AF64A-induced memory deficits in the performance on partially baited radial maze paradigm. Post-operative performances on a partially baited radial maze are shown in Fig. 2. The mean numbers for the three types of error (WM, RM, and WRM) of AF64A-treated rats increased significantly compared with preoperated rats, $F(3, 28) = 15.27$, for WM errors ($p < 0.001$), $F(3,28) = 17.11$, for RM errors ($p < 0.001$), and $F(3, 28) = 2.95$, for WRM errors ($p < 0.05$). ANOVA revealed that there were no significant differences in each type of error at 3, 5, and 7 weeks after treatment with AF64A, $F(2, 21) = 0.01$, for WM errors, $F(2, 21) = 0.23$, for RM errors, and $F(2, 21) = 0.07$, for WRM errors, all $p > 0.05$. This result demonstrated that the performance levels of AF64A-treated rats on the radial maze were stable.

Effects of huperzine A, E2020, and tacrine on working and reference memory impairments induced by AF64A. Analysis of postoperative performance using a one-way ANOVA revealed significant increases for three types of error, $F(1, 76) = 188.74$, for WM errors, $F(1, 76) = 187.69$, for RM errors, and $F(1, 76) = 59.78$, for WRM errors, all $p < 0.001$, compared with saline, non-lesioned alone. The effects of saline, huperzine A, E2020, and tacrine on WM deficits of AF64A-treated rats are presented in Fig. 3

One-way ANOVA on WM error indicated that there is a significant effect with huperzine A, $F(4, 74) = 5.46$, $p < 0.001$. The AF64A-induced WM deficit was also significantly reversed by E2020 and tacrine, $F(4, 76) = 4.66$ for E2020, $p < 0.01$, and $F(4, 72) = 2.52$ for tacrine, $p < 0.05$. Post hoc comparison utilizing Duncan's multiple test revealed that huperzine A at 0.5–0.8 mg/kg ($p < 0.05$–0.01), E2020 at 1.0–2.0 mg/kg ($p < 0.05$–0.01), and tacrine at 8 mg/kg ($p < 0.05$) significantly improved the AF64A-treated working memory deficit. However, huperzine A, E2020, and tacrine all can not improve RM deficit induced by AF64A, $F(4, 74) = 1.67$, for huperzine A, $F(4, 76) = 1.78$, for E2020, and $F(4, 72) = 1.80$, for tacrine, all $p > 0.05$ compared with saline, drug control alone (Fig. 4)

The effects of huperzine A, E2020, and tacrine on the deficit of WRM performance induced by AF64A are shown in Fig. 5. Huperzine A, at a dose of 0.8 mg/kg, significantly improved the WRM errors, $F(1, 51) = 4.13$, $P < 0.05$. Similarly, significant decreases in the number of WRM errors at a dose of 2.0 mg/kg for E2020 were observed, $F(1, 54) = 4.78$, $p < 0.05$. Tacrine was ineffective in decreasing the number of WRM errors induced by AF64A compared with saline, drug control alone.

Neurochemical analysis demonstrated that ChAT activity was significantly decreased (about 50%) in the hippocampus of AF64A-treated animals. In contrast, there were no significant reductions in ChAT activity in other brain regions (Table 1).

Biochemical Experiments

Comparison of the activity of AChE and BuChE in female and male rats. The activities of AChE and BuChE in both female and male rats are presented in Table 2. There were no significant differences in AChE activities of various brain regions between the female and male. However, there was a significant difference in BuChE activity in serum between the female and male ($p < 0.01$).

Inhibitory effects of oral administered huperzine A, E2020, and tacrine on brain AChE in vivo. Results of inhibition of huperzine A, E2020, and tacrine on AChE activity in the frontal cortex, hippocampus, striatum, and hypothalamus of rats expressed as percent change from saline-treated controls are shown in Fig. 6.

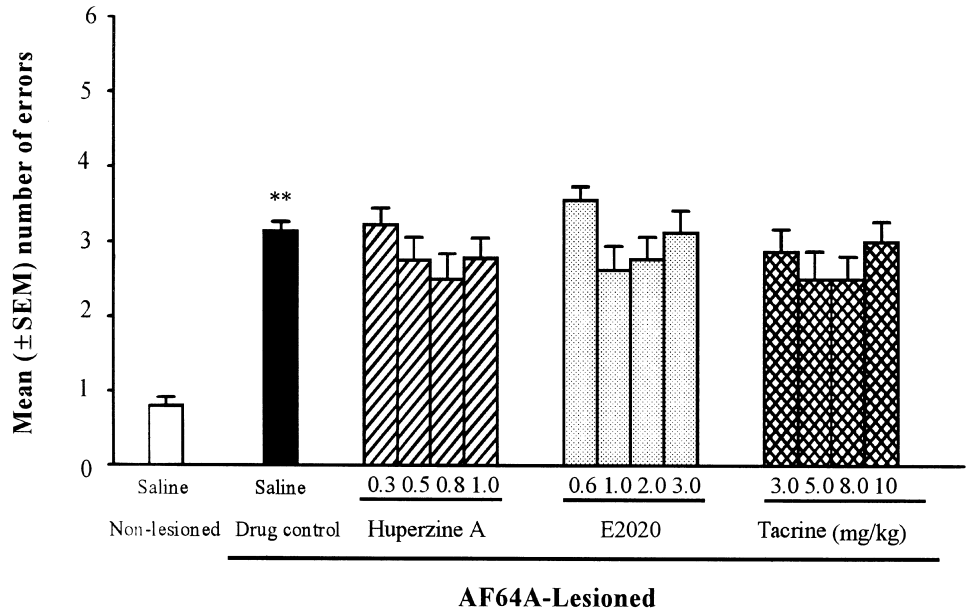

FIG. 4. Effects of oral huperzine A, E2020, and tacrine on AF64A-induced reference memory deficit in a partially baited radial maze paradigm. Data expressed as mean ± SEM indicated by vertical bars (n = 35 for saline, non-lesioned group, n = 43 for AF64A-lesioned, drug control, n = 8–12 for each drug). $^{**}p < 0.01$ vs. the saline, non-lesioned group.

Cortex. Significant inhibition of the AChE activity was observed in the cortex of rats that were sacrificed 30 min after oral administration of huperzine A at 4 μmol/kg (71.72% of control, $p < 0.01$) as well as 2 μmol/kg (82.99% of control, $p < 0.01$) compared with saline alone. AChE activity in the cortex was also significantly inhibited by E2020 at 16 μmol/kg (83.06% of control, $p < 0.01$), 8 μmol/kg (86.69% of control, $p < 0.05$), and tacrine at 128 μmol/kg (83.33% of control, $p < 0.05$). Their anti-acetylcholinesterase potencies in the cortex of rats were huperzine A > E2020 > tacrine.

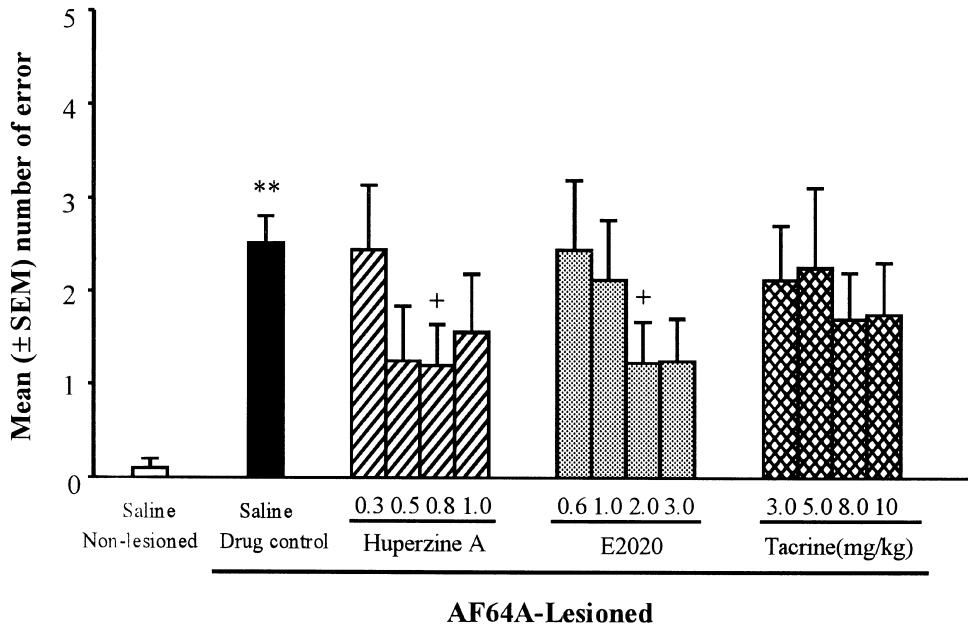

FIG. 5. Effects of oral huperzine A, E2020, and tacrine on AF64A-induced working reference memory deficit in a partially baited radial maze paradigm. Data expressed as mean ± SEM indicated by vertical bars (saline n = 35, AF64A-lesioned n = 43, n = 8–12 for each drug). $^{**}p < 0.01$ vs. the saline, non-lesioned group. $^{+}p < 0.05$ vs. AF64A-lesioned, saline drug control group.

TABLE 1

EFFECTS OF AF64A (3 nmol/sides ICV) ON CHOLINE ACETYLTRANSFERASE ACTIVITY IN DIFFERENT BRAIN REGIONS

Region	Lesion	Sham-Operated	Percentage of Sham
Hippocampus	19.6 ± 6.2**	39.7 ± 5.4	49.4
Striatum	107.4 ± 17.7	116.1 ± 14.3	92.5
Parietal cortex	35.5 ± 7.0	36.6 ± 7.7	97.0
Hypothalamus	27.0 ± 6.6	28.3 ± 8.3	95.4

Values represent means ± SD expressed as nmol ACh formed·mg protein $^{-1}$·h^{-1}.

**$p < 0.01$ vs. sham-operated group, independent *t*-tests.

Hippocampus. Administration of huperzine A significantly decreased AChE activity to 80.49 and 87.86% of control in the 4 μmol/kg ($p < 0.01$) and 2 μmol/kg group ($p < 0.01$) 30 min after oral administration. AChE activity also decreased significantly to 89.60 and 78.32% of control in the E2020 16 μmol/kg group ($p < 0.01$) and tacrine 128 μmol/kg group ($p < 0.05$). The anti-acetylcholinesterase potencies in hippocampus were huperzine A > E2020 > tacrine.

Hypothalamus. Huperzine A, E2020 and tacrine at the doses of 4, 16, and 128 μmol/kg significantly inhibited AChE activity in the hypothalamus, respectively (80.19% of control for huperzine A, 90.34% for E2020, and 84.97% for tacrine, all $p < 0.05$).

Striatum. At the dose of 4 μmol/kg, huperzine A had significant inhibitory effects on AChE in the striatum, and AChE activity remained at 81.6% of control ($p < 0.01$). Lower doses were ineffective (reference Fig. 6). All other groups had no significant effects on AChE activity in the striatum.

Inhibitory effects of ICV injection of huperzine A, E2020, and tacrine on brain AChE in vivo. The inhibitory effects of huperzine A, E2020, and tacrine on AChE expressed as percent change from saline sham controls in the frontal cortex, hippocampus, hypothalamus, and striatum of rats are shown in Fig. 7. There clearly is a dose-dependent inhibition of AChE in brain regions by huperzine A, E2020, and tacrine.

Cortex. At the doses of 16 and 8 μg, huperzine A (78.92 and 82.98% of control, $p < 0.01$) and E2020 (65.46 and 76.35% of control, $p < 0.01$) significantly decreased AChE activity in the cortex 30 min after the ICV injection, respectively. Tacrine at the doses of 64 μg and 32 μg significantly inhibited AChE activity to 89.6 and 90.8% of control ($p < 0.01$ and $p < 0.05$). The anti-acetylcholinesterase potencies were E2020 > huperzine A > tacrine.

Hippocampus. All groups of E2020 significantly inhibited AChE activity in the hippocampus 30 min after the ICV injection. AChE activity was decreased to 72.31, 76.58, and 84.63% of control in 16 μg ($p < 0.01$), 8 μg ($p < 0.01$), and 2 μg group ($p < 0.05$), respectively. Huperzine A at 16 and 8 μg, and tacrine at 64 μg also significantly decreased the AChE activity to 75.70% ($p < 0.01$), 80.78% ($p < 0.05$) and 79.78% ($p < 0.01$) of control, respectively. The anti-acetylcholinesterase potencies were E2020 > huperzine A > tacrine.

Hypothalamus. At the dose of 16 μg, E2020 and huperzine A significantly decreased AChE activity to 86.45% ($p < 0.05$) and 88.64% ($p < 0.05$) of control at 30 min following the ICV injection, respectively. At a dose of 64 μg, tacrine also decreased AChE activity to 81.12% of control ($p < 0.05$).

Striatum. E2020 and huperzine A at 16 and 8 μg significantly inhibited the AChE activity in the striatum 30 min after the ICV injection. AChE activity decreased to 70.07 and 76.94% of control (all $p < 0.01$) for E2020, 58.68% ($p < 0.01$), and 72.79% ($p < 0.05$) of control for huperzine A. At the dose of 64 μg, tacrine also significantly decreased AChE activity to 69.53% of control ($p < 0.01$).

Inhibitory effects of orally administered huperzine A, E2020, and tacrine on serum BuChE. To determine the oral effects of three ChEIs on BuChE activity in serum *in vivo*, rats were administered an oral dose that can significantly inhibit activity of brain AChE. The results indicated that tacrine at 100 μmol/kg significantly inhibited the BuChE activity compared with the saline controls in both female and male rats (all $p < 0.01$). No significant differences were observed in all other testing groups (Fig. 8).

DISCUSSION

A major problem in the drug development for AD is the lack of adequate animal models that can mimic this disease. Currently, the cholinergic deficiency hypothesis as a cause of dementia and cognitive deficits in AD is accepted. Therefore, using an animal model in which a long-term cholinergic hypofunction has been pharmacologically induced would be useful as a tool for evaluating new cholinergic therapies. Drugs that can reverse or improve an animal with a cholinergic hypofunction displaying memory and learning disorders might be applicable in the treatment of AD. At present, pharmacological long-term cholinergic hypofunction can be induced by such excitotoxins as cholinotoxin AF64A and immunotoxin

TABLE 2

DIFFERENCE OF AChE AND BuChE ACTIVITIES IN FEMALE AND MALE RATS

RAT	Activity of BuChE in Serum	Activity of AChE in Various Brain Regions			
		Cortex	Hippocampus	Hypothalamus	Striatum
Female	155.98 ± 9.55	1.47 ± 0.06	1.66 ± 0.05	1.84 ± 0.06	8.99 ± 0.39
Male	28.16 ± 2.83**	1.51 ± 0.04	1.78 ± 0.05	1.72 ± 0.06	9.03 ± 0.35

Data represent means ± SEM (n = 6–8 animals each group) expressed as OD values·mg protein^{-1} for activity of AChE and OD values·g protein^{-1} for activity of BuChE.

**$p < 0.01$ vs female.

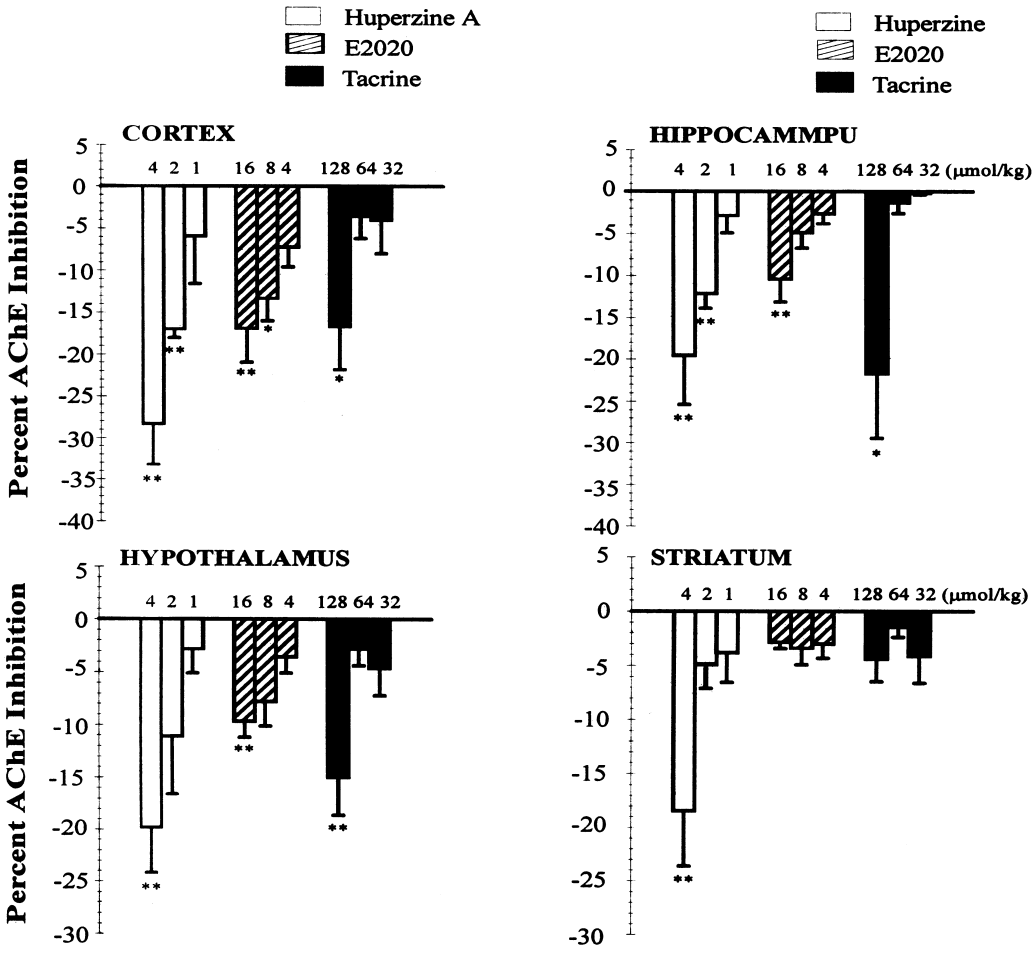

FIG. 6. Comparison of huperzine A, E2020, and tacrine on AChE inhibition in the frontal cortex, hippocampus, hypothalamus, and striatum of rats. Rats were killed 30 min after PO adminstration of huperzine A, E2020, and tacrine, respectively. Data are expressed as a percent of inhibition vs. control values ± SEM. n = 4–12 animals. AChE activity was expressed as o.d. values/mg protein. Basal saline control AChE values were: 1.46 ± 0.13 (cortex); 1.73 ± 0.14 (hippocampus); 1.78 ± 0.15 (hypothalamus); and 9.01 ± 0.87 (striatum) o.d. values/mg protein. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control. Huperzine A: 4 μmol = 968 μg, 2 μmol = 484 μg, 1 μmol = 242 μg. E2020: 16 μmol = 6656 μg, 8 μmol = 3328 μg, 4 μmol = 1664 μg. Tacrine: 128 μmol = 30,080 μg, 64 μmol = 15,040 μg, 32 μmol = 7520 μg.

192 IgG-saporin (12,23,25,30). The ICV injection of AF64A selectively causes a central cholinergic hypofunction in the hippocampus, and results in cognitive deficits in rodents (18,20,30). It has been reported that various pharmacological approaches have been successfully employed in the reverse of AF64A-induced cognitive deficits (17). The present experiments demonstrate that ICV AF64A (3 nmol/side) can produce significant impairment of the rat's performance in the partially baited radial maze paradigm. The impairment of spatial memory was paralleled by a significant decease in ChAT activity in the hippocampus. The results are consistent with the hypothesis that the cholinergic innervation of the hippocampus is necessary for performance of a radial arm maze task (2,26). Oral huperzine A exhibited a higher efficacy than did E2020 and tacrine on the improvement of AF64A-induced WM deficits. The relative potency of huperzine A on the improvement of WM deficit was found to be 2.5 and 10 times as potent as E2020 and tacrine, respectively. Additionally, huperzine A can improve the WRM deficit, which also reflects the impairment of WM. These effects may constitute a benefit for huperzine A in AD treatment, because the cognitive deficit in AD is severe for memory of recent events, whereas memory for the past remains relatively intact.

The effects of huperzine A, E2020, and tacrine on AChE activity in different brain regions in the rats were measured *in vivo* following administration of these drugs at several dose levels. They all produced a dose-dependent inhibition of AChE activity. The inhibitory potency of huperzine A differed from that of E2020 following different routes of administration. It was found that AChE inhibition induced by ICV injection of E2020 (8 μg = 0.019 μmol) was 3.5 times stronger than that of huperzine A (16 μg = 0.066 μmol). However, the converse efficacy was found following oral adminstration. The relative potency of oral huperzine A on the inhibition of brain

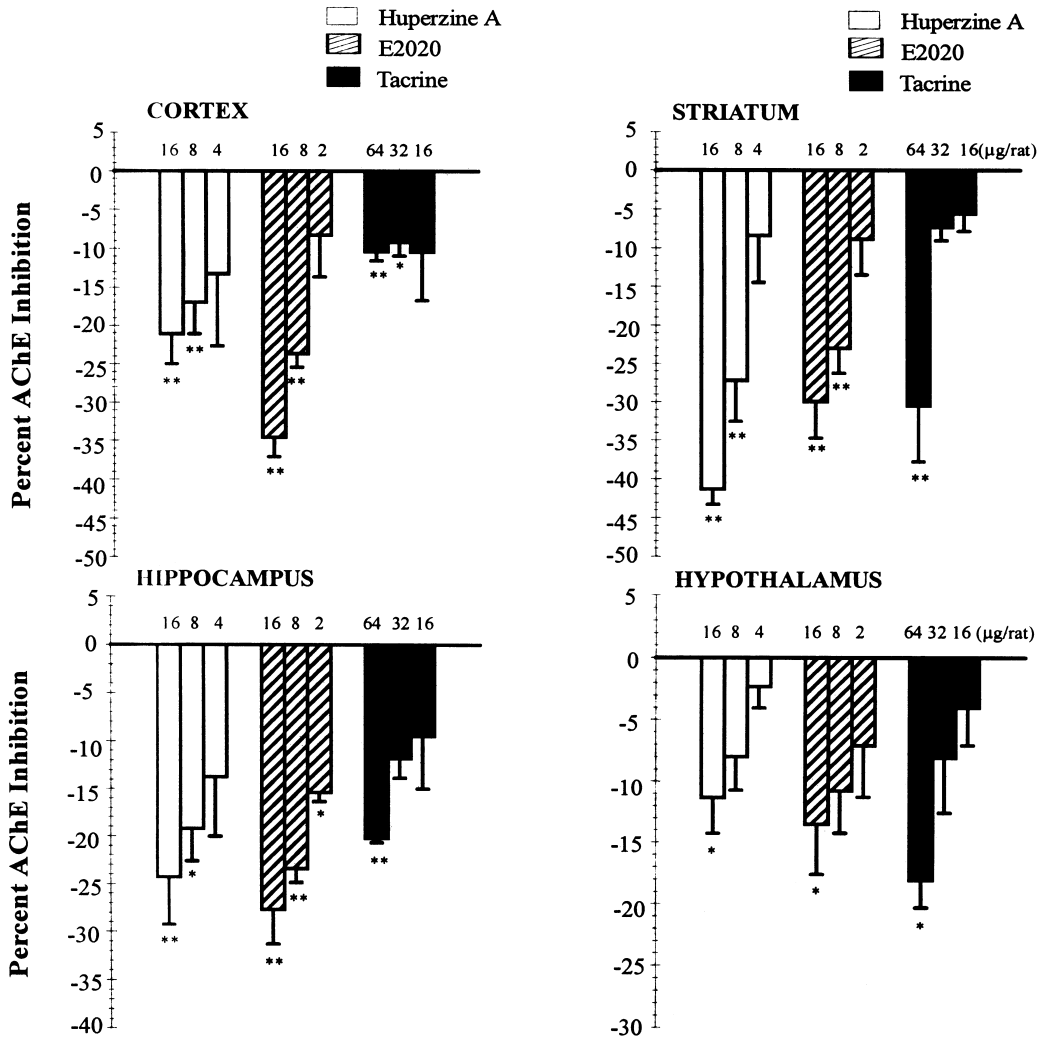

FIG. 7. Comparison of huperzine A, E2020, and tacrine on AChE inhibition in rat frontal cortex, hippocampus, hypothalamus, and striatum. Rats were killed 30 min after ICV injection of huperzine A, E2020, and tacrine, respectively. Data are expressed as percent inhibition vs. control values ± SEM. n = 4–12 animals. AChE activity was expressed as o.d. values/mg protein. Basal saline control AChE values were: 1.66 ± 0.16 (cortex); 1.99 ± 0.34 (hippocampus); 1.79 ± 0.22 (hypothalamus); and 10.68 ± 1.14 (striatum) o.d. values/mg protein. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control. Huperzine A: 16 μg = 0.066 μmol, 8 μg = 0.033 μmol, 4 μg = 0.017 μmol. E2020: 16 μg = 0.038 μmol, 8 μg = 0.019 μmol, 2 μg = 0.005 μmol. Tacrine: 64 μg = 0.272 μmol, 32 μg = 0.136 μmol, 16 μg = 0.068 μmol.

AChE activity was found to be 8 and 64 times as potent as E2020 and tacrine, respectively. The results indicated that huperzine A had higher bioavailability and/or more ability to penetrate the blood–brain barrier than did E2020 and tacrine, and can explain how huperzine A improved AF64A-induced WM deficit at a lower oral dose than did E2020 and tacrine. Furthermore, huperzine A inhibited AChE in cortex and hippocampus more strongly than did E2020 and tacrine. Because neurochemical studies have found that presynaptic cholinergic markers are significantly reduced in the cortex and hippocampus in AD (5,14), preferential inhibition of the cortex and hippocampus AChE activity could be beneficial in situations of cholinergic hypofunction and may constitute a therapeutic advantage for huperzine A. BuChE activity was measured in parallel with the brain, but was less affected by huperzine A or E2020 than by tacrine.

The present experiments showed that a dose of huperzine A (0.8 mg/kg), which improved AF64A-induced WM deficit, was correlated with about a 25–30% inhibition of AChE activity in the cortex and hippocampus. Previous studies have shown that huperzine A has no significant affinity for muscarinic and nicotinic receptors (32), was devoid of pre- and postsynaptic actions (21), as well as devoid of influence on either rate of ACh synthesis or release of ACh (19). The findings suggest that the improving effect of huperzine A on the AF64A-induced WM deficit was due primarily to its AChE inhibition in the brain.

Tacrine was much less potent in inhibiting brain AChE,

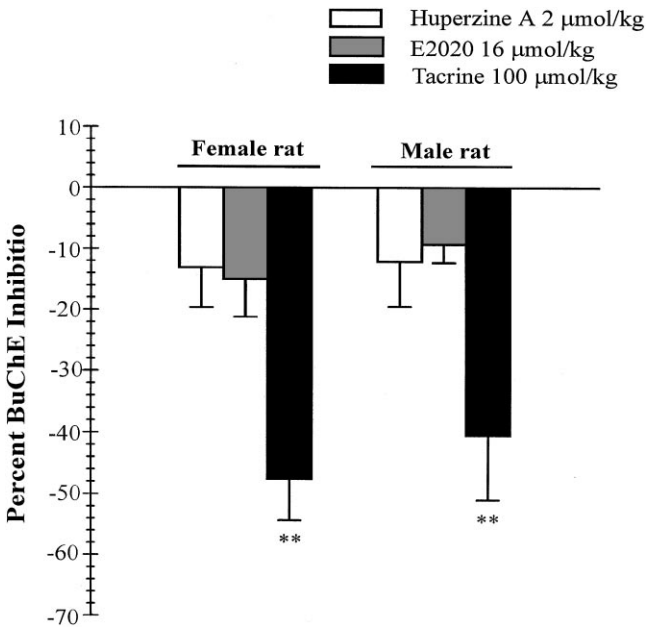

FIG. 8. Comparison of huperzine A, E2020, and tacrine on BuChE inhibition in rat serum *in vivo*. Rat blood was collected 30 min after oral huperzine A, E2020, and tacrine, respectively. Data are expressed as the mean ± SEM of 4–8 animals in each group. BuChE activity was expressed as o.d. values/g protein. Basal saline control values of serum were 155.98 ± 27.01 o.d. values/g protein for female and 28.16 ± 3.05 o.d. values/g protein for male. $^{**}p < 0.01$ vs. control.

and was more effective in inhibiting plasma BuChE than inhibiting brain AChE. The apparent inhibition constant (K_i value) for AChE is in the nM range *in vitro*, indicating tacrine has a high affinity for the enzyme (4). However, the dose of tacrine used orally is much higher than that needed to provide such an inhibitor concentration. The need for such a high dose might be explained by low bioavailability and/or by rapid metabolism. The peripheral adverse effects induced by tacrine may be related to its significant inhibition on peripheral BuChE activity. The behavior improvements induced by E2020 and tacrine were correlated to less than 10% inhibition of AChE activity in the cortex and hippocampus. These results indicate that, in addition to their ability to counteract AF64A-induced deficit, E2020 and tacrine beneficially influence WM, though it may be dependent on the other mechanisms (9,21,28).

The main disadvantages of the cholinesterase inhibitors investigated in clinical trials thus far are short duration of action in the case of physostigmine and potential liver toxicity in the case of the aminoacridine derivatives. The results obtained with huperzine A suggest that the disadvantages of AChE inhibitors might be overcome by improving CNS selectivity and thereby decreasing the peripheral cholinergic effects and toxicity. Huperzine A fits closely with the established criteria for an ideal cholinesterase inhibitor to be used in clinical studies.

ACKNOWLEDGEMENTS

We are grateful to Professor Da Yuan Zhu for preparation of huperzine A and to Professor Israel Hanin of Loyola University Chicago for his gift of acetylethylcholine mustard HCl. We also thank Miss Hui Hong Hu and Mr. Tie Wang for their technical help.

REFERENCES

1. Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S.: The colinergic hypothesis of geriatric memory dysfunction. Science 217:408–417; 1982.
2. Beatty, W. W.; Carbone, C. P.: Septal lesions, intramaze cues and spatial behavior in rats. Physiol. Behav. 24:675–678; 1980.
3. Bradford, M. M.: A rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
4. Cheng, H. C.; Ren, H.; Tang, X. C.: Huperzine A, a novel promising acetylcholinesterase inhibitor. Neuroreport 8:97–101; 1996.
5. Coyle, J. T.; Price, D. L.; DeLong, M. R.: Alzheimer's disease: A disorder of cortical cholinergic innervation. Science 219:1184–1190; 1983.
6. Davidson, M.; Stern, R. G.; Bierer, L. M.; Horvath, T. B.; Zemishlani, Z.; Markofsky, R.; R. C.: Cholinergic strategies in the treatment of Alzheimer's disease. Acta Psychiatr. Scand. Suppl. 366:47–51; 1991.
7. Davies, P.; Maloney, A. J. F.: Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2:1043; 1976.
8. Drachman, D. A.; Leavitt, J.: Human memory and the cholinergic system: A relationship to aging? Arch. Neurol. 30:113–121; 1974.
9. Drukarch, B.; Kits, K. S.; Van der Meer, E. G.; Lodder, J. C.; Stoof, J. C.: 9-Amino-1,2,3,4-tetrahydroaminoacridine (THA), an alleged drug for the treatment of Alzheimer's disease, inhibits acetylcholinesterase activity and slow outward K^+ current. Eur. J. Pharmacol. 141:153–457; 1987.
10. Ellman, G. L.; Courtney, K. D.; Andre, V., Jr.; Featherstone, R. M.: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95; 1961.
11. Fisher, A.; Hanin, I.: Potential animal models for senile dementia of Alzheimer's type, with emphasis on AF64A-induced cholinotoxicity. Annu. Rev. Pharmacol. Toxicol. 25:161–181; 1986.
12. Fisher, A.; Mantione, C. R.; Abraham, D. J.; Hanin, I.: Long-term central cholinergic hypofunction induced in mice by ethylcholine aziridinium ion (AF64A) *in vivo*. J. Pharmacol. Exp. Ther. 222:140–145;1982.
13. Fisher, A.; Mantione, C. R.; Grauer, E.; Levy, A.; Hanin, I.: Manipulation of brain cholinergic mechanisms by ethylcholine aziridinium ion (AF64A), a promising animal model for Alzheimer's disease. In: Spiegelstein, M. Y.; Levy, A.; eds. Behavioral models and analysis of drug action. Amsterdam: Elsevier; 1983: 333–342.
14. Francis, P. T.; Palmer, A. M.; Sims, N. R.; Bowen, D. M.; Davison, A. N.: Neurochemical studies of early onset Alzheimer's disease. N. Engl. J. Med. 313:7–11; 1985.
15. Giacobini, E.; Becker, R.: Development of drugs for Alzhimer therapy: A decade of progress. In: Giacobini, E.; Becker, R.; eds. Alzheimer disease: Therapeutic strategies. Boston: Birkhäuser; 1994:1–7.
16. Glowinski, J.; Iversen, L. L.: Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H]-norepinephrine, [^{3}H]-dopamine and [^{3}H]-DOPA in various regions of the brain. J. Neurochem. 13:655–659; 1966.
17. Hanin, I.: Pharmacological induction of cholinergic hypofunction as a tool for evaluating cholinergic therapies. In: Becker, R.; Giacobini, E.; eds. Alzheimer disease: From molecular biology to therapy. Boston: Birkhäuser; 1996:165–170.
18. Jarrard, L. E.; Jean Kant, G.; Meyerhoff, J. L.; Levy, A.: Behavioral and neurochemical effects of intraventricular AF64A administration in rats. Pharmacol. Biochem. Behav. 21:273–280; 1984.
19. Laganiére, S.; Corey, J.; Tang, X. C.; Wülfert, E.; Hanin, I.: Acute and chronic studies with the anticholinesterase huperzine A:

Effect on central nervous system cholinergic parameters. Neuropharmacology 30:763–768; 1991.
20. Leventer, S.; Mckeag, D.; Clancy, M.; Wulfert, E.; Hanin, I.: Intracerebroventricular administration of ethylcholine mustard aziridinium ion (AF64A) reduces release of acetylcholine from rat hippocampal slices. Neuropharmacology 24:453–459; 1985.
21. Lin, J. H.; HU, G. Y.; Tang, Xi. C.: Comparison between huperzine A, tacrine, and E2020 on cholinergic transmission at mouse neuromuscular junction *in vitro*. Acta Pharmacol. Sin. 18:6–10; 1997.
22. Liu, J. S.; Zhu, Y. L.; Yu, C. M.; Zhou, Y. Z.; Han, Y. Y.; Wu, F. W.; Qi, B. F.: The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. Can. J. Chem. 64:837–839: 1986.
23. Mantione, C. R.; Fisher, A.; Hanin, I.: The AF64A-treated mouse: Possible model for central cholinergic hypofunction. Science 213:579–580; 1981.
24. Mckinney, M.; Miller, J. H.; Yamada, F.; Tuckmantal, W.; Kozikowski, A. P.: Potencies and stereoselectivities of enantiomers of huperzine A for inhibition of rat cortical acetylcholinesterase. Eur. J. Pharmacol. 203:303–305;1991.
25. Nilsson, A.; Leanza, G.; Rosenblad, C.; Lappi, D. A.; Robertson, D.: Spatial learning impairments in rats with selective immunolesion of the forebrain cholinergic system. Neuroreport 3:1005–1008; 1992.
26. Olton, D. S. ; Becker, J. T.; Handelmann, G. E.: Hippocampus, space and memory. Behav. Brain Sci. 2:313–365; 1979.
27. Paxinos, G.; Watson, C.: The rat brain in stereotaxic coordinates. New York: Academic Press; 1982.
28. Perry, E. K.; Simith, C. J.; Court, J. A.; Bonham, J. R.; Rodwary, M.; Atack, J. R.: Interaction of 9-amino-1,2,3,4-tetrahydroaminoacridine (THA) with human cortical nicotinic and muscarinic receptor binding *in vitro*. Neurosci. Lett. 91:211–216; 1988.
29. Perry, E. K.; Tomlinson, B. E.; Blessed, G; Bergmann, K.; Gibson, P. H.; Perry, R. H.: Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br. Med. J. 2:1157–1159; 1978.
30. Potter, P. E.; Harsing, L. G., Jr.; Kakucska, I.; Gaal, G.; Vizi, E. S.: Selective impairment of acetylchloline release and content in the central nervous system following intracerebroventricular administration of ethylcholine mustard aziridinium (AF64A) in the rat. Neurochem. Int. 8:199–206; 1986.
31. Rogers, R. L.; Yamanishi, Y.; Yamatsu, K. E2020—The pharmacology of piperidine cholinesterase inhibitor. In: Becker, R.; Giacobini, E., eds. Cholinergic basis for Alzheimer therapy. Boston: Birkhäuser; 1991:314–320.
32. Tang, X. C.; De Sarno, P.; Sugaya, K.; Giacobini, E.: Effect of huperzine A, a new cholinesterase inhibitor, on the central cholinergic system of the rat. J. Neurosci. Res. 24:276–285; 1989.
33. Tang, X. C.; Xiong, Z. Q.; Qian, B. C.; Zhou, Z. F.; Zhang, C. L.: Cognition improvement by oral huperzine A: A novel acetylcholinersterase inhibitor. In: Giacobini, E.; Becker, R., eds. Alzheimer disease: Therapeutic strategies. Boston: Birkhäuser; 1994: 113–119.
34. Wang, Y. E.; Yue, D. X.; Tang, X. C.: Anti-cholinesterase activity of huperzine A. Acta Pharmacol. Sin. 7:110–113; 1986.
35. Watkins, P. B.; Zimmerman, H. J.; Knapp, M. J.; Gracon, S. I.; Lewis, K. W.: Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. JAMA 271:992–998; 1994.
36. Wilcock, G. K.; Esiri, M. M.; Bowen, D. M.; Smith, C. C. T.: Alzheimer's disease: Correlation of cortical choline acetyltransferase activity with severity of dementia and histological abnormalities. J. Neurol. Sci. 57:407–417; 1982.
37. Winbald, B.; Adem, A.; Bacman, L.; Nordberg, A; Elinder, F.; Arhem, P.: Cholinesterase inhibitors in Alzheimer's disease: Evaluation of clinical studies. In: Berker, B.; Giacobini, E., eds. Cholinergic basis for Alzheimer therapy. Boston: Birkhäuser; 1991: 238–243.
38. Wirsching, B. A.; Beninger, R. J.; Jhamandas, K.; Boegman, R. J.; El-Defrawy, S. R.: Differential effects of scopolamine on working and reference memory of rats in the radial maze. Pharmacol. Biochem. Behav. 20:659–662; 1984.