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Correlation of brain levels of 9-amino-1,2,3,4-tetrahydroacridine (THA) with neurochemical and behavioral changes

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9-Amino-1,2,3,4-tetrahydroacridine (THA) has been reported to cause improvement in patients with senile dementia of the Alzheimer's type. We have examined some effects of THA in vitro and in vivo to define its mechanism of action. In vitro, THA inhibits acetylcholinesterase (AChE) (IC_{50} = 223 nM) and blocks [^{3}H]AFDX-116 (M_2) and [^{3}H]telenzepine (M1) binding (IC_{50}s of 1.5 and 9.1 μM respectively). In vivo levels of THA were 10-fold higher in brain than plasma following 3.2 mg/kg i.p., a dose which was found to be active in reversing amnesia induced by scopolamine assessed in T-maze tests in rats and passive avoidance tests in mice. Additionally, these brain concentrations were above the IC_{50} of THA for AChE inhibition. THA (5.6-17.8 mg/kg i.p.) also elevated acetylcholine levels in the rat CNS. THA-induced side effects were blocked by the central muscarinic antagonist, scopolamine, but not by the peripheral antagonists methscopolamine and glycopyrrolate, nor by nicotinic antagonists. We conclude that brain AChE inhibition by THA is sufficient to explain its purported therapeutic activity in Alzheimer's disease and that its favorable brain/plasma distribution in vivo may account for its central cholinergic action without inducing the severe peripheral cholinergic effects typically seen with other AChE inhibitors.

THA (9-amino-1,2,3,4-tetrahydroacridine); Acetylcholinesterase inhibition; Brain acetylcholine; Cognition assays

1. Introduction

There are currently no therapeutic agents with proven efficacy for treating the cognitive deficits and memory loss associated with senile dementia of the Alzheimer's type (SDAT). Although many neurochemical markers have been reported to be altered in the brains of affected individuals, the most pronounced and consistent change in neurochemistry is in the cholinergic system as measured by decreases in acetylcholine (ACh) and in choline acetyltransferase (ChAT) (Bowen et al., 1976; Davies and Maloney, 1976). These decreases in cholinergic markers, along with the apparent involvement of ACh in memory processes (Bartus et al., 1982), have drawn attention to the central cholinergic system as a possible site for palliative therapy of SDAT. Recently, Summers et al. (1981; 1986) reported dramatic improvements in SDAT patients following administration of the potent cholinesterase inhibitor, THA. This effect is in marked contrast to that of another potent cholinesterase inhibitor, physostigmine, which yields only slight improvements in SDAT patients (Drachman and Sahakian, 1980; Thal and Fuld, 1983; Thal et al., 1983; Gustafson et al., 1987). The differences in efficacy of these two cholinesterase inhibitors suggests that THA may have non-cholinergic activities. Alternatively, THA may exhibit more favorable pharmacokinetics or fewer side effects than physostigmine.

In order to distinguish amongst these possible explanations for the clinical effects of THA, we

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have examined several neurochemical, behavioral and pharmacokinetic parameters in rats and mice following the administration of THA.

2. Materials and methods

2.1. Biochemical studies

Human acetylcholinesterase (AChE; EC 3.1.1.7) purified from red blood cells and equine butyrylcholine esterase (BuChE; EC 3.1.1.8) purified from serum were purchased from Sigma Chemical Company (St. Louis, MO). Assays were performed as described by Ellman et al. (1961), using 200 μM acetylthiocholine as substrate for AChE and 200 μM butyrylthiocholine as substrate for BuChE. Kinetic parameters were calculated from enzyme inhibition curves fitted with non-linear least squares using the programs COMP, UCOMP and NCOMP (Cleland, 1979).

Receptor binding assays were performed essentially as described by Yamamura and Snyder (1974), using rat forebrain as a source of receptor protein. Ligand concentrations and buffers are described in the legend to table 1. Tritiated ligands were purchased from New England Nuclear (Boston, MA).

Monoamine oxidase (MAO; EC 1.4.3.4) was assayed in rat brain homogenates by a modification of the method of Wurtman and Axelrod (1963), using [^{3}H]tryptamine as a substrate. Inhibition of ChAT (EC 2.3.1.6) was determined by the method of Fonnum (1975). High affinity choline uptake (HACU) into rat forebrain synaptosomes was measured as described by Boksa et al. (1988). Levels of monoamine metabolites in rat forebrain were determined by high performance liquid chromatography (HPLC) with electrochemical detection (EC) (Saller and Salama, 1984).

To determine brain and plasma THA concentrations, different protocols were used for rats and mice. For THA levels in mouse tissues, 7-bromo-THA was prepared and labelled by the tritium exchange method (New England Nuclear), the bromine acting as a leaving group to yield 7-[^{3}H]THA. The specific activity of [^{3}H]THA was 27.2 Ci/mmol, with radiochemical purity > 99%. Labelled THA was adjusted to a specific activity of 247.5 mCi/mmol by dilution with unlabelled THA hydrochloride and injected i.p. into male CD-1 mice at a dose of 3.2 mg/kg (4 mCi/kg). Animals were killed 20, 60 or 120 min later. Plasma was prepared from whole blood and stored at $-70\,^{\circ}$C until analysis. Whole brains (minus cerebellum) were frozen on dry ice. Tissues were extracted by adding 20 volumes of 0.1 M NaH_2PO_4, pH 3.0, containing 5 mM sodium octanesulfonic acid and homogenized with a polytron (Brinkman Instruments). Homogenates were centrifuged at $15\,000 \times g$ for 15 min and the supernatants used for analysis. Recoveries of total radioactivity were $86.7 \pm 2.3\%$ for brain and $101 \pm 3\%$ for plasma.

Chromatographic conditions were similar to those described by Park et al. (1986). A mobile phase consisting of 0.1 M NaH_2PO_4 with 5 mM sodium octanesulfonic acid (pH 3.0) was pumped through a cyano column (600-CN; 4.6 mm $\times$ 25 cm; Alltech) at 1 ml/min. A gradient of acetonitrile (0-30% in 5 min; 30-40% over 20 min) was used to elute THA and its metabolites; authentic THA eluted with a retention time of 15 min. Fractions (1 ml) were collected and assayed by liquid scintillation counting. The system was calibrated with unlabelled THA monitored at 327 nm.

For rat brain and plasma, a method was developed for the determination of unlabelled THA by HPLC with ultraviolet absorbance (UV) detection. Male Long-Evans rats were treated with 3.2 mg/kg THA i.p. and killed 20, 60 and 120 min after treatment. Brain tissue from fronto-parietal cortex (200 mg) was homogenized in 2 ml of distilled water. An internal standard, 9-amino-8-fluoro-1,2,3,4-tetrahydro-1,4-methanoacridine, was added to brain and plasma (0.25 ml) samples and the pH was adjusted to 10.5 with 0.1 N NaOH. Five milliliters of ethyl ether were then added; samples were vortexed and centrifuged. The ether layer was transferred to a tube containing 0.25 ml of 0.01 N HCl, vortexed and centrifuged again, the aqueous layer was neutralized with 0.1 M sodium phosphate (pH 7.0), and a 0.1 ml aliquot was assayed by HPLC. Drug separation and quantita-

tion was accomplished using a 4.4 mm × 15 cm cyano analytical column (5 μm Beckman Ultrasphere) connected to a LDC-CM 4000 programmable HPLC pump, which was equipped with a LDC-4000 UV detector and a Spectra-Physics 4200 integrator. Absorbance was monitored at 244 nm. Samples were introduced via a Perkin-Elmer ISS-100 autosampler. THA concentrations were determined by comparing the ratio of drug to internal standard peak area with similarly processed standards. The isocratic mobile phase consisted of 60% methanol and 40% 0.01 M sodium phosphate (pH 6.5), which was adjusted to pH 7.0 after mixing. The flow rate was 1.3 ml/min. Under these conditions, THA eluted at 8.3 min.

To determine ACh concentrations, THA was injected i.p. into male Long-Evans rats (225-275 g) which were killed 30 min later by microwave irradiation (1.3 kW, 5 s) (Litton Systems 70/50; General Medical, Peabody, MA). These conditions were shown to inhibit AChE activity completely in all brain regions assayed. Tissues (fronto-parietal cortex, hippocampus and striatum) were dissected and frozen on dry ice. Tissue was weighed and homogenized in 250 μl of 20 mM sodium phosphate (pH 5.3). The samples were centrifuged (15 000 × g) for 10 min and the supernatants were stored at −80 °C.

Samples were analyzed by HPLC with EC similar to the method described by Beley et al. (1987) using the ACh analysis system from Bioanalytical Systems, Inc. (West Lafayette, IN). Aliquots (10 μl) of tissue supernatants were injected and pumped at 0.5 ml/min in mobile phase (0.01 M sodium phosphate, pH 8.5 with 2 mM tetramethylammonium chloride and 0.5% tetrahydrofuran). ACh and choline (Ch) were resolved on a reverse-phase column and converted to betaine and hydrogen peroxide in a reactor column, which contained immobilized AChE and choline oxidase. Analytical and enzyme reactor columns were purchased from Bioanalytical Systems. The LC-4B amperometric detector (Bioanalytical Systems) was equipped with a platinum working electrode with the potential set at +0.5 V versus a Ag/AgCl reference electrode. The detection limit was 300 pg for ACh and 150 pg for Ch.

2.2. Behavioral studies

Male CD-1 mice (15-27 g depending on the procedure) and male Sprague-Dawley and Long-Evans rats (200-225 g) were purchased from Charles River. All animals were allowed tap water and Agway Pro Lab RMH 3000 chow (Agway, Syracuse, NY) ad libitum unless noted. The ambient temperature of the animal rooms was 21° C. Lights were on from 07:00 to 19:00. Animals were allowed to acclimate to the animal facility for at least four days before procedures began. Injection volumes were 10 ml/kg for mice and 1 ml/kg for rats.

The one-trial step-through passive avoidance procedure in mice was similar to that used by Bammer and others (for review see Bammer, 1982). The mouse was placed in the right side of the shuttle cage (Coulbourn Instruments Inc., Lehigh Valley, PA) and allowed to cross over to the other side where a constant current (unscrambled) footshock was delivered until the mouse escaped to the original side. Mice that did not cross over to the shock side within 60 s or that did not escape the footshock within 20 s were discarded from the experiment. Mice were tested for their retention of the avoidance task 24 h after the training procedure. Animals not entering the shock side within 300 s were removed and assigned a score of 300. THA (i.p.), scopolamine (s.c.) or vehicle (5% dimethyl sulfoxide (DMSO), 5% emulphor, 90% saline (0.9%)) was injected 15 min before the first session.

Learning and memory in rats was measured by using a transparent plexiglass T-maze (stem and side arms 7 × 18 × 70 cm) resting on 20 cm transparent boxes. The orientation of the maze in the room was constant. Black cylindrical (4 cm diameter) rubber cups containing the food reinforcement (approximately 50 mg pieces of chow) were placed 2 cm from the ends of the goal arms. Rats, which were food restricted until they weighed 80 ± 2% of their original weight, were placed at the base of the T with pieces of chow scattered about the maze floor and in the rubber cups during several 15 min acclimation sessions. Training sessions began after an acclimation session

occurred in which they ate all of the chow. One arm was baited and a rat was placed in the base facing the center of the T. After finding the food the animal was allowed to feed for 15 s and then removed from the arm and placed in a neutral transparent cage. Fifteen seconds later the animal was reintroduced to the maze with the opposite arm baited. This protocol was repeated three times per session. The first session of each day involved baiting the opposite arm to that which was baited during the last trial of the preceding day. Animals received i.p. and s.c. injections of vehicle (5% DMSO, 5% emulphor and 90% saline (0.9%)) 15 min before the first trial. Training sessions continued until the animals made at least 80% correct arm choices in five consecutive sessions. THA (i.p.) and/or scopolamine (s.c.) were injected 15 min prior to testing. Sessions in which vehicle was injected occurred between every drug test session. Animals were treated with vehicle (i.p.) and scopolamine once a month. Records were kept of the following parameters: (i) time taken to reach center, (ii) arm first chosen as defined by having all four paws in the arm, (iii) time to choose the baited arm and (iv) time to find the food.

Pain perception was monitored in mice (CD-1) using a hot plate thermostatically controlled at 55°C. Mice were treated with THA and/or scopolamine (N = 10/treatment) and then placed in individual plexiglass chambers on the hot plate. Jumping was monitored by photoelectric cells. Latency time was the interval between placing the animal in the apparatus and the first jump.

Food intake was measured by monitoring the amount of food consumed during a 60 min period immediately after treatment in 24 h food-deprived mice (CD-1) (N = 9/treatment) and Long-Evans rats (N = 6/treatment) treated with THA and/or scopolamine.

Side effects of THA were measured in food deprived (24 h) mice (CD-1) or rats (Long-Evans and Sprague-Dawley). Immediately after treatment (i.p.) they were placed in novel individual cages and locomotor activity, tremors, and salivation were rated, on a 0-4 scale, with 0 being none, 15, 30 and 60 min later. Fecal boluses were counted 15, 30 and 60 min after treatment. Results are presented as means of the cumulative ratings.

Drugs used were THA (Aldrich Chemical Co., Milwaukee, WI), glycopyrrolate (Robinul-V, A.H. Robins, Richmond, VA), mecamylamine hydrochloride, d-tubocurarine chloride and (−)-scopolamine hydrobromide (Sigma Chemical Co., Saint Louis, MO).

Data from the one-trial step-through passive avoidance, T-maze and side effect profiling were compared using the Mann-Whitney U test. Pain perception data were analyzed by one-way analysis of variance followed by Dunnett's test. Food intake data were analysed by Student's t-test. Brain ACh and Ch levels were analysed by two-way analysis of variance followed by Student's t-test.

3. Results

3.1. Cholinesterase inhibition

THA is a potent inhibitor of cholinesterase, with an IC_{50} of 223 ± 23 nM for the inhibition of AChE. Kinetics analysis showed that the data were best fitted by the following equation, $V = V_{max}[A]/(K_m(1 + [I]/K_{is} + [A](1 + [I])/K_{ii})$, indicating that THA possesses both competitive and uncompetitive components for the inhibition of AChE, with a competitive constant (K_{ii}) of 246 ± 66 nM and an uncompetitive component (K_{is}) of 145 ± 28 nM. THA was a much more potent inhibitor of BuChE, with an IC_{50} of 9 ± 2 nM.

3.2. Receptor binding assays

THA did not displace any of the ligands tested with high potency (table 1). In most cases, the IC_{50} for displacement was greater than 10 μM, the highest concentration tested. However, at three receptors, significant displacement was observed at less than 10 μM. Prazosin (α_2-adrenoceptor) was displaced with an IC_{50} equal to 3.3 μM and mepyramine (H_1 histamine) was displaced with an IC_{50} of 4.2 μM. Tritiated QNB was displaced from muscarinic receptors with an IC_{50} of 4.2 μM. However, QNB binding does not readily distinguish between subtypes of muscarinic receptors. To characterize further the binding of THA to these subtypes, ligand binding assays utilizing [^{3}H]telenzepine (a selective M_1 antagonist) (Schudt

TABLE 1

Binding of THA to a variety of CNS receptors.

Receptor	THA IC_{50} (μM)	Ligand (concentration)	Buffer *
ACh muscarinic	4.2	QNB (1 nM)	a
ACh muscarinic M_1	9.1	Telenzepine (2.5 nM)	b
ACh muscarinic M_2	1.5	AF DX-116 (10 nM)	c
ACh nicotinic	>10	Nicotine (4 nM)	d
α_1-Adrenoceptor	3.3	Prazosin (0.5 nM)	e
α_2-Adrenoceptor	>10	p-Aminoclonidine (0.13 nM)	e
β-Adrenoceptor	>10	Dihydroalprenolol (1 nM)	e
Dopamine D-1	>10	SCH 23390 (0.3 nM)	f
Dopamine D-2	>10	N-Propyl-norapomorphine (1 nM)	d
5-HT_{1A}	>10	8-Hydroxy-DPAT (1.5 nM)	e
5-HT_{1B}	>10	Serotonin (2 nM)	e
5-HT_2	>10	Ketanserin (1 nM)	e
Histamine H_1	4.2	Mepyramine (2 nM)	c

* Tritiated ligands were obtained from New England Nuclear and incubated with membranes prepared from rat whole brain minus cerebellum in the following buffers. a: 50 mM sodium phosphate, pH 7.5; b: 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.8; c: 50 mM sodium-potassium phosphate, pH 7.4; d: 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4; e: 50 mM Tris, pH 7.7; f: 50 mM Tris, 0.5 mM EDTA, 4 mM $MgSO_4$, pH 7.5.

et al., 1988) and [^{3}H]AFDX-116 (a selective M_2 antagonist) (Hammer et al., 1986) were performed. THA displaced [^{3}H]telenzepine with an IC_{50} of 9.1 ± 1.0 μM (N = 5) and [^{3}H]AFDX-116 with an IC_{50} of 1.5 ± 0.2 μM (N = 5).

3.3. Monoamine levels and MAO activity

To determine if THA was causing alterations in the levels of monoamines and their metabolites, THA (3 mg/kg) was injected into rats i.p. 1 h before brains were removed and prepared for analysis of 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and serotonin (5-HT) by HPLC-EC. In three separate experiments, only slight elevations (25-35%) were observed in DOPAC levels; these changes were statistically significant ($P < 0.05$) in two experiments. In one experiment, HVA was also significantly elevated by 28% ($P < 0.05$). No significant changes in dopamine, 5-HT or 5-HIAA were observed. MAO from brain or liver was not inhibited by THA at concentrations up to 100 μM.

3.4. ChAT and Ch uptake

THA had no detectable effect on ChAT activity in vitro up to a concentration of 100 μM. Similarly, THA was very weak at inhibiting high affinity Ch uptake in vitro, with an IC_{50} of 33 μM.

3.5. Brain and plasma levels of THA

The concentration of THA in mouse brain and plasma is shown in table 2. The highest concentration of THA in mouse brain, 2.52 ± 0.42 μM, was observed 20 min after i.p. dosing. Authentic THA, as judged by retention time on HPLC, comprised 56% of the total radioactivity in mouse brain at 20 min; this proportion fell to 31% at 60 min and to 18% at 120 min. Plasma THA levels were typically 8-12% of the corresponding brain levels at all times after injection. Furthermore, the fraction of total radioactivity present as THA was much lower in plasma than in brain, ranging from 12% at 20 min to 2% at 120 min (table 2). Thus, most of the label in plasma is present in metabolites even as early as 20 min after i.p. administration. The half-life of THA was similar (30-40 min) in both tissues, with apparent first-order kinetics for the elimination of parent drug.

A similar brain/plasma distribution was observed in rats treated with THA (3.2 mg/kg i.p.) (fig. 1). Brain levels of THA were highest at 20 min (4.89 ± 0.83 μM) and declined with a half-life of 1.4 h. Plasma levels remained well below 1 μM

TABLE 2

Concentration of THA in mouse brain and plasma. Data are expressed as the means ± S.E.M. from four animals at each time point following a dose of 3.2 mg THA/kg i.p.

Time (min)	Brain concentration		Plasma concentration		Brain/plasma THA ratio
	THA (μM)	%THA [a]	THA (μM)	%THA [a]	
20	2.52 ± 0.42	56 ± 2	0.266 ± 0.065	12 ± 1	9.9 ± 0.9
60	0.90 ± 0.06	31 ± 2	0.083 ± 0.017	3 ± 1	12.4 ± 3.2
120	0.34 ± 0.16	18 ± 6	0.039 ± 0.008	2 ± 1	8.0 ± 1.8

[a] This value represents the fraction of total radioactivity in the extract which was present as authentic THA determined by separation of parent drug from metabolites by HPLC.

throughout the sampling period; the plasma half-life was 1.1 h. The brain/plasma ratios of THA concentrations ranged from 7.4-9.2, demonstrating the relative accumulation of THA in brain.

In both rat plasma and brain extracts, a metabolite was observed that had an HPLC retention time of 5.8 min under the chromatographic conditions used to quantitate THA. The metabolite was isolated and purified by HPLC, and then characterized by thermospray mass spectrometry. A molecular ion corresponding to the parent ion +16 was observed. In addition, a fragment corresponding to the loss of H_2O was found. The same results were obtained when the metabolite was subjected to capillary gas chromatography-mass spectrometry. Taken together, these results indicate that the metabolite is a monohydroxylated species, and that the site of oxidation is the saturated ring. The metabolite has recently been isolated from rat urine and shown to be the 1-hydroxy-THA derivative by proton nuclear magnetic resonance spectrometry. Full details of its structural elucidation will be reported elsewhere (M.R. Nocerini, manuscript in preparation).

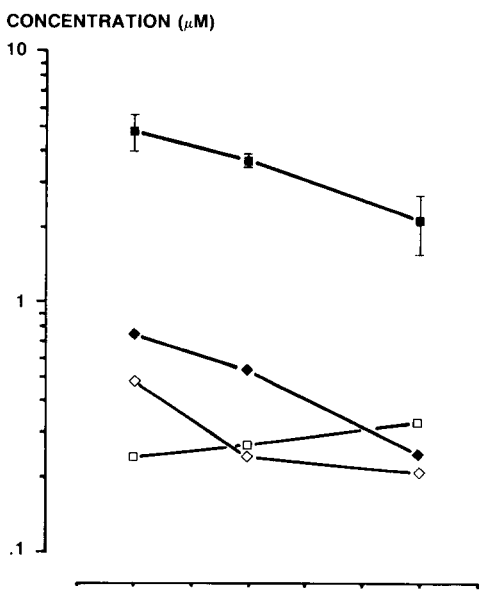

Fig. 1. THA and metabolite levels in rat brain and plasma. Male Long-Evans rats were injected with THA (3.2 mg/kg i.p.) and killed at 20, 60 and 120 min. Brain and plasma samples were extracted and analyzed as described in Methods. All values are the means ± S.E.M. of six animals at each time point. Brain THA (solid squares). Plasma THA (solid diamonds). Brain metabolite (open squares). Plasma metabolite (open diamonds).

Using a sample of synthetic 1-hydroxy-THA as a standard, concentrations of the hydroxy THA metabolite in plasma and brain were determined. Plasma levels of 0.5 μM were measured at 20 min post-dose, and declined to 0.2 μM by 2 h. In brain, concentrations of 0.2 μM were observed at 20 min, while levels at 2 h were 0.3 μM. The purified metabolite was not a potent inhibitor of AChE in vitro, having an IC_{50} of 4 μM, similar to that reported by Shutske et al. (1988).

3.6. Acetylcholine measurements

Since THA appeared to be a potent AChE inhibitor in vitro and sufficient brain levels could be achieved in vivo to inhibit the enzyme, the ability of THA to elevate brain ACh levels was determined. Three brain areas were investigated: striatum, hippocampus and cortex. THA (i.p.) produced a significant elevation in ACh in all three brain areas with a minimal effective dose of

TABLE 3

Levels of ACh and Ch in rat brain following THA treatment. Data (nmol ACh/g tissue) are expressed as the means ± S.E.M. of 8-37 animals. ACh: acetylcholine; Ch: choline.

Dose THA (mg/kg i.p.)	Striatum		Cortex		Hippocampus	
	ACh	Ch	ACh	Ch	ACh	Ch
Vehicle	66.6 ± 2.8	22.2 ± 1.3	10.2 ± 0.6	7.4 ± 0.6	15.9 ± 1.1	20.4 ± 2.1
3.2	76.1 ± 7.7	21.1 ± 3.6	11.7 ± 1.4	10.1 ± 1.6	16.9 ± 2.0	23.3 ± 5.3
5.6	80.5 ± 5.2 [a]	22.0 ± 2.4	11.5 ± 1.0	7.7 ± 1.1	16.9 ± 2.0	23.1 ± 2.2
10	90.0 ± 3.0 [a]	24.4 ± 1.4	13.3 ± 0.6 [a]	10.8 ± 0.6 [a]	19.5 ± 1.2 [a]	21.4 ± 2.2
17.8	83.4 ± 5.4 [a]	25.0 ± 2.4	14.4 ± 1.1 [a]	12.2 ± 1.1 [a]	18.1 ± 2.2	21.4 ± 4.1

[a] $P < 0.05$.

5.6 mg/kg in the striatum and 10 mg/kg in the other two brain regions (table 3).

3.7. T-Maze in rats

THA reversed scopolamine-induced amnesia in the rat T-maze paradigm within a narrow dose range (fig. 2). THA at 1 mg/kg was ineffective, 1.78 mg/kg produced marginal reversal, 3.2 mg/kg led to significant reversal, while 5.6 and 10 mg/kg resulted in ataxia.

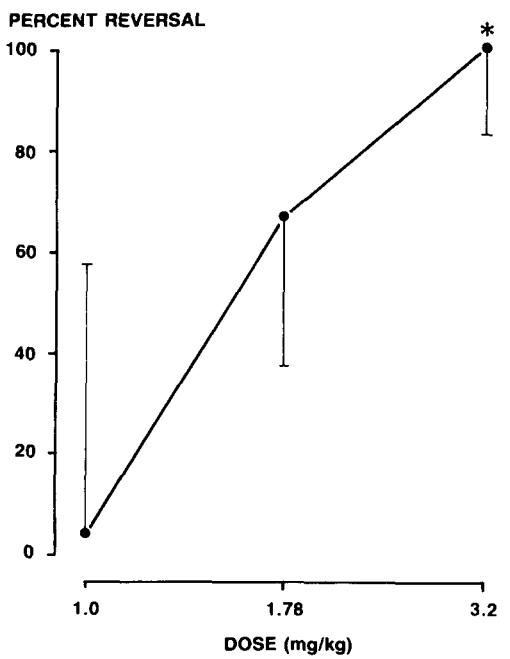

Fig. 2. Effect of THA on scopolamine amnesia in T-maze behavior in rats. Data are percent reversal of scopolamine effect on time to goal in second and third trials. Values are expressed as means ± S.E.M. of six animals at each point. THA (i.p.) and scopolamine (0.1 mg/kg s.c.) were administered 15 min before the session. A significant difference between THA and vehicle treatments is represented by an asterisk (* $P < 0.05$).

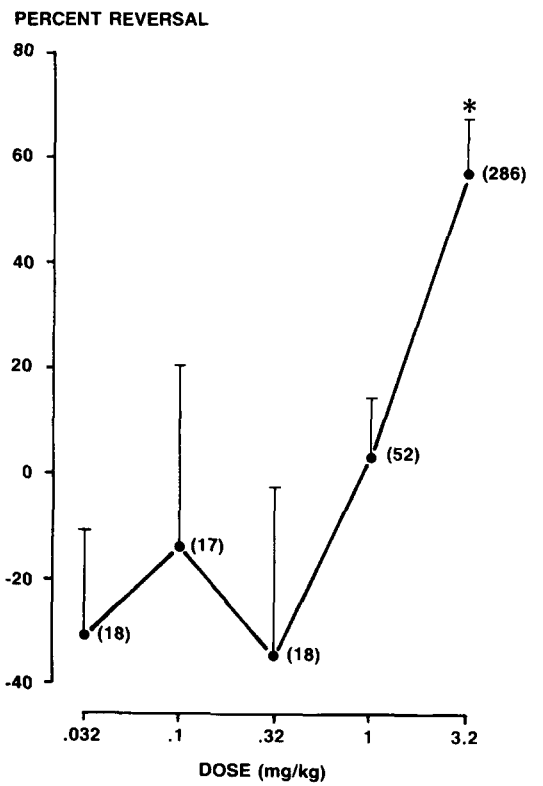

Fig. 3. Effect of THA on scopolamine amnesia in passive avoidance in mice. Data are percent reversal of scopolamine effect on step-through latency during the test session which was 24 h after the training session. Values are expressed as means ± S.E.M. The number of subjects are indicated in parentheses. THA (i.p.) and scopolamine (0.1 mg/kg s.c.) were administered 15 min before the training session. A significant difference between THA and vehicle treatments is represented by an asterisk (* $P < 0.05$).

3.8. One-trial step-through passive avoidance in mice

THA produced slight improvements in scopolamine-induced amnesia in mice (fig. 3). One dose (3.2 mg/kg i.p.) partially reversed ($P < 0.05$) a modest level of amnesia produced by a low dose of scopolamine (0.1 mg/kg s.c.) in 8 of 17 experiments at a low shock level (0.1 mA). Lower doses were ineffective, while higher doses (5.6 and 10 mg/kg) led to side effects that inhibited the animals from completing the task. Higher shock levels (0.15-0.35 mA) were tested to produce more robust learning and memory, and larger doses of scopolamine (0.25-1.5 mg/kg) were administered to produce greater amnesia. THA had no effect in various experiments where combinations of these shock levels and scopolamine were used.

3.9. Pain perception and food intake

To determine if THA was altering scopolamine-induced amnesia in the T-maze and passive avoidance through altered perception of the shock or food reward, the effect of THA and/or scopolamine on pain perception and food intake was studied. Neither THA nor scopolamine had any effect on food intake or pain perception at the doses used in the studies described above.

3.10. Side effect profiling

THA decreased locomotor activity in mice at doses of 5.6 mg/kg i.p. and higher (table 4). Slightly higher doses (17.8 and 32 mg/kg) produced tremors and salivation (table 4). These effects peaked at 30 min and were absent by 60 min. The LD_{50} for THA was 35.6 mg/kg i.p. The drug had no effect on the number of fecal boluses produced. THA produced side effects in Long-Evans and Sprague-Dawley rats very similar to those described above in mice.

In mice, both the peripheral and centrally active muscarinic receptor antagonists glycopyrrolate and scopolamine, respectively, prevented the salivation caused by THA (table 4). Scopolamine, but not glycopyrrolate, prevented low dose THA-induced hypolocomotion (table 4). At higher doses of THA, the effect was not antagonized by scopolamine. Scopolamine, but not glycopyrrolate, antagonized the tremors caused by THA (table 4). The peripheral muscarinic receptor antagonist methscopolamine had effects similar to glycopyrrolate. In mice, the peripheral and centrally active nicotinic receptor antagonists d-tubocurarine (0.32 mg/kg s.c.) and mecamylamine (1 mg/kg s.c.), respectively, had no effect on THA-induced salivation, hypolocomotion or tremors. Higher doses of d-tubocurarine (1 mg/kg s.c.) and mecamyla-

TABLE 4

Action of muscarinic antagonists on THA-induced side effects in mice. Means ± S.E.M. (N = 18-56). Control = vehicle (i.p. and s.c.). Veh. = 5:5:90 (DMSO-emulphor-saline). Glyco. = glycopyrrolate (0.01 mg/kg s.c.), Scop. = scopolamine (0.1 mg/kg s.c.). Tremor, salivation and locomotion were rated on a 0-4 scale with 0 being none.

THA mg/kg i.p.	Change from control								
	Tremors			Salivation			Locomotion		
	Veh.	Glyco.	Scop.	Veh.	Glyco.	Scop.	Veh.	Glyco.	Scop.
5.6	0±0	0±0	0±0	0±0	0±0	0±0	1.3 ±0.2	1.6 ±0.2	−0.1 ±0.1 [b]
10	0±0	0±0	0±0	0.2 ±0.1	0±0	0±0	2.5 ±0.2	2.4 ±0.3	2.2 ±0.2
17.8	1.0 ±0.1	0.9 ±0.1	0.5 ±0.1 [a]	0.9 ±0.2	0±0 [b]	0±0 [b]	2.4 ±0.2	2.5 ±0.1	2.1 ±0.3
32	2.0 ±0.3	1.6 ±0.3	0.7 ±0.2 [b]	1.2 ±0.1	0.5 ±0.2	0.1 ±0.1 [b]	2.4 ±0.3	2.2 ±0.2	2.2 ±0.2

[a] $P < 0.05$, [b] $P < 0.01$ versus THA vehicle.

mine (3.2 mg/kg s.c.) were not used because they produced hypolocomotion.

4. Discussion

THA has been reported to be effective clinically in the treatment of the cognitive deficits associated with Alzheimer's disease, and appears to be more efficacious than other cholinesterase inhibitors (Summers et al., 1981; 1986). The present study was designed to examine a variety of neurochemical, physiological and behavioral effects of THA and to correlate the potencies of THA in these assays with brain and plasma levels of the drug in rodents following behaviorally active doses.

The most striking neurochemical finding of this study was the rapid distribution of THA to the central nervous system, with brain concentrations typically exceeding plasma levels by 8 to 12-fold. The concentration of THA in the brain of mice and rats remained above the IC_{50} for inhibition of AChE for at least 2 h following i.p. administration of 3.2 mg/kg and was greater than 1 μM for the first hour. If the whole brain concentration reflects the concentration of THA at the enzyme, then this would indicate that AChE is at least 80-90% inhibited for 1 h post injection. This inhibition of AChE was shown to translate to an increase in brain ACh levels in striatum, hippocampus and fronto-parietal cortex. These elevations in brain ACh concentrations were observed at doses of 5.6-10 mg/kg i.p. Thus, it appears that at least 80-90% inhibition of AChE is necessary to elicit an increase in ACh content in brain. Although the metabolite, 1-hydroxy THA, was found in both plasma and brain, and was shown to have weak AChE inhibitory activity, its concentration at all times post-dose was too low for it to have a significant pharmacologic effect through inhibition of AChE. Other aspects of ACh synthesis, Ch levels in vivo, high affinity Ch uptake and ChAT activity in vitro, were not altered by THA at concentrations similar to those present in brain. Hence, the increases in brain ACh concentration cannot be explained by changes in Ch levels, HACU or ChAT activity, and appear to be due mainly, if not solely, to inhibition of cholinesterase.

The distribution of another cholinesterase inhibitor, physostigmine, does not show rapid accumulation in brain relative to plasma (Somani and Khalique, 1986), and physostigmine is only marginally beneficial in SDAT patients (Drachman and Sahakian, 1980; Thal and Fuld, 1983; Thal et al., 1983; Gustafson et al., 1987). Therefore, the high degree of brain exposure to THA after systemic administration of the drug does explain, in part, its apparent superiority to physostigmine as a therapeutic agent for SDAT.

That muscarinic receptors, but not nicotinic receptors, are involved with the behavioral and physiological actions of THA is supported by the reversal of the cholinergic effects of THA with selective ACh receptor antagonists. Neither centrally active (mecamylamine) nor peripherally active (d-tubocurarine) nicotinic antagonists were able to reverse any of the observed side effects caused by THA (tremor, salivation, hypolocomotion). In contrast, the centrally active muscarinic antagonist, scopolamine, blocked all of the behavioral effects and side effects induced by THA. Glycopyrrolate, a muscarinic antagonist that does not cross the blood-brain barrier, blocked only the increased salivation caused by THA. This indicates that other side effects, such as tremor and hypolocomotion, are centrally mediated. These results are consistent with the observation that glycopyrrolate is ineffective clinically at limiting the side effects associated with THA treatment (S. Gauthier, W.K. Summers, personal communications). However the data do not explain the low incidence of THA-induced tremors in man. If centrally mediated AChE inhibition is important for producing tremors, then the high brain levels of THA might be expected to lead to tremors. Some of the other side effects that we found are similar to those described by Rosic and Milosevic (1967), who reported that THA had an LD_{50} in rats after i.p. treatment of 30 mg/kg, while the THA-induced increase in salivation occurred for 30 min following 15 mg/kg i.p. and returned to control by 90 min.

As well as inhibiting AChE, another possible site for the action of THA is at ACh receptors.

Binding studies demonstrate that THA does not interact potently with nicotinic receptors (IC_{50} > 10 μM), but is somewhat more potent at muscarinic receptors, particularly the M_2 muscarinic receptor. Competition of THA with AF DX-116, a selective M_2 antagonist (Hammer et al., 1986; Wang et al., 1987), displayed an IC_{50} of 1.7 μM. This degree of inhibition is potent enough to expect significant interaction of THA with M_2 receptors at early times after injection, or after large doses of the drug. It is not yet known whether THA behaves as an agonist or an antagonist at the M_2 receptor, nor is it clear at present what effect this weak interaction may have on the behavioral actions of THA. However, M_2 receptors have been proposed to be involved with the regulation of ACh release in cortex (Raiteri et al., 1984) and striatum (Schoffelmeer et al., 1986). Such interaction could influence the activity of THA as a cholinomimetic.

In addition to its effects on cholinergic receptors, THA also interacts with a binding site in rat brain with K_d and B_{max} values of 420 ± 56 nM and $42.7 \pm$ pmol/mg protein (Mena, 1988). Furthermore, this binding is not blocked by ligands for several neurotransmitters, including compounds that interact with cholinergic receptors. The data presented here show that THA levels in the CNS are sufficient to interact with this binding site. However, because at this time the function of this site is unknown, it is impossible to determine if this interaction may contribute to the clinical results or behavioral effects of THA.

The most potent activity of THA is inhibition of BuChE (Rosic and Milosevic, 1967; this study). Normal human brain contains very low levels of BuChE activity, although brains from Alzheimer's disease patients contain relatively high levels of BuChE (Mesulam and Moran, 1987; Mesulam et al., 1987). The source and function of this enzyme in the brain is unknown; consequently the relevance of this activity of THA is difficult to ascertain.

THA has been reported to interact with monoamine metabolism in rat CNS. THA inhibits brain dopamine uptake with an IC_{50} of 7 μM (Drukarch et al., 1988). However, brain levels of THA 20 min after treatment were well below 7 μM, suggesting that THA does not directly inhibit dopamine uptake in vivo. In our experiments, acute THA treatment led to a slight but statistically significant elevation of dopamine brain metabolite levels. While DA antagonists are known to elevate dopamine metabolites, this effect of THA is not likely to be due to direct interaction with D-1 or D-2 receptors since THA brain concentrations were too low to interact significantly with these receptors. Muscarinic cholinergic agonists are known to increase dopamine metabolites in brain (Lehman and Langer, 1982; Raiteri et al., 1984; Schoffelmeer et al., 1986). Furthermore, the cholinesterase inhibitor diisopropylfluorophosphate has been shown to increase dopamine turnover in rat striatum (Potter et al., 1985). Presumably these slight changes following THA treatment result from inhibition of striatal AChE and subsequent elevation of ACh, with a corresponding enhancement of cholinergic transmission in the striatum.

THA has been shown to cause positive effects in animal cognition models. Neeleman and Kelly (1987) and Flood et al. (1985) found that THA had positive effects in mouse avoidance paradigms. THA had only marginal effects in our version of the passive avoidance shock paradigm. However, we did find that THA produced an effect in the rat T-maze model at 3.2 mg/kg i.p. This finding suggests that the rat T-maze test may be more predictive of cognitive enhancing activity of THA-like drugs than the mouse passive avoidance experimental design. Finally, Bartus and Dean (1988) as well as Fitten et al. (1988) found a very narrow window of efficacy for THA in monkeys just as we found a very narrow window of efficacy for THA in rats. They reported that doses one-half or two times the only active dose were ineffective. These very steep, inverted V shaped dose-response curves for the effects of THA in various cognition models suggest that accurate dose finding studies will be very important in treating SDAT patients with THA.

In conclusion, we find that THA rapidly accumulates in brain in vivo leading to inhibition of brain AChE and subsequent elevation in brain ACh levels in the absence of the production of severe peripheral cholinergic effects. Furthermore

the doses of THA which produce significant elevations in brain ACh are similar to those which produce behavioral activity in learning and memory paradigms. We conclude that inhibition of central AChE is the primary mechanism by which THA elicits these neurochemical and behavioral effects.

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References

Bammer, G., 1982, Pharmacological investigations of neurotransmitter involvement in passive avoidance responding: A review and some new results, Neurosci. Biobehav. Rev. 6, 247.

Bartus, R.T. and R.L. Dean, 1988, Tetrahydroaminoacridine, 3,4-diaminopyridine and physostigmine: Direct comparison of effects on memory in aged primates, Neurobiol. Aging 9, 351.

Bartus, R.T., R.L. Dean, B. Beer and A.S. Lippa, 1982, The cholinergic hypothesis of geriatric memory dysfunction, Science 217, 408.

Beley, A., A. Zekhnini, S. Lartillot, D. Fage and J. Bralet, 1987, Improved method for determination of acetylcholine, choline, and other biogenic amines in a single brain tissue sample using high performance liquid chromatography and electrochemical detection, J. Liquid Chromatogr. 10, 2977.

Boksa, P., S. Mykita and B. Collier, 1988, Arachidonic acid inhibits choline uptake and depletes acetylcholine in rat cerebral cortical synaptosomes, J. Neurochem. 50, 1309.

Bowen, D.M., C.B. Smith, P. White, A.N. Davidson, 1976, Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies, Brain 99, 459.

Cleland, W.W., 1979, Statistical analysis of enzyme kinetic data, Meth. Enzymol. 63, 103.

Davies, P. and A.J.F. Maloney, 1976, Selective loss of central cholinergic neurons in Alzheimer's disease, Lancet 2, 1403.

Drachman, D.A. and B.J. Sahakian, 1980, Memory and cognitive function in the elderly: A preliminary trial of physostigmine, Arch. Neurol. 37, 674.

Drukarch, B., J.E. Leysen and J.C. Stoof, 1988, Further analysis of the neuropharmacology profile of 9-amino-1,2,3,4-tetrahydroacridine (THA), an alleged drug for the treatment of Alzheimer's disease, Life Sci. 42, 1011.

Ellman, G., K.D. Courtney, V. Andrews, Jr. and R.M. Featherstone, 1961, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7, 88.

Fitten, L.J., K. Perryman, K. Tachiki and A. Kling, 1988, Oral tacrine administration in middle-aged monkeys: Effects on discrimination learning, Neurobiol. Aging 9, 221.

Flood, J.F., G.E. Smith and A. Cherkin, 1985, Memory enhancement: Supra-additive effect of subcutaneous cholinergic drug combinations in mice, Psychopharmacologia 86, 61.

Fonnum, F., 1975, A rapid radiochemical method for the determination of choline acetyltransferase, J. Neurochem. 24, 407.

Gustafson, L., L. Edvinsson, N. Dahlgren, B. Hagberg, J. Risberg, I. Rosen and H. Ferno, 1987, Intravenous physostigmine treatment of Alzheimer's disease evaluated by psychometric testing, regional blood flow (rCBF) measurement, and EEG, Psychopharmacologia 93, 31.

Hammer, E., E. Giraldo, G.B. Schiavi, E. Monferini and H. Ladinsky, 1986, Binding profile of a novel cardioselective muscarine receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat, Life Sci. 38, 1663.

Lehmann, J. and S.Z. Langer, 1982, Muscarinic receptors on dopamine terminals in the cat caudate nucleus: neuromodulation of ^{3}H-dopamine release in vitro by endogenous acetylcholine, Brain Res. 248, 61.

Mena, E.E., 1988, THA binding sites in rat brain, Soc. Neurosci. Abstr. 14, 208.

Mesulam, M.-M., C. Geula and M.A. Moran, 1987, Anatomy of cholinesterase inhibition in Alzheimer's disease: effect of physostigmine and tetrahydroaminoacridine on plaques and tangles, Ann. Neurol. 22, 683.

Mesulam, M.-M. and M.A. Moran, 1987, Cholinesterases within neurofibrillary tangles related to age and Alzheimer's disease, Ann. Neurol. 22, 223.

Neeleman, J. and P.H. Kelly, 1987, Reversal of scopolamine-impaired passive avoidance learning by tetrahydroaminoacridine, in: Alzheimer's Disease: Advances in Basic Research and Therapeutics, eds. R.J. Wurtman, S.H. Corkin and J.H. Growden (Center for Brain Science and Metabolic Charitable Trust).

Park, T.H., K.H. Tachiki, W.K. Summers, D. Kling, J. Fitten, K. Perryman, K. Spidell and A.S. Kling, 1986, Isolation and the fluorometric high performance liquid chromatographic determination of Tacrine, Anal. Biochem. 159, 358.

Potter, P.E., M. Hadjiconstantinou, J.S. Rubenstein and N.H. Neff, 1985, Chronic treatment with diisopropylfluorophosphate increases dopamine turnover in the striatum of the rat, European J. Pharmacol. 106, 607.

Raiteri, M., R. Leardi and M. Marchi, 1984, Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain, J. Pharmacol Exp. Ther. 228, 209.

Rosic, N. and M.P. Milosevic, 1967, The effect of tetrahydroaminoacridine on cholinesterase activity in brain of the rat, Jugoslav. Physiol. Pharmacol. Acta 3, 43.

Saller, C.F. and A.I. Salama, 1984, Rapid automated analysis of biogenic amines and their metabolites using reversed phase high performance liquid chromatography with electrochemical detection, J. Chromatogr. 309, 287.

Schoffelmeer, A.N.M., B.J. Van Vliet, G. Wardeh and A.H. Mulder, 1986, Muscarine receptor-mediated modulation of [^{3}H]dopamine and [^{14}C]acetylcholine release from rat neostriatal slices: selective antagonism by gallamine but not pirenzepine, European J. Pharmacol. 128, 291.

Schudt, C., C. Auriga, B. Kinder and N.J.M. Birdsall, 1988, The binding of ^{3}H-telenzepine to muscarinic acetylcholine receptors in calf forebrain, European J. Pharmacol. 145, 87.

Shutske, G.M., F.A. Pierrat, M.L. Cornfeldt, M.R. Szewczak, F.P. Huger, G.M. Bores, V. Haroutunian, and K.L. Davis, 1988, (±)-9-Amino-1,2,3,4-tetrahydroacrin-1-ol. A potential Alzheimer's disease therapeutic of low toxicity, J. Med. Chem. 31, 1278.

Somani, S.M. and A. Khalique, 1986, Distribution and pharmacokinetics of physostigmine in rat after i.m. administration, Fund. Appl. Toxicol. 6, 327.

Summers, W.K., L.V. Majovski, G.M. Marsh, K. Tachiki and A. Kling, 1986, Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer type, New Engl. J. Med. 315, 1241.

Summers, W.K., J.O. Viesselman, G.M. Marsh and K. Candelora, 1981, Use of THA in treatment of Alzheimer-like dementia: pilot study in twelve patients, Biol. Psychiat. 16, 145.

Thal, L.J. and P.A. Fuld, 1983, Memory enhancement with oral physostigmine in Alzheimer's disease, New Engl. J. Med. 308, 720.

Thal, L.J., P.A. Fuld, D.M. Masur and N.S. Sharpless, 1983, Oral physostigmine and lecithin improve memory in Alzheimer's disease, Ann. Neurol. 13, 491.

Wang, J.X., W.R. Roeske, K. Gulya, W. Wang and H.I. Yamamura, 1987, [^{3}H]AF-DX 116 labels subsets of muscarinic cholinergic receptors in rat brain and heart, Life Sci. 41, 1751.

Wurtman, R.J. and J. Axelrod, 1963, A sensitive and specific assay for the estimation of monoamine oxidase, Biochem. Pharmacol. 12, 1439.

Yamamura, H.I. and S.H. Snyder, 1974, Muscarinic cholinergic binding in the rat, Proc. Natl. Acad. Sci. U.S.A. 71, 1725.