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Low Dose Tetrahydroaminoacridine (THA) Improves Cognitive Function But Does Not Affect Brain Acetylcholine in Rats

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HODGES, H., A. M. RIBEIRO, J. A. GRAY AND R. M. MARCHBANKS. *Low dose tetrahydroaminoacridine (THA) improves cognitive function but does not affect brain acetylcholine in rats*. PHARMACOL BIOCHEM BEHAV **36**(2) 291–298, 1990.—Eight days of treatment with two low doses of tetrahydroaminoacridine (THA), given once daily, substantially improved radial maze performance in two groups of rats which showed persistent deficits either after ibotenic acid lesions at the source of forebrain cholinergic projections, or after 28 weeks treatment with alcohol (20% v/v) in drinking water. However, in immature, aged or aged and alcohol-treated rats, acetylcholine content was not significantly affected in any of the brain areas measured, even though the treatment regime had proved behaviourally effective. Inhibition of brain acetylcholinesterase activity was only marginally increased by this treatment regime. Thus, if THA influences behaviour by enhancing cholinergic transmission, its effects do not appear to be related to its activity as a cholinesterase inhibitor, and alternative mechanisms of action should be investigated.

Tetrahydroaminoacridine (THA) Radial maze Working memory Reference memory Acetylcholine Acetylcholinesterase inhibition Chronic alcohol Nucleus basalis, medial septal and diagonal band lesions Rat

9-AMINO-1,2,3,4-TETRAHYDROACRIDINE (THA) has been reported to be effective in treating Alzheimer's disease (14, 21, 24, 31), and to improve memory in animals (12, 13, 28), though not necessarily to a significant extent (7). Although the mechanism of action is far from clear, it has been suggested that THA acts through cholinergic (ACh) systems, either as a cholinesterase (AChE) inhibitor (19,28), or by binding to nicotinic or M2 muscarinic receptors (22, 23, 25) and so regulating release of ACh. A third possibility is that THA increases release of ACh by acting as a potassium channel blocker (10), but in this case it would also affect other transmitters such as dopamine, noradrenaline or serotonin, as has been reported (30). The potential complexity of THA's interactions with cholinergic systems makes it difficult to assess what the net effects might be, and these effects might differ according to the integrity of the cholinergic systems on which THA is thought to act.

If THA enhances memory by increasing the efficiency of cholinergic transmission, its effects would accord with the variety of findings which suggest that there is a causal link between cholinergic dysfunction and cognitive impairment (5, 6, 9, 20). Thus, neurodegenerative conditions involving memory loss, such as Alzheimer's disease or alcoholic dementias, display a variety of pathologies, but they have in common a loss of basal forebrain cholinergic cells, and low cortical levels of choline acetyltransferase (ChAT) activity (2,3). Moreover, postmortem studies have shown that there is a strong correlation between low levels of ChAT and degree of impairment in Alzheimer-demented patients (26). Cholinergic antagonists have been shown to impair, and agonists to improve, memory in both man and animals (6, 20, 27, 33). However, treatment of dementias with cholinergic agonists has not been therapeutically useful perhaps because of the narrow dose range, short duration of action and massive peripheral side effects involved (8). AChE inhibitors such as physostigmine have been occasionally successful (34), but do not appear to be as clinically effective as the initial evidence suggests that THA may be.

The present experiments had two purposes. The first was to examine the effects of two low doses of THA, given chronically (i.e., for eight days), in two groups of rats which displayed long-lasting deficits in radial maze performance after ibotenic acid lesions to cholinergic forebrain projection nuclei (nucleus basalis, projecting to cortex, and the medial septal/diagonal band area projecting to the hippocampal formation), or after 28 weeks treatment with 20% alcohol in drinking water. In both groups deficits were stable, but lesioned rats were more profoundly impaired than alcohol-treated animals. The doses of THA were chosen to minimise the risk of liver damage. They are equivalent to the top and bottom of the range reported by Summers *et al.* (31) to be effective in Alzheimer's disease patients, and have been reported (28) maximally to improve memory in mice. The second aim was to investigate the effects of this treatment regime on brain AChE activity and ACh levels, as a first step in clarifying the mode of action of THA.

METHOD

Behavioural Experiments

Animals. Male Sprague-Dawley rats with a mean weight of

431 ± 38 g, aged 8 months (Experiment 1, lesioned groups) or 516 ± 35 g, aged 14 months (Experiment 2, alcohol groups) at the start of the present experiments were fed a measured amount of food daily to maintain them at 85% of their free-feeding weight. They were kept on a 12-hr light/dark cycle and housed either four (lesioned groups) or two (alcohol groups) to a cage.

Previous treatments. The rats were part of a larger series of experiments in which effects of cholinergic lesions and alcohol treatment were examined prior to foetal cell transplants (Hodges *et al.*, in preparation). The rats had received two doses of a cholinergic agonist or antagonist, in addition to neurotoxic lesions or alcohol, before commencement of the experiment reported here.

Cholinergic lesions. Rats were anaesthetized with equithesin (3 mg/kg IP), and injected, under stereotaxic control, with ibotenic acid (Sigma), 10 mg/ml dissolved in phosphate buffered saline, at the following volumes and coordinates:

Nucleus basalis (NBM): Skull at 5° above interaural plane; V measured from dura
AP +1.0 L ±2.6 V −7.5 0.15 μl/2 min
AP +0.2 L ±3.2 V −7.0 0.20 μl/2 min

Medial septum/diagonal band (MSDB): Flat skull; V measured from dura
AP +0.2 L ±0.4 V −7.2 and 6.8 0.06 μl/2 min
AP +0.2 L 0 V −6.5, 6.0 and 5.8 0.15 μl/3 min

All injections were made over 2 or 3 min, using a Microject pump (Bioinvent HB, Sweden) and the needle was held in place for a further 4 min. For MSDB lesions the needle was slowly retracted and held at each of the specified depths for 1 min, while the pump was running, to spread the toxin over the medial septal area. Each rat was injected with ibotenate bilaterally in the NBM and MSDB. Controls received the same volumes of vehicle injected at the same sites.

Alcohol treatment. Rats were given concentrations of alcohol in drinking water increasing in steps of 2%/2 days till a 20% solution was reached, which was administered as the sole source of fluid for a further eight weeks. They were then allowed access to drinking water for 1 hr/day, and 20% alcohol for 23 hr/day. The alcohol solution was sweetened with sucrose (8.75 mg/ml) to increase palatability, so that a stable consumption, averaging 20 ml/rat/day was maintained for the rest of the 28-week treatment period. This yielded an average intake of 4.0 g/kg, and blood alcohol levels (taken in the second half of the dark period during week 24 of treatment) of the rats in the present experiment averaged 125 ± 22 mg%. Control rats were given 20 ml of the 8.75% sucrose solution daily, and consumed about 9 g/day more chow (which was available ad lib during the alcohol treatment period) than the alcohol group, so that the average daily calorific intake in the two groups was almost identical. After 28 weeks of treatment rats were given decreasing concentrations of alcohol (2%/2 days) and thus gradually withdrawn. Food deprivation for behavioural training was begun one month after withdrawal.

Behavioural testing. The radial maze had eight arms (62 cm long and 10 cm wide) radiating from a central circular platform (62 cm diameter). Two identical mazes were used, one placed exactly above the other, with the lower 62 cm and the upper 112 cm from the floor. The upper maze, with good visibility for room cues (posters, cupboards, etc.) was used for the place task, and the lower maze was used for the cue task, to enable the simultaneous testing of two rats. After pretraining, in which rats were encouraged with sucrose rewards to move freely around the maze, they were trained to find sucrose (30%) in 4 of the 8 arms, using the Jarrard (18) Place and Cue tasks. For the Place task rewards were always found in the same four arms. Our findings (Hodges *et al.*, in preparation) that rats were disrupted in the place, but not the cue task in conditions of dim light which obscured extra- but not intramaze cues, indicated that the animals utilized extramaze room cues in learning place task reward locations. For the Cue task each arm contained inserts of different textures (e.g., carpet, wire mesh, sandpaper), which were moved to a different arm after each trial, so that the rat had to learn the association between cue and reward, regardless of the location of the cue. Each rat received one of four subsets of four rewarded arms or inserts, which were distributed across rats (and all subsequent groupings) to spread possible odour cues. At the beginning of a trial a rat was placed in the centre of the maze, and allowed to explore freely, and was removed after food had been taken from all four arms, or after the elapse of 5 min for the lesioned group or 10 min for the slower moving alcohol group, while the experimenter noted each arm entered. Two types of error were scored: 1) Reference Memory errors when a rat entered a nonrewarded arm for the first time on each trial, and 2) Working Memory errors when a rewarded arm was revisited from which food had already been taken on that trial. Reference errors occurred for aspects of the task (which arms were rewarded) which were invariant from trial to trial, and thus represented a failure of long-term memory, whereas working memory errors exhibited a failure to remember which arms had been visited on a given trial, and thus taxed of short-term, trial-specific memory. Nonspecific errors of reentry into nonrewarded arm were noted separately, but were classified as working memory errors for analysis. Thus, the maze assessed four aspects of memory; spatial and associative, short- and long-term.

The cholinergic lesioned rats had been pretrained to asymptotic performance before lesioning, to assess effects of lesion on performance, whereas the alcohol-treated rats were trained after withdrawal from alcohol until controls had reached asymptotic performance, to assess effects of alcohol on acquisition. The effects of THA were tested four months postlesion, and six months after withdrawal from alcohol, when both groups had demonstrated persistent deficits in radial maze performance, relative to their controls. However, lesioned rats were far more impaired than alcohol-treated animals, with error rates approximately twice as high.

THA treatment. Rats in both treatment and control conditions were divided into three groups for treatment with saline or one of two doses of THA. There were 8 lesioned rats and 6 controls in each dosage group in Experiment 1, and 10 alcohol-treated rats and 6 controls per group in Experiment 2.

THA (Research Biochemicals Inc., 9 Erie Drive, Natick, MA 01760) was dissolved in saline and administered at doses of 3.0 and 0.3 mg/kg. Injections were given intraperitonally (IP) for eight consecutive days at 10–10.30 a.m., and rats were tested on days 4 and 8 from 12–6 p.m. on both tasks, half with the place and half with the cue task first, with an interval of ca. 3 hr between tasks. Thus, for each rat one task was performed 2–5 hr after THA treatment, and the second task within 5–8 hr after treatment. Alcohol-treated rats and their controls were run in two groups, offset by one day, to fit within the testing period. A fixed injection time was chosen, rather than a uniform treatment-test interval, because THA has been reported (28) to have a lengthy peak duration, and because of the potential clinical utility of long-lasting behavioural effects following from a single dose in the morning. Moreover, the correlations between total error scores and time of testing (i.e., injection-test interval for each rat) were found to be nonsignificant. All animals were given two trials on each task, with an intertrial interval of 10 min. Rats were also tested on days 4 and 8 after treatment.

Data Analysis

Total errors over two trials for Reference and Working memory

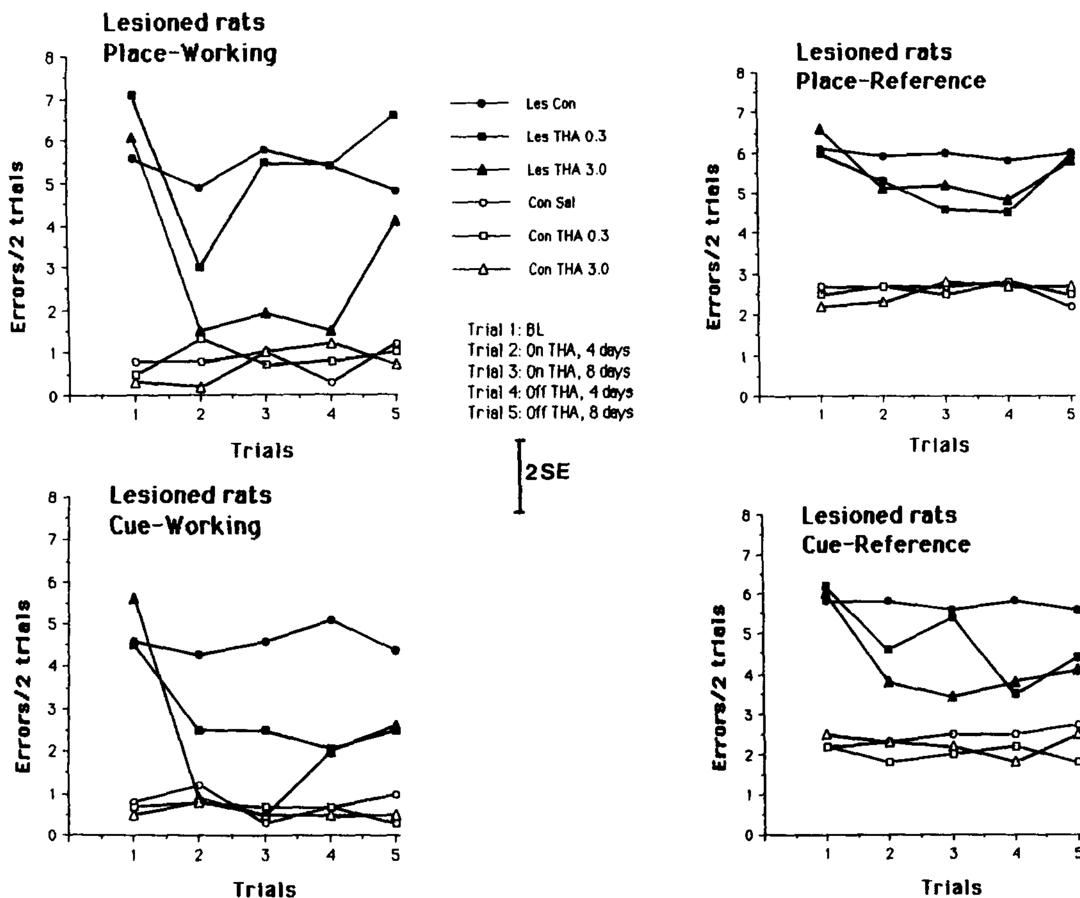

FIG. 1. Effects of THA on the radial maze performance of lesioned rats. Mean number of working and reference memory errors over 2 trials in place and cue tasks for baselines (trial 1), days 4 and 8 during treatment (trials 2 and 3) and days 4 and 8 after treatment (trials 4 and 5). Three groups of lesioned (Les) and control (Con) rats received doses of saline, 0.3 mg/kg, or 3.0 mg/kg THA once daily for 8 days.

in both Place and Cue tasks were analysed by a split plot analysis of variance, using the BMDP package. Groups (treatment, i.e., either lesion or alcohol, and control) and Dose of THA (0, 0.3 and 3.0 mg/kg) served as between S factors, and there were three within S factors of Trials (baseline, days 4 and 8 on THA, and days 4 and 8 after THA), Task (Place and Cue) and Error Type (Reference and Working). Variation in performance over trials, and its interactions were extracted by orthogonal components.

Biochemical Experiments

Animals. Ten immature rats (female Sprague-Dawley, weighing 200–270 g, aged 2–3 months) and 16 aged rats (male Sprague-Dawley, mean weight 579.8 ± 50.2 g, aged 20 months) were used. Eight of the aged rats had received chronic alcohol treatment. Animals were housed five (immature rats) or two (aged rats) to a cage and maintained in the same conditions as the rats used in the behavioural experiments, except that food was available ad lib for the immature rats. A further group of 7 immature rats was used to establish the time course of THA's effect on AChE activity.

Treatment. Immature rats were divided into two groups for IP injection with saline, or THA dissolved in saline, daily for eight consecutive days, using the higher dose (3.0 mg/kg) used in the behavioural experiments. The aged rats were drawn from alcohol-treated and control groups from which rats for the behavioural experiments with THA had also been taken. Hence the eight alcohol-treated rats had received the 28 weeks of treatment with the 20% solution followed by radial maze training, in which they had showed deficits in comparison with controls. Biochemical effects of THA were assessed 12 rather than 6 months after withdrawal, when behavioural effects were tested, as it was not possible to sacrifice animals before the end of the main series of experiments. However, groups of alcohol-treated rats which had been tested once a week for a full year after withdrawal showed consistent impairment in the radial maze throughout this period (Hodges *et al.*, in preparation), indicating that the detrimental behavioural effects of alcohol do not abate over this length of time, as Arendt *et al.* had previously shown (1,4). The two groups of aged rats (alcohol-treated and control) were each divided into two groups of four for treatment with either saline or 3.0 mg/kg of THA daily for 8 days.

Measurement of ACh. Rats were killed from 1 to 3 hr after the last THA injection. Focussed microwave irradiation was used for both immature and aged rats (30); the rats were first decapitated, and the heads irradiated immediately. The brain was rapidly removed, and dissected on ice, using a brain dissection block (16). Three regions were dissected from the brains of immature rats (frontal cortex, caudate nucleus and hippocampus) and four regions from the aged rat brains (frontal, parietal and

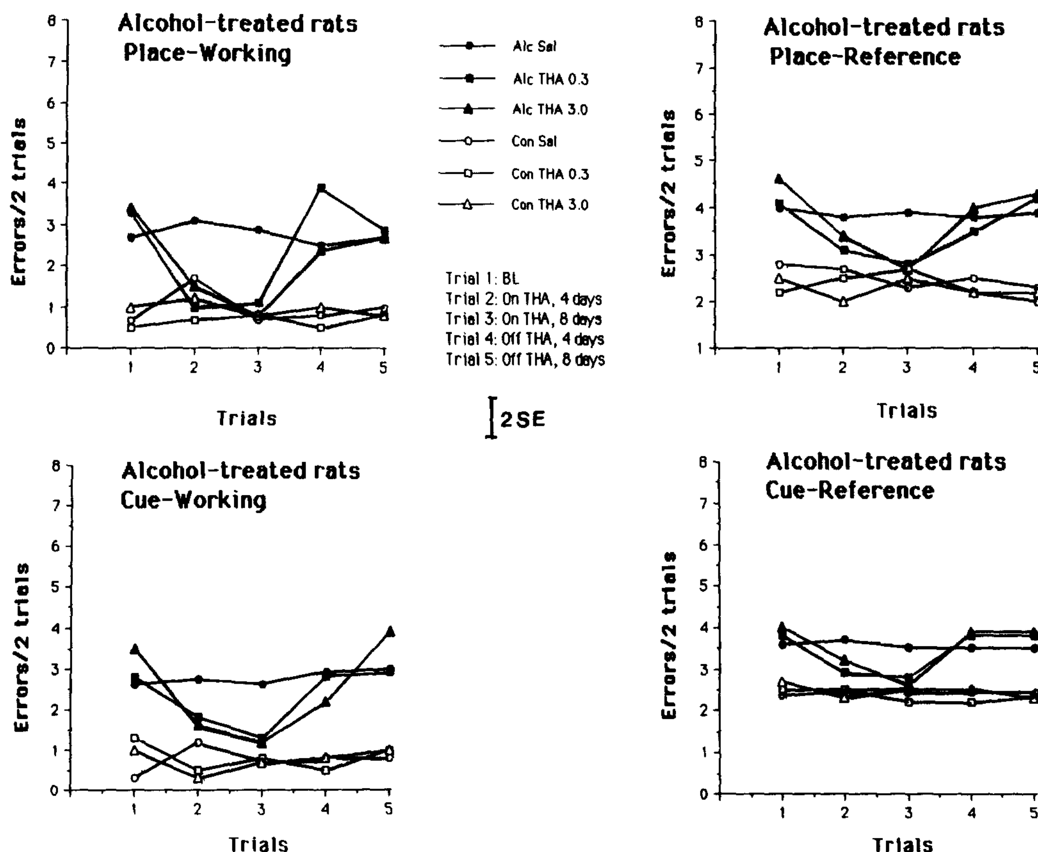

FIG. 2. Effects of THA on the radial maze performance of alcohol-treated rats. Mean number of working and reference memory errors over 2 trials in place and cue tasks for baselines (trial 1), days 4 and 8 during THA treatment (trials 2 and 3), and days 4 and 8 after treatment (trials 4 and 5). Three groups of alcohol-treated (Alc) and control (Con) rats received doses of saline, 0.3 mg/kg, or 0.3 mg/kg THA once daily for 8 days.

occipital cortex, and hippocampus).

Approximately 50–100 mg of tissue was transferred to an Eppendorf tube containing 0.5 ml of 0.5% trichloroacetic acid (TCA). After being homogenized it was centrifuged at 1000 × g for 10 min, and the supernatant separated. This procedure was repeated, and the combined supernatant (10 ml) was shaken with 4 ml ether three times to wash it, until the pH was 4, indicating that most of the TCA had been removed. The residual aqueous solution was evaporated and aliquots of 5–20 μl were used to assay ACh by the chemiluminescent method of Israel and Lesbats (17).

Measurement of AChE activity. To investigate the inhibitory effect of THA on brain anticholinesterase in situ, brain enzyme was taken from untreated rats and the inhibition of its activity by THA (or its metabolites) from the brains of rats injected daily with 3.0 mg/kg of THA for eight days was measured. Rats were killed by decapitation, from 1 to 3 hr after the last injection, and five brain areas (frontal, parietal and occipital cortex, caudate nucleus and hippocampus) were dissected as for ACh measurement. Weighed samples (40–100 mg) were then homogenized in approximately iso-osmotic 0.1 M Borate buffer pH 8.2, resulting in a final concentration of 330 mg of tissue per ml of buffer. Samples were then centrifuged at 11,000 × g for 10 min. The supernatant, essentially a brain extracellular extract, was removed and divided into two portions. One was boiled for 30 min at 100°C and the other was untreated. This manoeuvre is essential because THA is a reversible inhibitor, and if brain cholinesterase is simply measured in animals treated with THA, an incorrect low value will be obtained because of dissociation of the inhibitor from the enzyme on homogenization and dilution in the spectrophotometer cuvette. The boiled supernatant did not contain detectable AChE activity. In a further set of experiments the supernatant and a TCA extract of the pellet, subsequently neutralised, were tested for AChE inhibitory activity at 1–32 hr after injection of rats with 3 mg/kg THA, using purified AChE from Sigma. The AChE assay used a modified version of the method of Ellman *et al.* (11). A volume of 100–200 μl of boiled supernatant was added to a cuvette containing the following: 2.0–5.0 μl of untreated supernatant (containing brain cholinesterase activity), 2.5 μl of 5 mM dithiobisnitrobenzoic acid (DTNB), 3.5 μl of 50 mM acetylthiocholine; 0.1 M borate buffer, pH 8.2, was added to make the final volume 350 μl. Thus, the measurements were made with a dilution of no more than six-fold of the in situ extracellular concentration in the brain of THA or its metabolites. The development of colour was analysed at 440 nm, using a recording spectrophotometer.

RESULTS

Experiment 1: Radial Maze Performance of Lesioned Rats Under THA

Figure 1 shows that the performance of lesioned rats was improved after 4 and 8 days of THA treatment (trials 2 and 3)

TABLE 1

MEAN (± SEM) ACETYLCHOLINE LEVELS IN THREE BRAIN AREAS OF IMMATURE RATS TREATED FOR 8 DAYS WITH SALINE OR THA (3.0 mg/kg/day, N PER GROUP=5)

	nmol ACh/g Tissue	
Brain Area	Saline	THA
Frontal Cortex	14.4 ± 0.9	16.5 ± 2.2
Caudate Nucleus	30.8 ± 1.5	29.5 ± 3.8
Hippocampus	18.9 ± 1.4	19.4 ± 1.9

relative both to their own predrug baseline (trial 1) and to the performance of lesioned rats on saline. These lesioned controls maintained a stable high error rate throughout the experiment. There was no overall difference between doses of THA, but there is some indication that the decrease in errors was dose related (except for Place-Reference errors), in that by trial 3 the high dose group was making significantly fewer errors than the low dose group, and working memory errors had fallen to control levels. THA did not affect the performance of nonlesioned rats, so that all control groups showed a low and stable error rate throughout the experiment. The performance of lesioned rats on days 4 and 8 after withdrawal from THA (trials 4 and 5) deteriorated markedly. However, even eight days after treatment working memory errors were still significantly below predrug levels. By analysis of variance the difference between groups was substantial, $F(1,35)=80.53$, $p<0.0001$, indicating the extent of impairment in the lesioned rats relative to controls. Changes in performance of THA-treated lesioned rats over trials was shown by the interaction beween trials and groups, $F(4,140)=7.60$, $p<0.0001$, and the triple interaction between trials, groups and doses, $F(8,140)=2.43$, $p<0.01$. In particular, the close relationship between improvement and drug treatment in lesioned rats was shown by the significant quadratic trend over trials, $F(1,35)=8.05$, $p<0.01$. The fact that this improvement was not found in lesioned saline or any nonlesioned groups was shown by the interaction of groups with the quadratic trend of trials, $F(1,35)=9.75$, $p<0.05$, and the more marked improvement in the high dose group relative to the low dose group was indicated by the triple interaction between groups, doses and the quadratic trend of trials, $F(1,35)=3.61$, $p<0.05$. The persistence of improvement in lesioned THA-treated rats beyond the immediate treatment period was shown by the significant interaction between groups and the linear trend of trials, $F(1,35)=7.23$, $p=0.01$.

TABLE 2

MEAN (± SEM) ACETYLCHOLINE LEVELS IN CORTEX AND HIPPOCAMPUS OF AGED CONTROL AND ALCOHOL-TREATED RATS TREATED FOR 8 DAYS WITH SALINE OR THA (3.0 mg/kg/day, N PER GROUP=4)

	nmol ACh/g Tissue			
	Control		Alcohol-Treated	
Brain Area	Saline	THA	Saline	THA
Frontal Cortex	28.1 ± 5.0	26.2 ± 3.8	25.9 ± 1.7	21.5 ± 3.3
Parietal Cortex	21.1 ± 2.3	19.0 ± 0.8	21.9 ± 3.1	20.2 ± 2.0
Occipital Cortex	21.1 ± 2.2	23.8 ± 1.8	26.0 ± 1.1	22.3 ± 0.4
Hippocampus	31.1 ± 0.7	31.8 ± 1.9	32.4 ± 4.7	28.9 ± 0.6

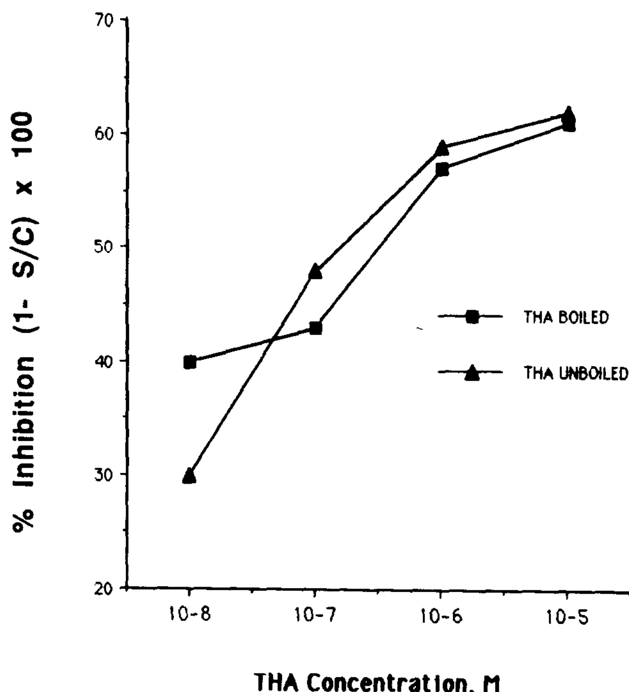

FIG. 3. The effect of THA on acetylcholinesterase activity. Standard curve for molar concentrations of THA. Acetylcholinesterase activity is expressed as moles of substrate hydrolysed/min in the presence (S) or absence (C) of THA.

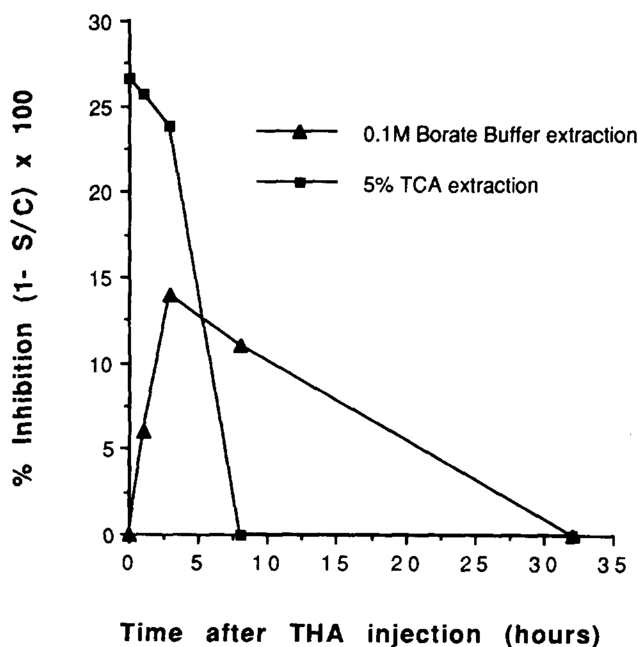

FIG. 4. Time course of the effect of THA on acetylcholinesterase activity. Rats were killed at the times shown after THA treatment (3.0 mg/kg, IP). The borate buffer extract is THA (or its metabolites) in the brain extracellular fluid, while the TCA extract is that remaining intracellularly (see the Discussion section).

TABLE 3

THE EFFECT OF THA ON BRAIN CHOLINESTERASE AND PURIFIED CHOLINESTERASE: % OF INHIBITION OF CHOLINESTERASE (PURIFIED AND FROM BRAIN) BY EXTRACT FROM RAT BRAIN 3–4 HR AFTER TREATMENT WITH THA

	% Inhibition* by Borate Extracts From THA-Treated Rats on:	
Brain Area	Brain AChE	Purified AChE
Frontal Cortex	2.4%	8.5%
Parietal Cortex	18.0%	0%
Occipital Cortex	25.0%	43.0%
Caudate Nucleus	20.0%	11.0%
Hippocampus	11.8%	20.0%

*% inhibition as $(1 - \frac{S}{C}) \times 100$. S = sample from brain treated with THA and C = effect on brain or purified AChE by extract from rat brain 3–4 hr after treatment with saline.

The effects of THA did not differ for the four aspects of memory in the radial maze. Overall, there were more errors on the Place than the Cue task, $F(1,35) = 5.51$, $p<0.025$, and substantially more Reference than Working memory errors, $F(1,35) = 23.21$, $p<0.0001$. Although the data suggest that Working memory was facilitated more than Reference memory, in that errors dropped to control level in both tasks in the high dose group, there was no significant interaction between doses and error type.

Experiment 2: Radial Maze Performance of Alcohol-Treated Rats Under THA

Figure 2 shows that both doses of THA treatment in alcohol-treated rats produced an improvement to control level in all four aspects of memory in the radial maze. Thus, there was no difference between the low and the high dose, and no interactions with type of error or task. Overall, there were more Reference than Working memory errors, $F(1,42) = 82.38$, $p<0.0001$, both types of error were maximally reduced during THA treatment. Improvement was most marked on day 8 of treatment (trial 3), but after the end of treatment error rates rapidly returned to pretreatment baseline levels. Error rates in control rats, given saline or THA, remained low and stable throughout testing, and the higher error rate of the alcohol-treated control group was also relatively stable over trials.

Analysis of variance indicated a substantial difference between groups, $F(1,42) = 58.77$, $p<0.0001$, showing that the alcohol-treated groups made more errors than the controls. There were also marked differences in performance over trials, with interactions between trials and groups, $F(4,168) = 9.20$, $p<0.0001$, trials and doses, $F(8,168) = 2.52$, $p<0.02$, and a triple interaction between trials, groups and doses, $F(8,168) = 2.53$, $p<0.025$. Trend analysis pinpointed the effects of treatment. Thus, the interaction of the quadratic trend of trials with groups, $F(1,42) = 29.58$, $p<0.0001$, doses, $F(2,42) = 9.23$, $p<0.001$, and the triple interaction of this trend with groups and doses, $F(2,42) = 5.21$, $p<0.01$, clearly demonstrate that the decrease in errors occurred only during drug treatment and was found solely in alcohol-treated rats. In contrast to the lesioned rats, the effects of THA did not persist beyond the immediate treatment period (compare Figs. 1 and 2), so that the linear trend of trials was not significant. In comparison with lesioned rats THA reduced errors, notably reference errors, more effectively in alcohol-treated than lesioned rats, but baseline error rates were much lower in the alcohol than in the lesion groups.

Acetylcholine Levels

Tables 1 and 2 show the effect of eight days of THA treatment on the levels of ACh in the areas dissected from the brains of immature, aged and aged alcohol-treated rats respectively. No significant alteration of ACh levels was observed in any group. THA treatment did not appear to modify the concentration of ACh in young or old rats with respect to their controls. In particular it did not increase ACh levels of alcohol-treated rats, even though behavioural performance was improved in such animals.

Acetylcholinesterase Activity

THA is known to inhibit erythrocyte acetylcholinesterase in vitro (19), but the lack of effect on ACh levels in brain prompted us to investigate the inhibitory effect of THA on the brain enzyme in situ.

Figure 3 gives a standard curve for THA and demonstrates that the essential step of boiling, used to destroy residual AChE activity which might compete with THA or its metabolites, had no effect on the inhibitory potency of THA.

Figure 4 shows that the inhibitory effect of THA (or its metabolites) reached a peak at 14% 3 hr after treatment.

Table 3 shows that only marginal inhibition (up to 25%) of AChE, by extracts from the brains of rats treated with THA, was found in any of the brain areas studied with supernatant fluid. This enables us to put an upper bound on the concentration of THA or its metabolites in the brain extracellular fluid of about 25 nM. This is about 1000 times less than the concentration of the dose of 3.0 mg/kg, if it had been distributed evenly throughout the body water.

DISCUSSION

The most striking feature of these experiments is that there were substantial decreases in error rates in poorly performing rats, but no increase in free ACh, and only marginal increases in inhibition of AChE activity in the five rat brain regions investigated, after 8 days of treatment with low doses of THA. Though the biochemical and behavioural parameters were not investigated in parallel groups or in the same rats, the biochemical data were so consistent across several replications in immature and aged rats, that we see no reason for the results to have differed, had we been able to conduct the experiments strictly in parallel.

There were some discrepancies in the behavioural effects of THA in the two groups. Improvement under THA was more prolonged in lesioned rats, but more pronounced in the alcohol-treated group. The apparently greater effect in the alcohol group (where there was maximal improvement even with the lower dose) may have occurred because of different error rates in the two groups. The lesioned rats were more impaired, so that a comparable degree of improvement would not bring them to control baseline, as occurred in the alcohol group. On the other hand, the lesioned rats, unlike the alcohol group, had been extensively pretrained before lesioning, so that the facilitative effects of THA may have interacted with prior learning to improve performance beyond the immediate drug treatment period. This improvement was not permanent. When tested again 20 days after the end of THA treatment, the lesioned rats were back to predrug baselines (data not shown). Alternatively, one could speculate that effects in alcohol-treated rats involved more than damage to cholinergic systems. Arendt *et al.* (4) have shown that 28 weeks of treatment with alcohol produces indices of decline in noradrenergic and serotonergic markers in rats that correlate as well with behavioural deficits as the decline in cholinergic markers. If THA exerts its behavioural effects through a blockade of potassium channels (10), its action on transmitter release would be general, and potentially enhance other systems that are damaged by alcohol, as well as the cholinergic system.

THA's lack of effect on ACh levels contrasts with the findings of Ueki *et al.* (33) who report substantial increases of ACh in cortex after chronic treatment with 3 mg/kg of THA, though their longer treatment period (21 days as opposed to our 8 days) may account for the discrepancy. Our findings are, however, in agreement with those of Hallack and Giacobini (15) who showed that after the first treatment, THA did not increase free ACh, even at a dose of 15 mg/kg, which produced a 40% inhibition of AChE activity. Thus, THA differed from AChE inhibitors such as physostigmine where both inhibition of AChE activity and elevated levels of ACh were found with repeated treatment. However, our data suggest that elevation of brain ACh content is not necessary to sustain improved performance in a memory task, and thus is not likely to account for the behavioural effects of THA.

Previous work has suggested that the behavioural effects of THA are related to its activity as an inhibitor of AChE (28), though extracellular activity in brain has not been measured. Our measurements of AChE inhibition by brain extracts would indicate that the concentration in the brain extracellular fluid of THA or any metabolite retaining AChE inhibitory potency is too low to have more than a marginal effect on brain AChE. By using an iso-osmotic homogenizing medium we have chosen to distinguish intracellular from extracellular THA. Intracellular THA will not affect outward facing acetylcholinesterase, so that only the extracellular THA concentration is relevant with respect to functional cholinesterase inhibition. Our procedure would estimate extracellular concentration with only 6-fold dilution, and we find it to be of the order of 10–25 nm. This would provide only a slight (10–25%) inhibition of brain cholinesterase, which, in view of the great functional excess of AChE, may fairly be regarded as marginal. The intracellular concentration is hardly higher. The peak concentration of both intracellular and extracellular inhibitory potency is ca. 3–6 hours, which is the period within which the bulk of the behavioural measures were made. This unexpectedly low extracellular brain concentration of THA could have several explanations. THA may be rapidly metabolised to noncholinesterase-inhibiting derivatives, or it might not penetrate the blood-brain barrier. A further possibility is that THA is sequestered in some brain cells. Further studies would be required to identify which of these conjectures is correct. Sherman and Messamore (28) report a greater degree of inhibition of AChE activity than we obtained (up to 40% in some brain regions) with comparable low doses of THA. Since it was not clear whether total or functional (i.e., extracellular) AChE inhibitory potency was being measured, this may account for the discrepancy.

Although we have not been able to detect changes in ACh, and only small changes in AChE activity as a consequence of behaviourally effective THA injections, the cholinergic hypothesis of memory function can be retained by supposing interactions with nicotinic or muscarinic autoreceptors (23,25), or potassium channel blockade (10), which might affect ACh release, but would not be detected by the crude concentration measures used here. More sophisticated in vitro procedures to detect effects on ACh dynamics in the THA concentration range of 10 to 100 nm would be required to sustain this view. In the groups of rats which provided the subjects for THA treatment, behavioural deficits occurred after lesions to projection areas containing cholinergic cells or chronic alcohol treatment which has been found to deplete cortical choline acetyltransferase (ChAT) activity along with other neurotransmitter markers (4). Deficits in both groups of rats were found to be ameliorated by low doses of cholinergic agonists (nicotine and arecoline) which did not affect controls (Hodges *et al.*, in preparation). Subsequently, improvements were found with cholinergic-rich, but not noncholinergic foetal cell implants, though of course there are other types of cell as well as those expressing ACh in the injected suspensions. These findings (Hodges *et al.*, in preparation) might suggest that behavioural impairment was related to damage to cholinergic systems in these rats, since it was alleviated by treatments which augmented cholinergic output. However, the present results with THA indicate that this may be a simplistic view.

In conclusion, these experiments show that chronic treatment with low doses of THA improves the radial maze performance of lesioned or alcohol-treated rats dramatically. However, there was no evidence that THA increased rat brain levels of ACh, and inhibition of AChE activity was only marginally increased after the treatment regime that had proved behaviourally effective. Therefore, THA would not appear to act in the same way as cholinesterase inhibitors such as physostigmine, which have been shown both to increase ACh levels and to maintain a high degree of inhibition of AChE activity throughout chronic treatment (15). Further work is required to show if THA interacts more subtly with cholinergic systems in chronic treatment, and the extent to which it interacts with other systems. Moreover, it is important to assess the reliability of treatments over several occasions (7), and over a longer treatment period, to investigate any possible development of tolerance to effects on behaviour.

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