Measurements of tacrine and monoamines in brain by *in vivo* microdialysis argue against release of monoamines by tacrine at therapeutic doses

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1 The concentration of tacrine (tetrahydroaminoacridine or THA) in plasma, regions of brain and cerebral extracellular fluid has been studied in the rat at various times following injection of a dose of 5 mg kg⁻¹, i.p.

2 The peak plasma THA concentration was 2.46 nmol ml⁻¹, and occurred 30 min post injection and clearance was first order ($t_{1/2}$ = 90 min). The concentration in the brain peaked between 30–60 min, and was around 30 times plasma concentration (striatum peak concentration = 65 ± 3 nmol g⁻¹). Extracellular cerebral concentration measured by *in vivo* microdialysis was similar to plasma concentration with the peak occurring 100 min post-injection.

3 No evidence was obtained by *in vivo* dialysis for THA inducing dopamine release from striatum or 5-hydroxytryptamine (5-HT) release from the frontal cortex. Enhanced release of dopamine did occur after (+)-amphetamine (5 mg kg⁻¹, i.p.) injection, while KCl (100 mM) in the probe released both dopamine and 5-HT.

4 Since the minimum plasma THA concentration achieved in this study was at least twice that found in the plasma of patients given THA for the treatment of dementia, these results suggest that monoamine release in the brain does not occur during therapy.

Keywords: Tacrine; *in vivo* microdialysis; monoamine release *in vivo*; 5-hydroxytryptamine release; dopamine release; cholinesterase inhibition; tacrine drug-distribution; dementia

Introduction

Tacrine (tetrahydroaminoacridine or THA) is a cholinesterase inhibitor that has been reported to be of therapeutic benefit to some patients with clinically diagnosed Alzheimer-type dementia (see for example Summers *et al.*, 1986). Whilst several other cholinesterase inhibitors and cholinceptor agonists have been investigated in senile dementia, none has been found to be as effective as THA (for review see Whalley, 1989). This has led to the suggestion that THA may possess other pharmacological properties of benefit in dementia.

In view of the structural similarity of THA to 4-aminopyridine, a potassium channel antagonist, Summers *et al.* (1986) suggested that THA might block these channels and there is some electrophysiological evidence for this (Drukarch *et al.*, 1987; Osterreider, 1987; Rogawski, 1987; Schauf & Sattin, 1987; Stevens & Cotman, 1987) albeit only at high concentrations of THA (0.1–1 mM).

Studies *in vitro* on rat brain tissue slices (Robinson *et al.*, 1989) have shown that THA can induce the release of endogenous 5-hydroxytryptamine (5-HT) and dopamine and do so by a mechanism that does not require cholinergic mechanisms since cholinceptor blockade does not affect the response. Whilst the concentrations of THA required to enhance monoamine release are lower than those required to block potassium channels, it is not clear whether such an effect occurs *in vivo* following doses of THA necessary to inhibit acetylcholinesterase. In the present study the technique of intracerebral dialysis has been used to examine 5-HT and dopamine release from rat brain *in vivo*.

Initial experiments examined the concentration of THA in both plasma and brain to ensure that doses given were sufficient to achieve concentrations at least as great as those seen in plasma during clinical studies.

Methods

Animals

Male Lister hooded rats (Harlan Olac, Bicester, U.K.) weighing 250–400 g were housed in groups of 6 in conditions of constant temperature (21°C) and controlled lighting (light period 07 h 00 min–19 h 00 min) and fed a diet of RMI expanded pellets and tap water *ad libitum*.

Determination of tacrine concentration in plasma, brain and cerebral extracellular fluid

THA was determined in plasma, brain and cerebral extracellular fluid at various times after injection of THA (5 mg kg⁻¹, i.p.).

To determine the drug in plasma or brain regions, animals were killed and blood collected or the brain removed.

Plasma was diluted 1:4 in perchloric acid (final concentration 0.3 M) and centrifuged at 10,000 *g* for 15 min. The supernatant was neutralised with K₂CO₃ (5 M) and stored overnight on ice. Samples were then centrifuged at 10,000 *g* for 15 min and the supernatant taken for determination of THA.

The brain was dissected and cortex, striatum and cerebellum isolated. Regions were homogenized in 0.4 M perchloric acid, centrifuged at 10,000 *g* for 5 min and the supernatant stored at –20°C prior to analysis.

For studies using intracerebral dialysis, probes were implanted in the striatum (see below) 24 h before drug administration. At the start of the experiment animals were anaesthetized with chloral hydrate (350 mg kg⁻¹, i.p.) and anaesthesia maintained through sample collection. The perfusate (artificial CSF, composition mM: NaCl 125, KCl 2.5, MgCl₂ 1.18, CaCl₂ 1.26) was pumped at a rate of 1 μ l min⁻¹ and 20 or 40 μ l fractions collected. The efficacy of each dialysis probe was determined before implantation by perfusion in standard solutions of THA in artificial CSF.

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THA concentrations were determined by a modification of the high performance liquid chromatography (h.p.l.c.) method of Forsyth *et al.* (1988). Dialysis samples, plasma extracts and tissue extracts were injected directly into a 250×4.6 mm Hypersil ODS ($5\mu\text{m}$) column with a Spheri-5 RP18 guard column, and eluted with 33% methanol in H_2O containing 1% triethylamine pH 5.0 at a flow rate of 1.0 ml min^{-1} . Peaks were detected with a fluorescence detector (Spectrovision, U.K.) excitatory wavelength 254 nm, emission wavelength 375 nm.

Monoamine release in vivo

Basically two protocols were used. The first involved implantation of a dialysis probe into the striatum in order to examine dopamine release, which was measured in anaesthetized animals since perfusion with a high K^+ concentration (which was used to confirm the viability of the release mechanisms) led to behavioural stimulation. The second protocol examined the release of 5-HT from the frontal cortex which was performed in freely moving rats as behavioural stimulation was not observed following perfusion with a high K^+ concentration.

Implantation of dialysis probes

Rats were anaesthetized using sodium pentobarbitone (Sagatal, 60 mg kg^{-1} i.p.) and secured in a Kopf stereotaxic frame with the tooth-bar at -3.3 mm below interaural zero. In the dopamine-release and THA concentration studies rats were implanted with a 4.0 mm dialysis probe into the striatum: $+0.8\text{ mm}$ anterior to bregma, $\pm 3.0\text{ mm}$ lateral and -7.0 mm below the surface of the brain. In the 5-HT release studies rats were implanted with a 5.0 mm dialysis probe into the frontal cortex: $+3.2\text{ mm}$ anterior to bregma, $\pm 2.0\text{ mm}$ lateral and -5.8 mm below the surface of the brain. Concentric dialysis probes (diameter, $200\mu\text{m}$) essentially as described by Hutson *et al.* (1985) were used except that they were implanted without the use of a guide assembly and the internal glass capillary tubes were replaced by fused silica tubes.

The probes were secured to the skull with 2 screws and dental cement. Two pieces of plastic tubing (Portex pp10) were attached to the inlet and outlet tubes of the dialysis probe and secured in place with quick-setting glue. The rats were then placed into individual cages and left to recover for approximately 20 h.

Collection of dialysate samples

In the investigation on dopamine release, rats were anaesthetized with chloral hydrate (350 mg kg^{-1} , i.p.) and anaesthesia maintained throughout sample collection. However in the study on 5-HT release, rats were placed in $30 \times 30 \times 25\text{ cm}$ Perspex cages where they were allowed to move freely during sample collection. Dialysis probes were perfused with artificial CSF at a rate of $1\mu\text{ l min}^{-1}$. The perfusate also contained citalopram ($1\mu\text{M}$) when 5-HT release was being investigated.

After a 1–2 h equilibration period, successive 30 or 60 min samples were collected into tubes containing 30 or $60\mu\text{ l}$, respectively, perchloric acid (0.01 M) and cysteine hydrochloride (0.2%). After collection of two baseline samples, animals received an injection of either THA (5 mg kg^{-1}) or vehicle. One hour later the concentration of KCl in the perfusate was increased to 100 mM for 30 min in half of the animals in each treatment group. Probes were then perfused with normal CSF for another 90 min. All dialysis samples from the study examining 5-HT release were analysed immediately. Samples from the study on dopamine release were frozen in solid CO_2 until analysis. Dopamine and 5-HT were stable in these conditions.

Determination of dialysate dopamine and 5-hydroxytryptamine concentrations

Dialysis samples were analysed for either dopamine or 5-HT by h.p.l.c. with coulometric detection as described previously (Robinson *et al.*, 1989). The minimum levels of detection were 20 pg for dopamine and 5 pg for 5-HT.

Behavioural observations

After injection of THA or vehicle, animals were observed for signs of chewing, flat body posture and tremor.

Drugs

Drugs were obtained from the following sources (in parentheses): 9-amino-1,2,3,4-tetrahydroacridine HCl hydrate (THA) (Aldrich Chemical Co., Dorset); sodium pentobarbitone (Sagatal) (May and Baker Ltd., Dagenham); chloral hydrate and (+)-amphetamine (Sigma Chemical Co., Dorset). Citalopram was a gift from Lundbeck, Copenhagen, Denmark. THA was dissolved in a vehicle of dimethylsulphoxide (20% v/v) and saline. All drug concentrations refer to free base.

Statistical analyses

Concentrations of dopamine and 5-HT have been expressed as pg/30 min dialysate sample. The dialysate concentrations of the first two samples from each rat were averaged to give a baseline value. The effect of drug treatments was investigated by two-way analysis of variance (ANOVA) with drug treatment as the between subjects factor and time as the repeated measure. The effects of THA and amphetamine were analysed separately. Individual means were compared by Newman Keul's post-hoc tests.

Results

The concentration of tacrine in plasma following its administration

In initial experiments rats were given THA (5 mg kg^{-1}) and the concentration measured in plasma at various times thereafter. The THA concentration peaked by 30 min and then declined and analysis of clearance revealed a first order decline with a $t_{1/2}$ of 90 min (Figure 1). THA concentration had reached around 2 nmol ml^{-1} by 15 min and peaked at 2.46 nmol ml^{-1} (Figure 1) while at 240 min it was at least twice the maximum plasma concentration of the drug seen in several clinical studies (see Discussion). The dose of 5 mg kg^{-1} was therefore chosen for subsequent experiments since it produced drug concentrations that were significantly greater than those seen clinically and thus sufficient to reveal if THA would be likely to have effects on monoamine release when used therapeutically.

The distribution of tacrine in the brain

Following THA (5 mg kg^{-1}) administration the drug could be readily detected in regions of rat brain and dialysate samples. Peak tissue concentrations occurred 30–60 min post-injection and were in the range $50\text{--}70\text{ nmol g}^{-1}$ (Figure 2). The decline was clearly not first order; however, there were insufficient data to allow further analysis.

In contrast, the extracellular concentration of the drug did not peak until around 100 min and its clearance was similar to plasma ($t_{1/2} = 75\text{ min}$). The extracellular concentration was also some 30 times lower than the tissue concentration (Figure 3).

Figure 1 Plasma tacrine (THA) concentration in the 240 min following injection of THA (5 mg kg^{-1}). Results shown as mean with s.e.mean shown by vertical bars ($n = 4$). Insert graph shows log concentration versus time curve; $t_{1/2}$ of the decline = 90 min.

Effect of tacrine on dopamine release in striatum

Administration of THA (5 mg kg^{-1}) had no effect on dopamine release in the striatum as measured by *in vivo* dialysis (Figure 4a). However amphetamine (5 mg kg^{-1} , i.p.) significantly increased dialysate dopamine concentrations compared with baseline concentrations (ANOVA $F(2,6) = 4.0$; $P < 0.05$) and the effect was similar in both the THA and saline pretreated groups (Figure 4a).

Perfusion of a high K^+ solution (100 mM KCl in artificial CSF) in animals pretreated with either saline or THA

Figure 2 Concentration of tacrine (THA) in regions of rat brain following injection of THA (5 mg kg^{-1}). Mean concentration (with s.e.mean shown by vertical bars) ($n = 4$) shown in striatum (●), cortex (□) and cerebellum (■).

Figure 3 Extracellular tacrine (THA) concentration in rat brain following injection of THA (5 mg kg^{-1}). Results shown as mean with s.e.mean indicated by vertical bars ($n = 4$).

(5 mg kg^{-1}) markedly enhanced dopamine release (ANOVA $F(5,60) = 12.5$; $P < 0.0001$) (Figure 4b) and the magnitude of this response was not altered by pretreatment with THA compared with saline.

Figure 4 (a) Dialysate dopamine concentration following injection of tacrine (THA, 5 mg kg^{-1}) (□) or vehicle (▲) and (+)-amphetamine (5 mg kg^{-1}). Results shown as mean with s.e.mean indicated by vertical bars ($n = 3$ for both groups). (b) Dialysate dopamine concentration following injection of THA (5 mg kg^{-1}) (□) or vehicle (▲) and (+)-amphetamine (5 mg kg^{-1}) and addition of KCl (100 mM) to the artificial CSF. Results shown as mean with s.e.mean indicated by vertical bars. (THA-treated, $n = 8$; vehicle, $n = 6$).

Figure 5 Dialysate 5-hydroxytryptamine (5-HT) concentration following injection of tacrine (THA, 5 mg kg^{-1}) ($\square$) or vehicle ($\blacktriangle$) and addition of KCl (100 mM) to the artificial CSF. Results shown as mean with s.e. mean indicated by vertical bars ($n = 5$ for both groups).

Effect of tacrine on 5-hydroxytryptamine release

Administration of THA (5 mg kg^{-1}) had no effect on 5-HT release from the frontal cortex either 30 min immediately following injection (Figure 5) or over a period of 2 h (data not shown). However, perfusion of CSF containing high K^+ (100 mM) markedly increased 5-HT release (ANOVA $F(5,40) = 20.1$; $P < 0.001$) and the response was similar in both THA and saline pretreated rats (Figure 5).

Behavioural observations

All the conscious animals injected with THA (5 mg kg^{-1}) showed chewing, flat body posture and tremor. These responses were seen within 5 min of administration of THA and remained constant over the next 2 h. Some tremor could still be observed 4 h later. Responses observed were about half the maximal occurring after a dose of 20 mg kg^{-1} as has previously been reported by Hunter *et al.* (1989). None of the saline-injected rats displayed any of these behaviours.

Discussion

A dose of THA (5 mg kg^{-1} , i.p.) to rats produced a concentration of the drug in the plasma at 240 min that was at least twice the steady state concentration seen in the clinical study of Summers *et al.* (1986) or the peak values seen following a dose of THA of 50 mg given to patients in the studies of Nybäck *et al.* (1988) and Forsyth *et al.* (1988). All these workers recorded plasma levels of around 50 ng ml^{-1} ($= 0.25 \text{ nmol ml}^{-1}$). A dose of THA of 5 mg kg^{-1} in rats therefore seemed suitable in order to study what concentration of drug might occur in cerebral tissues and whether it would enhance monoamine release in the brain *in vivo* as has been observed *in vitro* (Robinson *et al.*, 1989; Drukarch *et al.*, 1988). This in turn would indicate whether such a mechanism might occur when the drug was being given to Alzheimer's disease patients.

As has been observed previously (Nielsen *et al.*, 1989) THA is concentrated in cerebral tissue following peripheral administration. The concentration in the striatum and cortex being some 25–35 times greater than in plasma 60 min after injection (compare Figures 1 and 2). The tissue concentration of THA at 2 h was also considerably higher (around 25 nmol g^{-1}) than that found by Tachiki *et al.* (1988) who reported a maximum value of 1142 ng g^{-1} ($= 5.8 \text{ nmol g}^{-1}$) after a dose of THA of 3 mg kg^{-1} . In contrast, the cerebral extracellular THA concentration as measured by *in vivo* microdialysis was rather similar

to that of plasma. The clearance rate was also similar but the peak occurred later.

Following THA (5 mg kg^{-1}) all animals displayed marked central cholinergically mediated behaviour (tremor and chewing) and this is to be expected since the K_i for acetylcholinesterase inhibition by THA is $0.06 \mu\text{M}$ (Hunter *et al.*, 1989) which is considerably less than both the peak tissue concentration (around $60 \mu\text{M}$) and also the extracellular concentration ($1.5 \mu\text{M}$). However, following this dose of THA no evidence was obtained for a release of dopamine from the striatum. In contrast, amphetamine (5 mg kg^{-1}) induced a marked release of dopamine which was similar in THA-treated and control rats. Our *in vitro* data had indicated that amphetamine and THA were approximately equipotent in releasing dopamine from slices (Robinson *et al.*, 1989). The current results therefore suggest both that the dialysis technique is adequate for detecting release and also that THA, unlike amphetamine, does not induce dopamine release *in vivo* at pharmacologically relevant doses. The addition of 100 mM KCl to the dialysis medium also induced a substantial dopamine release which returned to basal levels on removal of the high KCl. This 'potassium-induced' release of dopamine was also unaffected by THA administration, consistent with previous observations *in vitro* on brain slices (Robinson *et al.*, 1989). Overall the data suggest that the dopamine-releasing action of THA observed *in vitro* does not occur *in vivo*. Nevertheless all these experiments were performed on animals under chloral hydrate anaesthesia and it seemed possible that the anaesthetic might have masked the monoamine releasing actions of THA. It was possible, however, to study 5-HT release from the frontal cortex in unanaesthetized animals. In these experiments, THA was again without effect on the release of 5-HT while a high K^+ concentration evoked a marked enhancement of transmitter release which was unaffected by prior THA administration. Since it had been previously shown that THA was equally effective in stimulating 5-HT and dopamine release *in vitro* (Robinson *et al.*, 1989), it seems unlikely that the failure of THA to enhance dopamine release was due to the anaesthesia.

A further indication that THA does not alter monoamine function *in vivo* was the observation that a high dose of THA (15 mg kg^{-1}) did not affect the concentration of dopamine or its metabolite DOPAC and HVA in the striatum or 5-HT or its metabolite 5-HIAA in the frontal cortex (Robinson *et al.*, 1989). Taken together the present results suggest strongly that THA does not affect monoamine release in the brain at doses which inhibit acetylcholinesterase and which are relevant therapeutically.

The lack of effect on monoamine release is probably explained by examination of the concentration of THA in the brain. The intracellular or tissue concentration seen in the current study if present in the incubation medium would have enhanced monoamine release from brain slices *in vitro*. However, the extracellular concentration *in vivo* was about 30 times lower and would have been ineffective in releasing monoamines from the slices (Robinson *et al.*, 1989). The lack of effect *in vivo* might be explained therefore by the low extracellular concentration that occurs. Similar arguments might also be used to render less likely the suggestions based on *in vitro* studies that *in vivo* THA might interact with the phencyclidine binding site (Albin *et al.*, 1988) and alter γ -aminobutyric acid (GABA) release (de Belleruche & Gardiner, 1986). Indeed one *in vivo* study has already failed to detect altered GABA release following THA administration to rats (Palmer *et al.*, 1990).

It has been proposed that the efficacy of THA in the treatment of demented patients might be due not only to cholinesterase inhibition but also to some other pharmacological effect. The present results make it unlikely that one of these pharmacological effects is enhanced monoamine release. It therefore remains possible that the efficacy of the drug in dementia results solely from its cholinomimetic action coupled with a good pharmacokinetic profile.

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