Effect of thyrotropin releasing hormone (TRH) on acetylcholine release from different brain areas investigated by microdialysis

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1 The effect of thyrotropin releasing hormone (TRH) administration upon acetylcholine (ACh) release in freely moving rats was investigated by means of transversal microdialysis coupled to h.p.l.c. TRH administered either s.c. or via local perfusion increased the ACh release from the cortex and hippocampus but not from the striatum. The increase in ACh release was maintained after 7 days of s.c. administration of TRH.

2 After s.c. injection of the neuropeptide, the increase in ACh release was dose-dependent and reached a maximum at 40 min after administration. The maximal percentage increases were 18, 52, 66 and 89% at doses of 1, 2.5, 5 and 10 mg kg⁻¹ and 35, 48 and 54% at doses of 2.5, 5 and 10 mg kg⁻¹ in the cortex and hippocampus, respectively. The effect of TRH was dependent on neuronal activity since it was completely inhibited by perfusion with tetrodotoxin (TTX), 5 × 10⁻⁷ M.

3 Perfusion with TRH, 2.5 µg µl⁻¹, caused 198% and 150% increase in ACh release 60 and 80 min after the beginning of the perfusion in the cortex and hippocampus, respectively. After this initial peak, a 100% increase in ACh release persisted throughout the perfusion.

4 Systemic TRH administration was followed by marked hyperactivity and stereotyped behaviour that showed a time course shorter than that of the increase in ACh release.

5 These findings demonstrate that TRH exerts a strong stimulant action on cortical and hippocampal cholinergic pathways.

Introduction

Thyrotropin-releasing hormone (L-pyro-glutamyl-L-histidyl-L-prolineamide, TRH) is present not only in the hypothalamus where it participates in the process of releasing thyrotropin and prolactin from the anterior pituitary (Boler *et al.*, 1969) but is also localized in neurones of the septal nuclei, preoptic area (Brownstein *et al.*, 1974), raphe nuclei of the medulla oblongata (Palkovits *et al.*, 1984) and spinal cord (Harkness & Brownfield, 1985). TRH is synthesized in neuronal cell bodies as a high molecular weight precursor which is then processed via post-translational modifications (Lechan *et al.*, 1986) to make the mature peptide ready to be released from the nerve endings where it is contained in large dense core vesicles (Johansson *et al.*, 1980). Mendez *et al.* (1987) demonstrated that TRH is released from slices of different brain structures in response to depolarizing stimuli. TRH receptors have been found in many areas of the CNS, the highest densities being present in limbic structures such as the amygdala, hippocampus and hypothalamus, and lower densities in the brain stem and cerebellum (Pilotte *et al.*, 1984; Simasko & Horita, 1982). These findings suggest that TRH, in addition to its hormonal activity, plays a role as a neurotransmitter and/or modulator of other neurotransmitter agents (Horita *et al.*, 1986). In particular TRH stimulates the turnover of acetylcholine (ACh) in the parietal cortex of freely moving rats (Malthe-Sorensen *et al.*, 1978), and in the hippocampus of pentobarbitone-anaesthetized rats (Brunello & Cheney, 1981). Since intraseptal injections of TRH antagonize pentobarbitone-induced depression of ACh turnover and the anaesthetic action of this barbiturate (Brunello & Cheney, 1981), it appears that TRH would stimulate the activity of forebrain cholinergic neurones to elicit behavioural arousal (Horita *et al.*, 1986). A role of cholinergic mechanisms in the behavioural effect of TRH is also indicated by the finding that the TRH-induced arousal in pentobarbitone-anaesthetized rats is blocked by atropine (Kalivas & Horita, 1983). Furthermore, a TRH analogue reverses the learning deficits and hip-

poampal decrease in high affinity choline uptake induced by lesioning the medial septal cholinergic neurones (Horita *et al.*, 1989).

In the present study we investigated whether the action of TRH on cholinergic neurones was equally present in various brain areas and its characteristics in terms of dose-dependence, time-course and reproducibility. We studied the release of endogenous ACh, which is a reliable index of the functional activity of cholinergic neurones (Pepeu, 1973). ACh release was investigated by the intracranial dialysis technique (Ungerstedt, 1984) in freely moving rats in order to correlate behavioural changes with those in ACh release. TRH was administered either systemically or by intracerebral perfusion.

Methods

Animal housing and surgery

Male adult Wistar rats (Charles River, Italy) weighing 250–300 g were used. They were housed in groups of three with free access to food and water and kept on a 12 h light/dark cycle. The rats were anaesthetized with chloral hydrate (400 mg kg⁻¹, i.p.) and placed in a stereotaxic frame (Stoelting, Stellar). Microdialysis tubes were inserted transversely in the parietal cortex, dorsal hippocampi and both striata following the procedure described by Wu *et al.* (1988). The microdialysis tubing (AN 69 membrane, Dasco, Italy; of 220 µm internal diameter and 310 µm external diameter, molecular weight cut-off >15,000 Da) was covered with Super-Epoxy glue along the whole of its length except for a region corresponding to the brain areas to be studied (8 mm for the parietal cortex, 6 mm for the dorsal hippocampus and two sections of 3.5 mm separated by a glued central zone 2.5 mm long for the striata). The coordinates used for the implantation of the microdialysis tubing were as follows (Paxinos & Watson, 1982): for the parietal cortex, AP – 0.5 mm and H 2.5 mm from bregma; for the dorsal hippocampi, AP – 3.3 mm and H 3.4 mm from bregma; for the striata, AP 0.0 mm and H 5 mm from bregma.

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Microdialysis procedure

One day after surgery each rat was placed in a Plexiglas cage and the inlet of the microdialysis probe was connected to a microperfusion pump (Carnegie Medicine, mod. CMA/100, Sweden) while the outlet was inserted into a 200 μ l test tube containing 5 μ l of 0.5 mM HCl to prevent hydrolysis of ACh. The microdialysis tubing was perfused at a constant flow rate (2 μ l min⁻¹) with Ringer solution (composition mM: NaCl 147, CaCl₂ 3.4, KCl 4.0, pH 7.0) containing physostigmine sulphate, 7 μ M. The molecular weight cut-off of the membrane allows low molecular weight solutes, such as choline (Ch) and ACh, to cross the dialysis membrane according to their concentration gradients and to be collected at the outlet for quantitative analysis.

After 1 h settling period the perfusate was collected at 20 min intervals and directly assayed for ACh and Ch. After collecting the first three samples to measure the basal release of ACh and Ch, TRH (dissolved in normal saline) was injected subcutaneously (1–10 mg kg⁻¹) into a group of rats. In another group, 2.5 μ g μ l⁻¹ TRH (dissolved in Ringer solution containing 7 μ M physostigmine sulphate) was perfused through the dialysis tubes. In a third group of rats TRH (5 mg kg⁻¹, s.c.) was administered once daily for 6 days; on the 7th day, the microdialysis membrane was implanted in the parietal cortex after TRH administration. On the following day the release of ACh and Ch was measured before and after the last injection of TRH (5 mg kg⁻¹).

Histological control

At the end of the experiment the rats were anaesthetized with urethane (1.2 g kg⁻¹, i.p.) and killed by decapitation. The brain was rapidly removed and placed in a vial containing 10 ml of 9% phosphate buffered formaldehyde solution. Two or three days later the brain was frozen with liquid CO₂ and coronal slices were cut with a freezing microtome. The 50 μ m thick slices were observed under light microscopy (Leitz, Dialva 20EB) to verify the positioning of the dialysis membrane. Data obtained from rats in which the membrane tubing was not correctly positioned were discarded.

In vitro recovery experiment

In order to evaluate the recovery of ACh and Ch through the microdialysis tubing *in vitro* recovery experiments were performed as follows. Dialysis tubing of varying exposed length (prepared as described under 'surgery') was placed in a glass beaker containing Ringer solution with Ch and ACh at concentrations similar to those found in the cortex, hippocampus or striatum of rats killed by microwave irradiation (Spignoli *et al.*, 1986). The membrane was then perfused at room temperature for 20 min with Ringer solution at a flow rate of 2 μ l min⁻¹. The content of ACh and Ch of perfused samples and of standard Ringer solution was then measured. Average recovery from two tubes of equal length was then calculated, during three 20 min periods, as a percentage of the amount of ACh and Ch present in the standard solution. The recovery of ACh and Ch from the dialysis tubing was: 56% $\pm$ 3.3 and 61% $\pm$ 3.4 for the tubing used for the cortex, 56% $\pm$ 0.6 and 60% $\pm$ 0.7 and 58% $\pm$ 2.9 and 64% $\pm$ 3.2 (mean $\pm$ s.e. mean) for the tubing used for the hippocampus and striatum, respectively. ACh and Ch values reported here were not corrected for recovery.

Assay of acetylcholine and choline in the dialysate

ACh and Ch were directly assayed in the dialysate by a high performance liquid chromatography (h.p.l.c.) method with an electrochemical detector as described by Damsma *et al.* (1987a). The h.p.l.c. apparatus consisted of a Perkin Elmer Series 10 pump (Perkin Elmer, Norwalk, CT, U.S.A.), a presaturation column (Chromspher 5 C18, 100 $\times$ 3 mm, Chrompack, Middleburg, The Netherlands), an injector (Rheodyne

7125, Rheodyne, Cotati, U.S.A.) equipped with a 32 μ l loop, a guard column (reverse phase) and an analytical column (Chromspher 5 C18, 100 $\times$ 3 mm, Chrompack), an enzyme reactor (10 $\times$ 2.1 mm, Chrompack) containing Lichrosorb-NH₂ activated with glutaraldehyde to which the enzymes acetylcholinesterase (AChE, E.C. 3.1.1.7) and choline oxidase (E.C. 1.1.3.17) were covalently bound, an electrochemical detector (BioAnalytical System, Electroanalytical Instruments) and a Perkin Elmer chart recorder. The mobile phase consisted of 0.1 M phosphate buffer (pH 8.0) with 5 mM KCl added; the solution was filtered through a 0.45 μ m nylon membrane (Supelco Inc., Bellefonte, PA, U.S.A.) and degassed by ultrasonic vibration under vacuum. The flow rate was 0.6 ml min⁻¹.

ACh and Ch were separated on a cation exchange column prepared by loading the reverse phase column with sodium lauryl sulphate (0.5 mg ml⁻¹) as described by Damsma *et al.* (1987b). ACh was hydrolyzed by AChE to acetate and Ch in the postcolumn enzyme reactor; Ch was then oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was electrochemically detected by a platinum electrode at +250 mV.

For the quantitative analysis of ACh and Ch we constructed a calibration curve by spiking the Ringer solution with standard ACh and Ch in the concentration-range we expected to find in the dialysates. Three or four concentrations for each calibration curve were then injected at the beginning and the end of the analysis and the heights of the recorded peaks were then plotted against the concentrations. A regression line was calculated and quantitation of unknown samples was carried out by the method of inverse prediction. Under these experimental conditions the sensitivity limit (s/n ratio 3/1) was 30 fmol μ l⁻¹ and 15 fmol μ l⁻¹ for ACh and Ch, respectively.

Chemicals

All reagents were of analytical grade. Physostigmine sulphate, AChE (E.C. 3.1.1.7., grade VI-S) and choline oxidase (E.C. 1.1.3.17) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TRH (bitartrate salt) was kindly supplied by Cyanamid Italia (Catania).

Statistical analysis

ACh and Ch release rates were expressed as percentage variation over basal output which in each group of experiments was the mean ($\pm$ s.e. mean) of all pre-drug determinations. Significant differences were evaluated on the percentage values by use of one-way analysis of variance, followed by Dunnett's test. *P* values of less than 0.05 were considered significant.

Results

Single administrations

The release of ACh and Ch at rest in freely moving rats is shown in Table 1. There was no significant difference in ACh release between the cortex and the hippocampus, while the

Table 1 Acetylcholine (ACh) and choline (Ch) release from different brain regions in freely moving rats at rest

	Cortex	Hippocampus	Striatum
ACh	4.4 $\pm$ 0.24 (40)	4.5 $\pm$ 0.24 (48)	10.4 $\pm$ 0.63* (17)
Ch	111.8 $\pm$ 4.8 (32)	114.7 $\pm$ 5.1 (41)	105.9 $\pm$ 5.7 (12)

Release expressed as pmol/20 min $\pm$ s.e. mean. Number of determinations in parentheses. **P* < 0.01 with respect to the cortex and hippocampus determined by Student's *t* test.

release from the striatum was significantly larger. No significant differences were found among the release of Ch from these three brain structures. The subcutaneous administration of TRH at doses of 1, 2.5, 5 and 10 mg kg^{-1} brought about an increase in ACh release from the cerebral cortex and hippocampus (but not from the striatum) 1 h after the beginning of sample collection. Ch efflux was not modified, regardless of the doses used and the regions investigated. Figure 1 shows the effect of TRH on cortical release of ACh. The curves represent the time-course of the increase for each dose tested, expressed as percentage changes from the means of three collection periods before TRH administration, taken as controls. TRH elicited a dose-dependent increase in ACh release that peaked 40 min after its administration and returned to basal values within 100 min. While 1 mg kg^{-1} was ineffective, the peak response to the 2.5, 5 and 10 mg kg^{-1} administration was significantly different from controls. The largest effect ($+87 \pm 20\%$) was observed following 10 mg kg^{-1} TRH. Figure 2 shows the effect of TRH on the release of ACh from the hippocampus. The maximal increase in ACh release occurred between 40 and 60 min after TRH administration, and was statistically significant for all doses of TRH used (2.5, 5 and 10 mg kg^{-1}). Altogether, the increases observed in the hippocampus were lower than those observed in the parietal cortex (see Figure 1) and saturated at 5 mg kg^{-1} TRH. Injection of the vehicle alone (normal saline, 1 ml kg^{-1}) did not change the release of ACh from the cortex and hippocampus, ruling out an effect upon ACh release due to the stress of the injection. Finally, at a dose of 5 mg kg^{-1} TRH did not change the release of either ACh or Ch from the striatum ($-4.5 \pm 2\%$; $n = 3$).

A few minutes after a s.c. injection of TRH, stereotyped behaviours such as wet-dog shaking, sniffing, forepaw licking, tail elevation, face washing and hyperactivity were observed. Although no quantitative analysis of the behavioural changes was made, it was apparent that these effects developed within the first 20 min after TRH administration and then gradually disappeared.

It is well established that locally applied tetrodotoxin (TTX) blocks the release of ACh (Damsma *et al.*, 1988). In the

Figure 1 Effect of s.c. administration of thyrotropin releasing hormone (TRH) on the release of acetylcholine (ACh) from the parietal cortex expressed as percentage variation over basal output: TRH, 1 mg kg^{-1} (○); 2.5 mg kg^{-1} (●); 5 mg kg^{-1} (△); 10 mg kg^{-1} (▲). Each point represents the mean of at least 4 independent experiments; s.e. mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation vs the mean $\pm$ s.e. mean of all pre-drug determinations. * $P < 0.05$; ** $P < 0.01$, Dunnett's test.

Figure 2 Effect of s.c. administration of thyrotropin releasing hormone (TRH) on hippocampal acetylcholine (ACh) release expressed as percentage variation over basal output: TRH 2.5 mg kg^{-1} (●); 5 mg kg^{-1} (△); 10 mg kg^{-1} (▲). Each point represents the mean of at least 4 independent experiments; s.e. mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation vs the mean $\pm$ s.e. mean of all pre-drug determinations. * $P < 0.05$; ** $P < 0.01$, Dunnett's test.

present experiments we studied whether TRH (5 mg kg^{-1}) could reverse the action of TTX on the ACh release from the cerebral cortex. Figure 3 shows that following local application of TTX ($5 \times 10^{-7} \text{ M}$) via the dialysis tubing the s.c. injection of TRH (5 mg kg^{-1}) did not enhance the release of ACh which gradually fell to undetectable values.

Repeated administration

In order to verify whether tolerance may develop after repeated administration of TRH, cortical ACh release was investigated in rats injected daily with TRH (5 mg kg^{-1} s.c. for 7 days). On day 6 the microdialysis tubing was implanted and on day 7 release of ACh and Ch was investigated before and after the last administration of 5 mg TRH . The basal ACh and Ch outputs from the parietal cortex were similar to those of untreated animals. The size and time-course of the increase in ACh release were not significantly different from those observed in untreated animals ($+78 \pm 14\%$; $n = 4$).

Intracerebral perfusion

Figure 4 shows the effect of perfusing $2.5 \mu\text{g } \mu\text{l}^{-1}$ TRH through the dialysis tubing on ACh release from the cortex, hippocampus and striatum. Each curve represents the time-course of the increase, expressed as percentage change from the mean of three collection periods before TRH. TRH perfusion started 60 min after the beginning of the collection period and lasted throughout the experiment. TRH perfusion was accompanied by a significant increase in ACh release from the cortex and hippocampus but not from the striatum. The maximal increase of 200% for the cortex and 150% for the hippocampus occurred 60 and 80 min, respectively, after the beginning of TRH administration. Both in the cortex and the hippocampus, the increase was significantly different from the mean of the basal values at 40 min after the beginning of TRH perfusion (Dunnett's test, $P < 0.05$). After a peak value, the increase in ACh release settled at about 100% rise which persisted throughout the perfusion period. In three experiments a dose of $0.5 \mu\text{g } \mu\text{l}^{-1}$ of TRH was used. This lower dose

Figure 3 Effect of tetrodotoxin (TTX) perfusion on acetylcholine (ACh) release elicited by s.c. thyrotropin releasing hormone (TRH) administration to the cortex: (O) 5 mg kg^{-1} TRH only; (Δ) 5 mg kg^{-1} TRH administered 20 min after the beginning of $5 \times 10^{-7} \text{ M}$ TTX perfusion. Each point represents the mean of at least 3 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage of variation vs the mean $\pm$ s.e.mean of all pre-drug determinations. * $P < 0.05$; ** $P < 0.01$, Dunnett's test.

Figure 4 Effect of thyrotropin releasing hormone (TRH) perfusion ($2.5 \mu\text{g } \mu\text{l}^{-1}$) on acetylcholine (ACh) release from the cortex (●), hippocampus (Δ) or striatum (○). TRH perfusion started after three basal values had been collected and lasted throughout the experiment. The three individual basal values are shown and expressed as percentage values $\pm$ s.e.mean. Each point represents the mean of at least 4 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation with the mean $\pm$ s.e.mean of all pre-drug determinations. * $P < 0.05$; ** $P < 0.01$, Dunnett's test.

elicited a $+66 \pm 20\%$ increase in the release of ACh from the parietal cortex, showing a dose-dependence in the effect.

Stereotyped behaviours were not seen after local perfusion of TRH.

Discussion

The technique of microdialysis *in vivo* has enabled us to evaluate directly the effects of TRH on the release of endogenous ACh from the brain of freely moving rats. Such a release is considered to be a reliable index of the functional activity of central cholinergic neurones (Pepeu, 1973).

Most importantly, our results demonstrate that TRH selectively activated the cholinergic neurones of the cortex and the hippocampus, while being ineffective on those of the striatum, as shown by the marked and long lasting increase in the release of endogenous ACh from the first two brain areas. These differences in regional cholinergic activation may be related to the uneven distribution of TRH receptors in the central nervous system (Simasko & Horita, 1982; Pilotte *et al.*, 1984) with the striatum showing the lower density among the three brain regions under investigation (Ogawa *et al.*, 1983). Our results provide direct evidence that TRH modulates central cholinergic transmission and accord with other circumstantial evidence in favour of it. In fact, intraseptal injection of TRH increases the turnover of ACh in the hippocampus of pentobarbitone-anaesthetized rats (Brunello & Cheney, 1981) and i.c.v. administration of TRH increases the turnover of ACh in the parietal cortex of freely moving rats but not in other areas investigated (Malthe-Sørensen *et al.*, 1978). Muscarinic antagonists also block some central effects induced by TRH, including hypothermia and reversal of the hypnotic action of barbiturates (Breese *et al.*, 1975; Kalivas & Horita, 1979). No direct demonstration of the ability of TRH to enhance ACh release from the central nervous system has been reported before. In this respect, Hedley & Vaughan (1985) failed to demonstrate any effect of TRH on ACh release from rat nucleus accumbens and septal *in vitro* cubes both in the presence and absence of depolarizing concentrations of K^+ . One can only surmise that these areas are, like the striatum, comparatively insensitive to TRH.

It has been shown that TTX infusion (10^{-6} – 10^{-7} M) through the microdialysis probe causes a drop in the output of basal ACh to undetectable values (Damsma *et al.*, 1988). In our experiments with local application of TTX ($5 \times 10^{-7} \text{ M}$) the s.c. injection of TRH (5 mg kg^{-1}) was unable to enhance the release of ACh which gradually fell to undetectable values. This result indicates that the effect of TRH depends on propagated electrical activity of the cholinergic neurones themselves or of afferent fibres that in turn activated the cortical cholinergic pathways. The possibility that TRH may exert an indirect action on cortical neurones is supported by the findings of Burov *et al.* (1989) who showed that while systemic administration of TRH induces an increase of the electrical activity of sensorimotor cortical neurones, microiontophoretic application of TRH causes a decrease in the spike rate of most of them. They assumed that the excitation of cortical neurones by systemic administration of TRH was due to activation of extracortical neurones exerting a stimulatory effect on the cortex. On the other hand, direct stimulation by TRH of rat cholinergic neurones such as motoneurones has been demonstrated (Takahashi, 1985; Lacey & Nistri, 1989).

In our experiments after one week of daily administration of TRH (5 mg kg^{-1}) no tolerance to its effects was observed. On the contrary, Ogawa *et al.* (1983) found a down regulation of TRH receptors in the cortex, hippocampus and hypothalamus of rats treated daily for 14 days with TRH (6 mg kg^{-1} , i.p.). It appears therefore that in our experiments either the duration of treatment was too short and/or the dose used too low to develop tolerance to the effect of TRH. It might also be the case that a down regulation of TRH recep-

tors develops, but that it is too small to influence the release of brain ACh if the system possesses adequate spare capacity.

Systemic administration, but not local infusion, of TRH was followed by marked behavioural effects, such as wet-dog shaking, sniffing, forepaw licking, tail elevation, face washing and hyperactivity. These effects reached a maximum 20 min after TRH administration and then gradually disappeared, showing a shorter time-course than the increase in the release of ACh. Thus, the different time-course of the biochemical and behavioural effects and the lack of the latter during the increase in ACh output elicited by local TRH infusion indicate that the two effects are not simply related to each other. Although Sills *et al.* (1988) reported that the wet-dog shaking caused in the rat by an analogue of TRH (MK-771) is blocked, in a noncompetitive manner, by atropine or scopolamine, other evidence indicates that the stereotyped behaviour and the locomotor effects elicited by TRH rely also on other neurotransmitter systems, particularly the dopaminergic system of the nucleus accumbens (Roth *et al.*, 1976; Kalivas *et al.*, 1987). In this respect, TRH stimulates the release of [^3H]-

dopamine from slices of rat nucleus accumbens but not nucleus caudate *in vitro* (Kerwin & Pycocock, 1979); bilateral injections of TRH into the nucleus accumbens of rats increase locomotor activity and promote behavioural activities similar to those produced by activation of the dopaminergic system (Heal & Green, 1979; Heal *et al.*, 1981).

Since in the present study no close correlation between increased release of brain ACh and stereotyped behaviour was observed, the question arises as to whether the enhanced activity of cholinergic neurones may have other functional consequences. The recent demonstration that an analogue of TRH can reverse learning deficits caused by cholinergic impairment (Horita *et al.*, 1989) might indicate that cognitive rather than motor behaviour is more susceptible to the interaction between ACh and TRH.

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