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EFFECTS OF MORPHINE, PHYSOSTIGMINE AND RAPHE NUCLEI STIMULATION ON 5-HYDROXYTRYPTAMINE RELEASE FROM THE CEREBRAL CORTEX OF THE CAT

PETRA AIELLO-MALMBERG, A. BARTOLINI, ROSALIA BARTOLINI & A. GALLI

Department of Pharmacology, University of Florence, I 50134 Firenze, Italy

1 The release of 5-hydroxytryptamine (5-HT) from the cerebral cortex and caudate nucleus of brainstem-transected cats and from the cerebral cortex of rats anaesthetized with urethane was determined by radioenzymatic and biological assay.

2 The stimulation of nucleus linearis intermedius of raphe doubles the basal 5-HT release in the caudate nucleus and increases it 3 fold in the cerebral cortex. The effects of the electrical stimulation of the raphe are potentiated by chlorimipramine.

3 Brain 5-HT release is greatly increased by morphine hydrochloride (6 mg/kg i.v.) and by physostigmine (100 μg/kg i.v.), but not by DL-DOPA (50 mg/kg i.v.).

4 It is suggested that the 5-HT releasing action of physostigmine can contribute to some of its pharmacological effects such as the analgesic effect so far attributed exclusively to its indirect cholinomimetic activity.

5 The 5-HT releasing action of physostigmine seems unrelated to its anticholinesterase activity.

Introduction

Several recent reports suggest the participation of 5-hydroxytryptamine (5-HT, serotonin) in some of the pharmacological effects of morphine. For instance, the analgesic effect of morphine can be antagonized by *p*-chlorophenylalanine (Tenen, 1968), by lesions of midbrain raphe nuclei (Samanin, Gumulka & Valzelli, 1970) and by cyproheptadine (Görlitz & Frey, 1972). Moreover the analgesic effect of morphine can be enhanced by pretreatment with 5-hydroxytryptophan (Sigg, Caprio & Schneider, 1958; Saarnivaara, 1969) and morphine is known to enhance the cerebral turnover of 5-HT (Görlitz & Frey, 1972; Yarbrough, Buxbaum & Sanders-bush, 1972; 1973; Haubrich & Blake, 1973; Sawa & Oka, 1976). On the other hand, Way, Loh & Shen (1968), Loh, Shen & Way (1969) and Shen, Loh & Way (1970) failed to show a morphine-induced increase in brain 5-HT turnover in mice.

In the present work the 5-HT releasing effect of morphine was investigated by measuring the 5-HT released from the cerebral cortex and caudate nucleus, and by comparing the morphine effect with the effect of the electrical stimulation of the midbrain raphe nuclei.

The effect of physostigmine was also investigated not only because of its very good analgesic properties (Flodmark & Wramner, 1945; Harris, Dewey, Howes, Kennedy & Pars, 1969; Sitram, Buchsbaum & Gillin, 1977), but also because of its structural analogy with 5-HT.

Midbrain-transected cats were used in order to avoid the use of anaesthetics during experiments.

Preliminary accounts of this work have been given by Bartolini & Aiello-Malmberg (1975) and by Bartolini, Galli, Bartolini, Aiello-Malmberg & Renzi (1976).

Methods

Forty-three mongrel adult cats of either sex, weighing between 2 and 5 kg, and 15 Wistar male albino rats weighing about 300 g were used.

Cats

All surgery was performed under halothane-air anaesthesia. Suitable cannulae were inserted into the trachea, vein and artery for adequate ventilation, intravenous injections and recording of arterial blood pressure. The animal's head was placed in a stereotaxic apparatus. The atlanto-occipital membrane was opened to allow free drainage of cerebrospinal fluid. A stereotaxically oriented spatula was used for brainstem transections. Most of the transections were

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placed at the midpontine pretrigeminal level; others were placed at a collicular prepontine level. Shortly following the transection, the skull was opened over a large part of the frontoparietal area. After opening the dura, in order to determine the 5-HT release, a collecting cylinder covering 0.78 cm^2 and constructed according to Mitchell (1963), was lowered with the aid of an adjustable electrode carrier onto the sensorimotor cortex and its posterior association area of one side of the brain.

After surgery, the cats were allowed to recover from halothane anaesthesia for 3 h. During this time, they resumed spontaneous respiration. Body temperature was maintained as close to 37°C as possible, by means of a heatpad connected via a relay to a rectal probe.

Results from experiments with cats in which either brain haemorrhage or oedema occurred were discarded. Blood pressure was recorded from the femoral artery by means of a Statham pressure transducer connected to a preamplifier (MARB 776) driving a potentiometric recorder (Rikadenki).

The collecting cylinder on the cortex was filled with one of the following two solutions, depending on the method used for the 5-HT assay: solution A, used for biological assay recommended by Uuspää & Uuspää (1962): (g/l) NaCl 8.0, KCl 0.4, $CaCl_2$ 0.17, $NaHCO_3$ 0.3 and glucose 0.3; solution B, used for radioenzymatic assay: (g/l) $NaH_2PO_4.H_2O$ 0.95 and $Na_2HPO_4.2H_2O$ 16.62.

Every 10 min, the solution was aspirated from the collecting cup and either immediately bioassayed or frozen for the radioenzymatic assay.

In some cats a push-pull cannula (Gaddum, 1961) was also inserted into the nucleus caudatus head in the opposite side of the brain. The cannula was positioned stereotaxically, by using the following coordinates: A 17.5; L 3.5; H 3.0, according to the atlas of Snider & Niemer (1970). Caudate perfusion was performed with solution A at the rate of 0.1 ml/min. Every 10 min the collected perfused fluids were bioassayed for 5-HT. After collection, each sample was carefully examined for traces of blood both before and after centrifugation. If there was blood in some samples or a difference in the volume between infused and collected fluid, the whole experiment was discarded. The electrical activity of the cortex was recorded by means of a monopolar silver ball electrode placed inside the collecting cylinder and screw electrodes inserted in the skull. An indifferent electrode was placed on the midline of the frontal bone. A concentric bipolar stimulating electrode was placed in the nucleus linearis intermedius of raphe (Ashkenazi, Holman & Vogt, 1973). The nucleus was stimulated via the bipolar electrode inserted according to the following stereotaxic co-ordinates: A 1.5; L 0.3; H −4.5 to −5.5. Periods of 10 min stimulation at a frequency of 10 Hz with pulses of 0.5 ms duration, were delivered through a Tektronix stimulator by changing polarity every 3 min. Submaximal voltage was used for optimal effects. At the end of the experiments, the brain was removed and fixed in 10% formalin. The position of the push-pull cannula and stimulating electrode and the level of the brainstem transection were verified histologically.

Rats

The experiments were performed under ethyl urethane anaesthesia (1 g/kg i.p.) without any brainstem transection. The collecting cylinder covered 0.26 cm^2 of cortex. The remaining experimental technique was the same as that used for the cat although the caudate nucleus was not cannulated in the rat.

5-Hydroxytryptamine assay

Biological assay. The biological assay of 5-HT was performed on the fundus strip of the rat stomach (Vane, 1957). A long (≈13 cm) thin strip of stomach fundus was hung in a suitable thermoregulated bath, containing 4.5 ml of bathing solution (solution A with the addition of scopolamine hydrobromide 1 μg/ml and diphenhydramine hydrochloride 0.1 μg/ml). The contractions were recorded with an isotonic lever. The prewarmed bathing solution was perfused through the organ bath at a rate of about 1 ml/min for the duration of the experiment. The assay was performed by injecting standard and unknown solutions in the perfusing stream. The sensitivity limit of the system was 10 pg. At the end of the assay, all samples were always retested in the presence of the 5-HT antagonist, methysergide bimaleate 0.1 μg/ml.

Radioenzymatic assay. A slight modification of the procedure described by Saavedra, Brownstein & Axelrod (1973) for the microassay of 5-HT in tissues was used. Aliquots (50 μl) of the fluid from the collecting cylinder (solution B), accounting for about 25% of the total sample volume, were incubated in duplicate with 10 μl of a preparation of N-acetyl-transferase (ES 2.3.1.5) from rat liver and 10 μl of a solution of acetyl-coenzyme A (1 mg/ml) at 37°C for 30 min. All subsequent steps, viz., incubation with beef pineal hydroxyindole-*O*-methyltransferase (ES 2.1.1.4) and [^{3}H]-methyl-*S*-adenosyl-l-methionine, extraction in toluene of the tritiated melatonin, evaporation overnight of the organic solvent at 80°C and counting of the recovered radioactivity, were performed as in the original procedure. The sensitivity limit of this method was 50 pg/50 μl of the sample. All values of 5-HT are expressed in terms of the base. Biological and radioenzymatic assays were in good agreement.

The drugs, unless otherwise stated, were adminis-

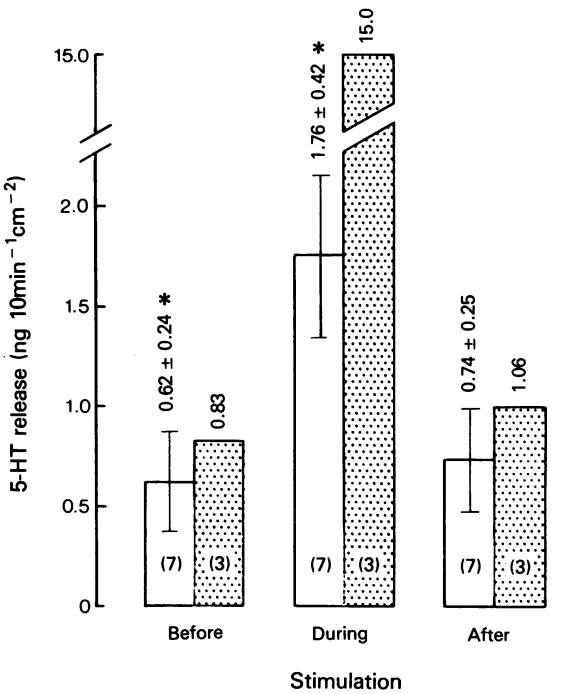

Figure 1 Effect of raphe stimulation on 5-HT release as measured by bioassay from the cerebral cortex of brainstem-transected cats. The nucleus linearis intermedius of raphe was stimulated for 10 min with 10 Hz and 0.5 ms pulses of alternating polarity every 3 min. The number of experiments are shown in parentheses. The vertical lines give s.e. mean. Numbers above each column indicate the release (ng 10 min^{-1} cm^{-2} ± s.e.). *Significant difference between the two values, $P < 0.05$. Stippled columns represent the 5-HT release after intravenous injection of chlorimipramine hydrochloride 1 mg/kg.

tered through a polyethylene cannula inserted in the femoral vein.

The following drugs were used: morphine hydrochloride (Carlo Erba), physostigmine sulphate (Sigma), methysergide bimaleate (Sandoz), scopolamine hydrobromide (BDH), diphenhydramine hydrochloride (Benadryl, Parke Davis), DOPA (DL-3,4-dihydroxyphenylalanine (BDH), 5-hydroxytryptamine creatinine sulphate complex (Sigma), chlorimipramine hydrochloride (Geigy), *S*-adenosyl-L-methyl-[^{3}H]-methionine sp. act. 8.9 Ci/mmol (The Radiochemical Centre, Amersham), melatonin (Sigma), acetylcoenzyme A (Sigma).

Results

Baseline levels of spontaneous 5-hydroxytryptamine release

The spontaneous release of 5-HT from the cortex ranged between 20 and 1550 pg 10 min^{-1} cm^{-2}. In 29 animals, 3 h posthalothane anaesthesia and brainstem transection, the mean (±s.e.) was 710 (±80) pg 10 min^{-1} cm^{-2}.

The factors involved in the wide range observed in baseline release of 5-HT probably included post-surgical brain trauma and diet difference between cats from various sources.

The spontaneous release of 5-HT from the caudate nucleus varied between 20 and 500 pg/10 min, with a mean (±s.e.; $n = 12$) of 160 (±30) pg.

No difference in the 5-HT basal release was observed between midpontine and prepontine transected cats, that is between EEG activated and EEG synchronized cats.

Effect of raphe stimulation

In most instances, stimulation of the raphe did not induce any change in the EEG. EEG slowing was

Table 1 Effect of the raphe stimulation on 5-hydroxytryptamine (5-HT) release from caudate nucleus of brainstem-transected cats

5-HT release (ng/10 min)		*Increase*
At rest	*During stimulation*	*(ng/10 min)*
0.07	0.33	0.26
0.50	0.70	0.20
0.02	0.26	0.24
0.23	0.36	0.13
0.20* ± 0.10	0.41* ± 0.09	0.20 ± 0.02

The nucleus linearis intermedius of raphe was stimulated for 10 min with 10 Hz and 0.5 ms pulses of alternating polarity every 3 min.
* Significant difference between the two values, $P < 0.01$

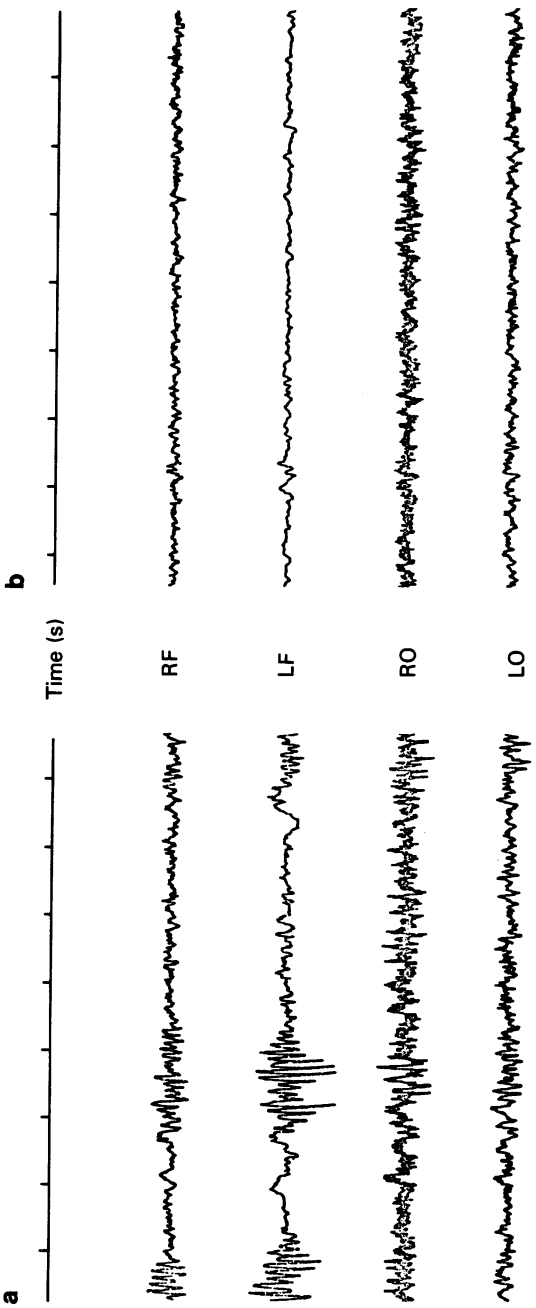

Figure 2 Effect of morphine on EEG in a collicular prepontine-transected cat. Neocortical EEG tracings are shown (a) before and (b) 10 min after morphine. Cats prepared under halothane-air anaesthesia and after brainstem transection were allowed to breath room air. Before morphine administration the neocortical EEG showed synchronization in the frontal and occipital leads and cortical 5-hydroxytryptamine (5-HT) release was 0.9 ng 10 min^{-1} cm^{-2}. After intravenous morphine hydrochloride 6 mg/kg (b) a generalized neocortical activation was observed in spite of the fact that the cortical 5-HT release as measured by bioassay was increased to 1.5 ng 10 min^{-1} cm^{-2}. RF: right frontal somatosensory cortex; LF: left frontal somatosensory cortex; RO: right occipital and LO: left occipital cortex.

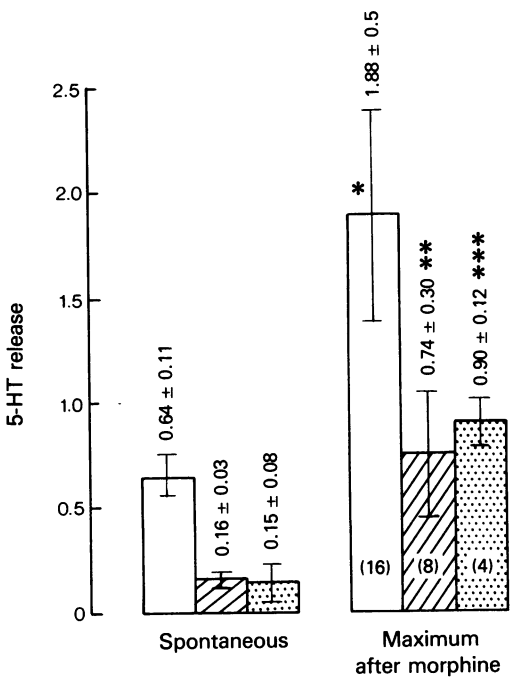

Figure 3 Effect of morphine on cerebral 5-hydroxytryptamine (5-HT) release as measured by bioassay. Morphine hydrochloride 6 mg/kg was injected intravenously. Open, hatched and stippled columns represent 5-HT release from cat cerebral cortex (ng 10 min^{-1} cm^{-2}), cat caudate nucleus (ng/10 min) and rat cerebral cortex (ng 15 min^{-1} cm^{-2}) respectively. Cats were brainstem-transected while rats were anaesthetized with urethane. The increase in 5-HT release reaches its maximum generally during the second collecting period after morphine administration. The number of experiments are shown in parentheses. The vertical lines give s.e. mean. Numbers above each column indicate the ng 10 min^{-1} cm^{-2} ± s.e. for cats and the ng 15 min^{-1} cm^{-2} ± s.e. for rats. The 5-HT release after morphine is significantly increased in all three experimental conditions: $*P < 0.02$; $**P < 0.05$; $***P < 0.01$.

seen only in those cats in which raphe stimulation induced a cortical 5-HT release above 1.5 ng 10 min^{-1} cm^{-2}.

As shown in Figure 1 and Table 1, the stimulation of nucleus linearis intermedius of raphe produced a consistent increase in 5-HT release from both cerebral cortex and caudate nucleus. The intravenous injection of 1 mg/kg of chlorimipramine, 80 min before the raphe stimulation, increased only slightly the basal release of 5-HT. However, pretreatment with chlorimipramine caused a marked increase in the amounts of 5-HT released by raphe stimulation (Figure 1).

Effect of morphine

The effect of morphine on the EEG of brainstem-transected cats varied with the EEG pattern present prior to morphine administration. In EEG activated cats, no obvious EEG modification was observed following morphine, while in EEG synchronized animals, morphine caused an activation (Figure 2).

The effect of morphine (6 mg/kg) on release of 5-HT from the cerebral cortex of cats and rats and from caudate nucleus of cats is shown in Figure 3. The release of 5-HT from the cerebral cortex after morphine administration was about 3 and 6 times greater than the basal release in cat and rat respectively. The release from the caudate nucleus of the cat was 4.6 times greater than the basal release.

Effect of physostigmine

The administration of physostigmine (100 μg/kg i.v.) in brainstem-transected cats induced an activation of the EEG. This was observed only in those cats with a previously synchronized EEG.

The mean percentage increase of 5-HT release due to physostigmine, observed in the 11 cats examined, was 88% (Figure 4).

The release of 5-HT from the cortex with time after physostigmine administration is shown in Figure 5. Following 3 control periods in which the basal level of spontaneous release was established, physostigmine was injected and 5 samples were withdrawn and assayed. The effect of physostigmine was maximal between 20 and 30 min.

Effects of DL-DOPA

DL-DOPA was administered by slow intravenous injection in order to avoid large blood pressure variations. Under these conditions, DOPA caused mydriasis, slight changes in blood pressure, an increase of muscular rigidity, vomiting and activation of the EEG in those animals with a synchronized EEG. DOPA administration did not cause any substantial variation in 5-HT release (Figure 6).

Discussion

Our results clearly show that both morphine and physostigmine increase brain 5-HT release. However, while the effect of morphine, because of indirect evidence (Sigg *et al.*, 1958; Tenen, 1968; Samanin *et al.*, 1970; Görlitz & Frey, 1972), was expected, the effect of physostigmine was completely new.

As far as the 5-HT release is concerned, whether basal or that evoked by raphe stimulation, our results are in very good agreement with those reported by

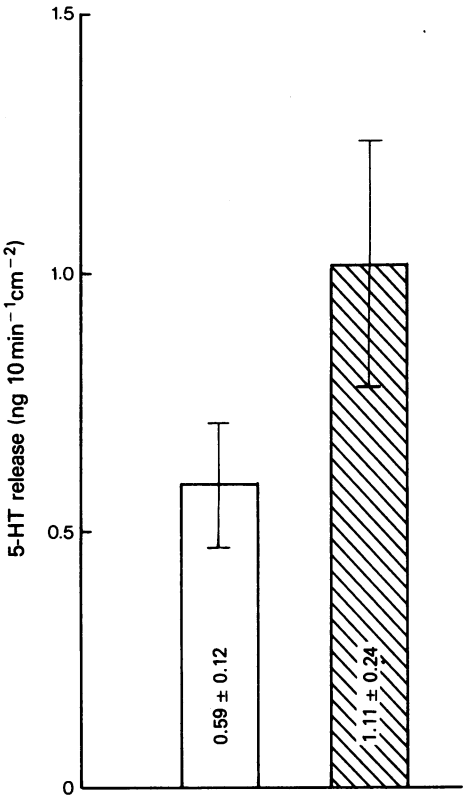

Figure 4 Effect of physostigmine on 5-hydroxytryptamine (5-HT) release from cerebral cortex of brainstem-transected cats ($n = 11$). Radioenzymatic assay checked by bioassay. Open column: basal release before physostigmine. Hatched column: maximum release after the intravenous injection of physostigmine sulphate 118 μg/kg corresponding to physostigmine base 100 μg/kg. The vertical lines give s.e. mean. Numbers within each column indicate release (ng 10 min^{-1} cm^{-2} ± s.e.). The 5-HT release after physostigmine is significantly increased, $P < 0.05$.

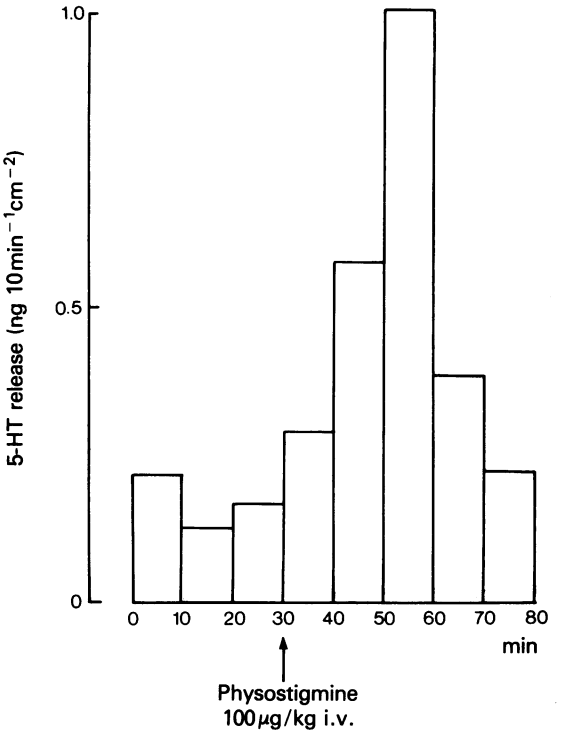

Figure 5 Effect of intravenous administration of physostigmine sulphate (118 μg/kg) on spontaneous 5-hydroxytryptamine (5-HT) release from cerebral cortex of a brainstem-transected cat. Radioenzymatic assay.

Holman & Vogt (1972), by Ashkenazi *et al.* (1973) and by Chiueh & Moore (1976), although they were obtained through different perfusion techniques and from different brain areas.

The smaller release of 5-HT from the cerebral cortex of rats in comparison with the release from the cerebral cortex of cats is probably due to the ethyl urethane anaesthesia of rats, rather than to brainstem transection. Moreover the smaller release of 5-HT obtained from the caudate nucleus in comparison with that from the sensorimotor cortex derives from the much smaller brain surface perfused by the push-pull cannula in comparison with 1 cm^2 of cortex. In fact, in agreement with the greater number of 5-hydroxytryptaminergic endings (Fuxe, Hökfelt & Ungerstedt, 1968) and with the larger quantity of 5-HT contained in the caudate nucleus (Bogdanski & Udenfriend, 1956; Kuntzman, Shore, Bogdanski & Brodie, 1961; Pscheidt, Molpurgo & Himwich, 1964), morphine induced a larger release of 5-HT from the caudate nucleus than from the sensorimotor cortex.

We believe that the 5-HT collected from the cerebral cortex and from the caudate nucleus originates from nerve endings. This view is in accordance with the specific increase of 5-HT release evoked by raphe stimulation and with the marked increment in the amounts of 5-HT released by raphe stimulation after chlorimipramine. It is relevant that electrical stimulation gave rise to an increase of 5-HT release only if specific raphe nuclei were stimulated; however, we cannot rule out the possibility that 5-HT derives from other anatomical structures.

We would stress the fact that among the drugs tested, only morphine and physostigmine were able

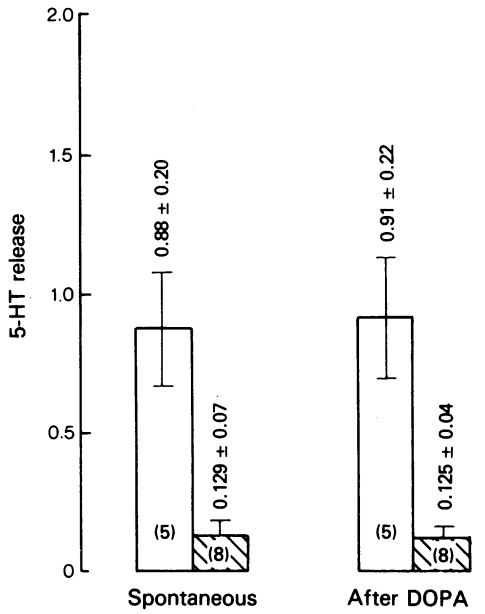

Figure 6 Lack of effect of intravenous administration of DL-DOPA (50 mg/kg) on 5-hydroxytryptamine (5-HT) release from cerebral cortex. Open columns: 5-HT release from brainstem-transected cats (ng 10 min^{-1} cm^{-2}). Hatched columns: 5-HT release from rats anaesthetized with urethane (ng 15 min^{-1} cm^{-2}). The spontaneous and after DOPA release represent the mean of the previous two and the subsequent four collected samples respectively. 5-HT was determined by bioassay. The number of experiments are shown in parentheses. The vertical lines give s.e. mean. Numbers above each column indicate ng 10 min^{-1} cm^{-2} ± s.e. for cats and the ng 15 min^{-1} cm^{-2} ± s.e. for rats.

to increase 5-HT release from the brain; neither DL-DOPA nor homotaurine (Bartolini, Galli, Bartolini, Adembri, Zilletti & Giotti, 1975) modified the basal 5-HT release.

The following two considerations induced us to try DL-DOPA: (1) morphine can stimulate the chemoreceptor trigger zone in the area postrema of the medulla as does apomorphine, the well known dopamine receptor agonist; therefore it was possible that the effect of morphine on 5-HT release resulted from the stimulation of dopamine receptors; (2) the data in the literature are controversial; in fact while Algeri & Cerletti (1974), Antoniaccio & Robson (1974), Fuller & Perry (1975), Grabowska (1975), Smialowska (1975), reported indirect evidence showing that DOPA or apomorphine can increase cerebral 5-HT release, Corrodi, Farnebo, Fuxe and Hamberger (1975), Snider, Hutt, Stein & Fahn (1975) reported that the dopamine receptor agonist, bromocriptine, reduces 5-HT release *in vitro* and *in vivo*.

Moreover Chiueh & Moore (1976) reported direct evidence that all three dopamine receptor stimulating agents, (+)-amphetamine, apomorphine and L-DOPA, were without effect on 5-HT release from the cat brain. Our results concerning the effect of DOPA on 5-HT release were in agreement with those of Chiueh & Moore. It seems therefore that the effect of morphine on 5-HT release is independent of stimulation of dopamine receptors.

The results showing that morphine is able to increase the release of 5-HT from cortex and caudate nucleus are in good agreement with much indirect evidence which suggests the participation of 5-HT in some pharmacological effects of morphine (see introduction). On the other hand we have shown, for the first time, that physostigmine also is able to increase the 5-HT release from cerebral cortex *in vivo*. It is possible that the analgesic effect of physostigmine is related to its 5-HT releasing action in addition to the indirect cholinomimetic property. However, it may be significant that physostigmine (250 μg/kg s.c.), like morphine, induces a 'Straub tail' response in the mouse, while other cholinomimetics such as DFP (1 mg/kg s.c.) and arecoline (5 mg/kg s.c.) do not (unpublished observations).

Recently Héry, Bourgoin, Hamon, Ternaux & Glowinski (1977) have found that physostigmine at a concentration of 2×10^{-4} M increases the spontaneous release of newly synthesized [^{3}H]-5-HT from rat hypothalamic slices. The concentration of physostigmine used by these authors is about 500 times higher than the dose which we have used *in vivo* (100 μg/kg). This concentration is very close to the K_i of physostigmine (2.6×10^{-4} M) on [^{3}H]-5-HT uptake in hypothalamic synaptosomes (Héry *et al.*, 1977). Thus, in our experiments, it is unlikely that 5-HT release evoked by physostigmine was due to the inhibition of 5-HT uptake. In support of this it is noteworthy that in our experiments, the effect of the 5-HT uptake inhibitor, chlorimipramine, on spontaneous 5-HT cortical release was much weaker than that obtained with physostigmine.

Bartolini, Renzi, Galli, Malmberg & Bartolini (1977) have recently reported that molecules such as (−)eseroline (1,3a,8-trimethyl-1,2,3,3a,8,8a-hexahydropyrrole [2,3-b]-indol-5-ol), are devoid of anticholinesterase activity but are structurally related to physostigmine and retain the 5-HT releasing and the analgesic effects of physostigmine. Thus it is probable that the 5-HT releasing action of physostigmine is independent of its anticholinesterase activity. Moreover, Cox & Tha (1972) reported that dyflos at a dose of 2 mg/kg has no antinociceptive activity although the induced inhibition of the whole brain cholinesterase was similar to the inhibition induced by physostigmine at analgesic doses.

In conclusion, our results have shown that both

morphine and physostigmine increase the release of 5-HT from the brain. The 5-HT releasing effect of physostigmine may be related to some of the pharmacological actions of physostigmine, such as the analgesic and the antiaggression effects in raphe-lesioned rats (Vergnes & Penot, 1976), which up to now have been ascribed to its indirect cholinomimetic activity.

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