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EFFECT OF NERVE ACTIVITY ON THE IN VIVO RELEASE OF [^{3}H]SEROTONIN CONTINUOUSLY FORMED FROM L-[^{3}H]TRYPTOPHAN IN THE CAUDATE NUCLEUS OF THE CAT

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SUMMARY

A new isotopic approach has been developed to study the in vivo release of serotonin (5-HT). 'Encéphale isolé' cats were implanted with a push-pull cannula in the ventrocaudal part of the head of the caudate nucleus to estimate the release of [^{3}H]5-HT continuously synthesized from L-[^{3}H]tryptophan. Both [^{3}H]5-HT and [^{3}H]tryptamine were found in superfusates. Resting steady state in the release of [^{3}H]indoleamines was observed as soon as 20 min after the beginning of the superfusion with L-[^{3}H]tryptophan; the levels of [^{3}H]5-HT in superfusates were 2.5 times those of [^{3}H]tryptamine and about 6 times the blank value. They were markedly enhanced in the presence of fluoxetine (5×10^{-6}M), a blocker of the 5-HT uptake process.

A marked increase in the release of [^{3}H]5-HT was seen during the local depolarization of 5-HT terminals with potassium chloride (60 mM) or batrachotoxin (10^{-6}M) or during the stimulation of 5-HT cell bodies in the nucleus raphe dorsalis with L-glutamic acid (5×10^{-5}M). These treatments did not enhance the efflux of [^{3}H]tryptamine. The potassium-evoked release of [^{3}H]5-HT was reduced by LSD (10^{-5}M). LSD added alone in the superfusing fluid was without effect. The batrachotoxin-evoked release of [^{3}H]5-HT was inhibited in the presence of tetrodotoxin (9×10^{-6}M).

The spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine was markedly reduced in the presence of a calcium-free medium containing cobalt (10 mM). A transient slight reduction in the spontaneous release of [^{3}H]5-HT was observed in the presence of tetrodotoxin (9×10^{-6}M). The local cooling of 5-HT cell bodies with a cryoelectrode induced a slight reversible decrease in [^{3}H]5-HT release. These last two treatments were without significant effect on [^{3}H]tryptamine efflux in superfusates.

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These results indicate that the release of [^{3}H]5-HT endogenously formed from [^{3}H]tryptophan is dependent on nerve activity and that this is not the case for [^{3}H]tryptamine. The advantages of the isotopic approach for in vivo studies on the release of 5-HT are discussed.

INTRODUCTION

Despite numerous studies made to analyze the properties of central serotoninergic (5-HT) neurons, the in vivo release of the transmitter has been directly investigated in only a few cases. Ventricular perfusions or push-pull cannulae were used in anesthetized[3,4,18,32] or unanesthetized cats or monkeys[5,15,26,27,44] and 5-HT was estimated with a biological assay[43] or a spectrofluorimetric method[14]. During the last few years, we have also attempted to study the in vivo release of 5-HT from the caudate nucleus. In our first experiments, 5-HT was measured with a radioenzymatic assay in perfusates of the lateral ventricle in rats anesthetized with halothane[40]. Since anesthesia was shown to decrease the rate of 5-HT turnover[11], the 'encéphale isolé' cat preparation was then used[33]. In this case, radioenzymatic estimations of 5-HT were made in superfusates from a cup placed on the ependymal surface of the caudate nucleus[42]. Then, a detailed topographical analysis of the distribution of 5-HT terminals in the caudate nucleus of the cat revealed that they were mainly located in the ventrocaudal part of the structure[41]. To reach this area rich in 5-HT terminals, we chose to use a push-pull cannula with an 'open pulling' system developed in our laboratory by Nieoullon et al.[30]. Due to the limited size of the area superfused, it was not possible to routinely use the radioenzymatic 5-HT assay since 5-HT levels in superfusates were less than 30 pg/15 min fraction, that is, less than twice the blank value. For this reason, and to avoid contamination by 5-HT originating from platelets[4], a new method was developed. It consisted of measuring the release of [^{3}H]5-HT continuously synthesized from L-[^{3}H]tryptophan ([^{3}H]TRP) of high specific activity.

This methodology is described in the present report. It allowed the study of the release of endogenously synthesized [^{3}H]5-HT from the caudate nucleus with a push-pull cannula in the 'encéphale isolé' cat preparation. It will be shown that [^{3}H]tryptamine is also released in superfusates. Attempts were made to define the characteristics of the release process of [^{3}H]5-HT by examining the depolarizing effects of potassium and batrachotoxin and of the pharmacological stimulation of the 5-HT neurons which originate in the nucleus raphe dorsalis[9]. The release of [^{3}H]5-HT was also studied under conditions which are generally used to reduce nerve activity, that is, in the presence of a calcium-free medium or of tetrodotoxin (TTX) or while locally cooling the 5-HT nerve cell bodies.

MATERIALS AND METHODS

The following compounds were used: halothane (fluothane, ICI Pharma), fluoxetine (Lilly 110140, Eli Lilly and Co.), tetrodotoxin (citrate, Sankyo Co., Ltd.),

batrachotoxin (generous gift of J. Daly, Bethesda, U.S.A.), L-glutamic acid (Sigma), LSD 25 (D-lysergic acid diethylamide bitartrate, Sandoz Ltd, Basel), L-[5-^{3}H]TRP (20–30 Ci/mmol., Radiochemical Centre, Amersham).

Physiological procedures

Animals and surgery

Sixty cats of both sexes weighing 2.2–2.8 kg were used. The release of the [^{3}H]indoleamines was studied in 'encéphale isolé' unanesthetized animals maintained under physiological conditions[33]. However, the surgery (tracheotomy, femoral catheterization, implantation of push-pull cannulae) was made under deep halothane anesthesia and the transection of the spinal cord at the C_1–C_2 level was realized after a local injection of Xylocaine (2%). Then the head of the cat was stereotaxically immobilized in an Horsley Clarke apparatus using two steel pieces fixed by acrylic resin in the frontal sinus and on the occipital bone to avoid nociceptive stimulations.

Alveolar CO_2, monitored by a Beckman LB2 capnometer, was maintained between 3.8 and 4%. Femoral blood pressure, heart rate and body temperature were also continuously controlled.

In some experiments, the activity of the 5-HT neurons was modified by local cooling of the nucleus raphe dorsalis using a cryoelectrode (0.9 mm diameter) inserted at P —1.5, L 0, H —2 according to the cat atlas of Snider and Niemer[39]. The minimal temperature reached at the tip of the cryoelectrode was 0 °C; the gradient of temperature (up to 38 °C) extended spherically (1 mm diameter)[7,8]. The nucleus raphe dorsalis was also superfused with L-glutamic acid (5×10^{-5}M) using a push-pull cannula inserted at stereotaxic coordinates identical to those used for local cooling.

The localization of each push-pull cannula or of the cryoelectrode was histologically examined at the end of each experiment.

Local superfusion procedure

The superfusion of the ventrocaudal part of the head of the caudate nucleus was made using a push-pull cannula with an open pulling system[30] which prevents the local suction at the tip of the cannula and thus limits tissue damage. The characteristics of the push-pull cannula were: outer tube, external diameter, 1.9 mm; inner tube, external diameter, 0.5 mm; lateral hole for pulling, 1 mm. The length of the inner tube exceeded by 1.5 mm that of the outer tube.

The push-pull cannula with its mandrel was stereotaxically introduced into the caudate nucleus (A 14.5, L 5, H +5) (ref. 39) using an angular (60°) direction (in a frontal plane) to avoid the lateral ventricle. Then the mandrel was replaced by the internal cannula allowing the continuous delivery of an artificial cerebrospinal fluid (CSF) (in mM: NaCl, 126.5; $NaHCO_3$, 27.5; KCl, 2.4; KH_2PO_4, 0.5; $CaCl_2$, 1.1; $MgCl_2$, 0.85; Na_2SO_4, 0.5; glucose, 5.9 adjusted to pH 7.3 with an O_2–CO_2 95:5 v/v mixture) using a peristaltic pump. The artificial CSF contained 50–60 μCi/ml of L-[5-^{3}H]TRP. Superfusate fractions (15 min) were collected in tubes containing 5-HT (2 μg), tryptamine (2 μg) and tryptophan (20 μg). The optimal concentration of [^{3}H]TRP

in the artificial CSF was 60 μCi/ml and the optimal flow rate was 0.5 ml/15 min. Under these conditions, the quantities of [^{3}H]5-HT and [^{3}H]tryptamine spontaneously released were 5–8 times the respective blank values (0.25 and 0.11 nCi). All treatments were made 140 min after the beginning of the superfusion with [^{3}H]TRP.

Biochemical methods

[^{3}H]tryptophan purification

To obtain a low blank, [^{3}H]TRP was purified just before each experiment. The original ethanol/water solution (50%) of [^{3}H]TRP was diluted with an equal quantity of water and adjusted to pH 2 before its passage through a Dowex column (Dowex AG 50 WX4, buffered at pH 6.5 with Na/Na$_2$ phosphate buffer; 1 cm high, 0.4 cm diameter). The column was washed with 2 ml of 0.1 N HCl and [^{3}H]TRP was eluted with 9 ml of NaCl (0.9%). The final artificial CSF used was prepared taking into account the presence of NaCl in the purified [^{3}H]TRP solution.

Isolation of [^{3}H]indoleamines from [^{3}H]tryptophan

At the end of the superfusion experiments, samples were passed through a Dowex resin (identical to that used for the purification of [^{3}H]TRP) introduced into a plastic tip of an automatic pipette. The size of this conic microcolumn was 1.3 cm high, and 1 mm and 3 mm in diameter in its low and high parts, respectively. The passage of the samples through the microcolumns was made by centrifugation (8 min at 200 $\times$ *g*) in a cold room. The Dowex microcolumns were washed during the night with 1 ml of a solution containing 0.2 M sodium acetate and 0.1% Triton X-100 in water. [^{3}H]-amines were then eluted with 1 ml of a 2 N perchloric acid/ethanol solution (1:2, v/v). To diminish the contamination by the remaining [^{3}H]TRP (less than 1% of the initial amount), the Dowex eluates were passed through an Amberlite column. They were first neutralized (6.4 < pH < 6.8) with 0.7 ml of a 0.2 M K1/K2, phosphate buffer, pH 5.5, containing 7.5% potassium hydroxide. The insoluble potassium perchlorate was discarded by centrifugation. Finally, the samples were diluted with 5 ml of an aqueous solution of Triton X-100 (0.1%, v/v) and passed through the Amberlite column (CG50 buffered at pH 6.1; 1.5 cm high, 0.4 cm diameter). The columns were successively washed with 2 ml of an aqueous solution of Triton X-100 (0.1%, v/v) and 9 ml of a mixture of ethanol, water and Triton X-100 (200:100:0.03 in volumes). They were then pre-eluted with 1 ml of a 0.5 M sodium borate buffer (pH 10) and the [^{3}H]indoleamines were finally eluted with 2 ml of this buffer.

Since the addition of cobalt in the calcium-free medium increased the blank value, it was eliminated before the isolation of the [^{3}H]indoleamines by first passing the CSF superfusates through a Sephadex G10 column[10]. Formic acid eluates of the Sephadex column were neutralized at pH 7.3 with 2 M Tris and then passed through the Dowex column as previously described.

Separation of [^{3}H]5-HT from [^{3}H]tryptamine

[^{3}H]Tryptamine was selectively extracted from the Amberlite eluates (2 ml) with

benzene (10 ml repeated twice). An aliquot (16 ml) of the benzene layer (containing [^{3}H]tryptamine) was mixed with Instafluor (4 ml) (Packard), whereas [^{3}H]5-HT was finally estimated by mixing 1.5 ml of the aqueous solution with Lumagel (10 ml) (Lumac) for radioactivity counting. Corrections were made to take into account the overlap of [^{3}H]5-HT into [^{3}H]tryptamine samples ($< 2\%$) and the overlap of [^{3}H]tryptamine into [^{3}H]5-HT samples ($< 2\%$). After all these steps, the final recoveries ranged between 60 and 70% for [^{3}H]5-HT and [^{3}H]tryptamine.

Identification of [^{3}H]5-HT and [^{3}H]tryptamine

After their isolation from superfusate fractions, the identity of [^{3}H]5-HT and [^{3}H]tryptamine was checked by cochromatography of their acetylated derivatives. For this purpose, the [^{3}H]indoleamines respectively contained in Amberlite eluates and in the aqueous layer after organic extraction were acetylated according to the technique of Laverty and Sharman[23].

To identify [^{3}H]tryptamine, the benzene layer was evaporated and the dry residue was then extracted with 5 ml of warm methanol. This solution was concentrated to 1 ml under a stream of nitrogen and acetone (5 ml) was finally added. After 1 h at —20 °C, the precipitate was discarded by centrifugation and the supernatant concentrated to 40 μl for application on a thin layer silicagel plate (Merck). The solvent system was a mixture of chloroform–methanol–NH_4OH (12:7:1 in volumes)[46].

Expression of data

In steady state, the quantities of [^{3}H]5-HT and [^{3}H]tryptamine spontaneously released from the caudate nucleus varied slightly from one cat to another. The limits of these variations for [^{3}H]5-HT were 0.7 and 2.5 nCi for 0.5 ml/15 min. Therefore, each cat was used as its own control. The mean value of the quantities of [^{3}H]5-HT measured in the 4 fractions preceding each experimental treatment was used as the control value (100%). [^{3}H]5-HT content in each successive fraction was then expressed as a percentage of this basal value. Similar calculations were made for [^{3}H]tryptamine. Means $\pm$ S.E.M. of data obtained in 5–8 experiments were calculated.

A variance analysis of the spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine in the successive fractions revealed that the quantities of [^{3}H]amines were never statistically different from one fraction to another during the 5 h of the superfusion (F 19/367 = 0.287 and F 19/384 = 0.19 for [^{3}H]5-HT and [^{3}H]tryptamine respectively). Therefore, statistical analysis of data were made using the Student's t-test[36] by comparing the value of [^{3}H]amines in each successive fraction after various treatments with the mean content of [^{3}H]amines in the 4 fractions preceding the treatments. When the P value was higher than 0.05, the difference was not considered to be significant.

RESULTS

(1) Spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine synthesized from [^{3}H]-tryptophan in the caudate nucleus of the cat

A steady state release of [^{3}H]indoleamines was observed as soon as 15 min after

the beginning of the superfusion of the caudate nucleus with [^{3}H]TRP. Both [^{3}H]5-HT and [^{3}H]tryptamine were found in superfusates (Fig. 1). The content of [^{3}H]5-HT was about 2.5 times that of [^{3}H]tryptamine during the 5 h of superfusion and represented about 6 times the blank value. After their isolation and separation, [^{3}H]5-HT and [^{3}H]tryptamine were further identified by cochromatography (Fig. 2). This confirmed that [^{3}H]tryptamine is selectively extracted in the benzene layer whereas [^{3}H]5-HT represents the only [^{3}H]indoleamine which remains in the aqueous layer.

When introduced for 2 h in the artificial CSF delivered to the push-pull cannula, fluoxetine (5×10^{-6}M), a potent blocker of the 5-HT uptake process, markedly increased the release of newly synthesized [^{3}H]5-HT (Fig. 3). Indeed, the quantity of [^{3}H]5-HT in the two first 15 min fractions was already twice that of the spontaneous release and reached about 300% of control levels at the end of the drug's application. [^{3}H]5-HT levels remained elevated for at least 1 h after the removal of fluoxetine from the artificial CSF. The efflux of [^{3}H]tryptamine was much less affected (Fig. 3).

(2) Effects of a calcium-free medium and of tetrodotoxin on the spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine

In a first experiment, calcium was omitted from the artificial CSF 2 h after the beginning of the superfusion and cobalt (10 mM) was added to the superfusion medium to further compete with calcium influx in nerve terminals. Under these

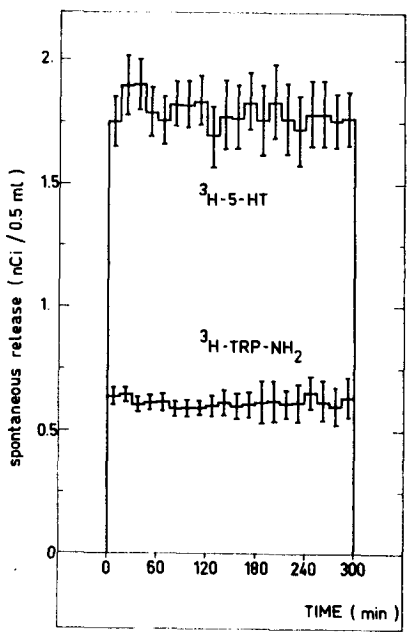

Fig. 1. Spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine from the caudate nucleus of the cat. L-[^{3}H]tryptophan (60 μCi/ml) was continuously delivered (0.5 ml/15 min) to a push-pull cannula with an open pulling system implanted in the ventrocaudal part of the caudate nucleus in the 'encéphale isolé' cat preparation. The release of [^{3}H]5-HT and [^{3}H]tryptamine ([^{3}H]TRP-NH$_2$) were estimated in serial 15 min superfusate fractions 20 min after the onset of the superfusion with L-[^{3}H]tryptophan. Results are the mean $\pm$ S.E.M. of values obtained in 15 experiments.

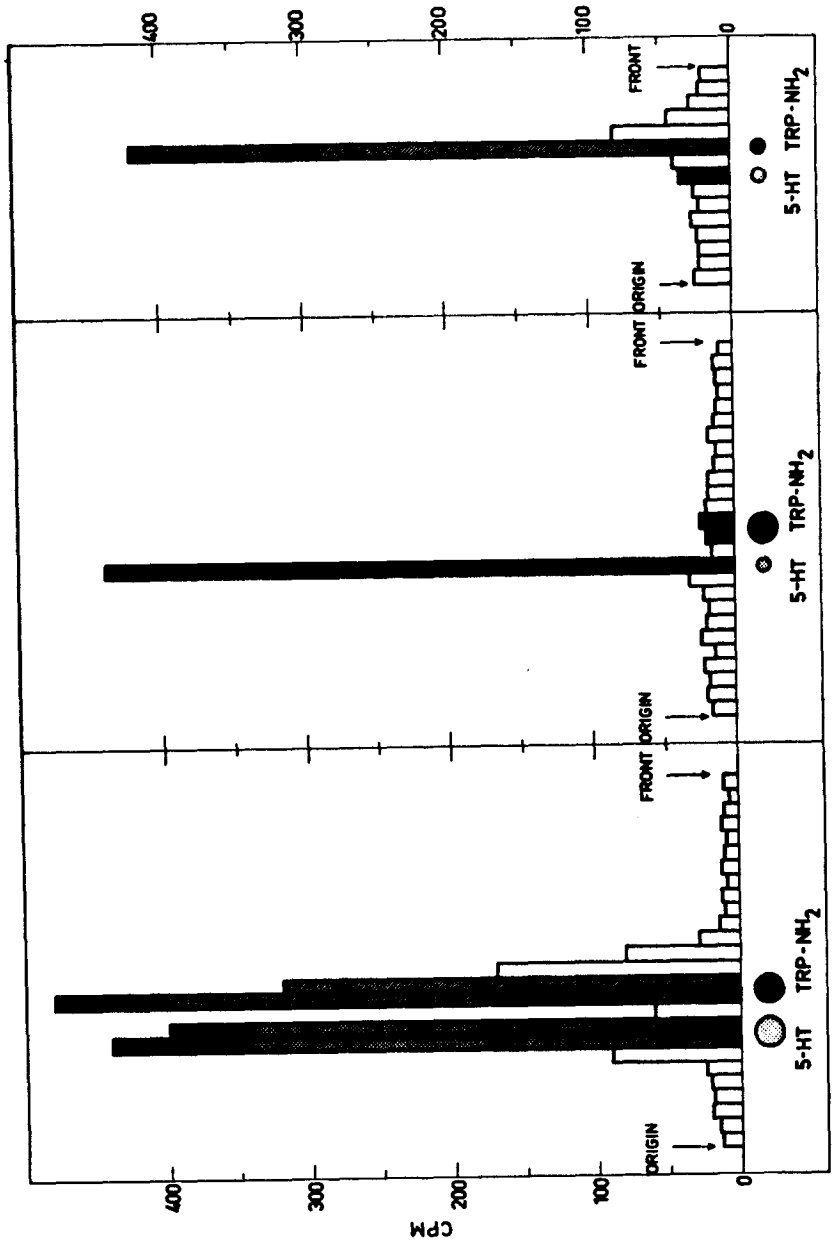

Fig. 2. Identification of endogenously synthesized [^{3}H]indoleamines released in superfusates. Eluates of Amberlite columns corresponding to samples of 6 successive 15 min superfusate fractions collected during continuous labeling of 5-HT terminals with L-[^{3}H]tryptophan were pooled for acetylation of the [^{3}H]indoleamines. The left part of the figure illustrates the co-chromatogram of the acetyl derivatives of [^{3}H]5-HT and [^{3}H]tryptamine released in superfusates. The cochromatogram of the acetyl derivative of [^{3}H]5-HT obtained after elimination of [^{3}H]tryptamine in Amberlite eluates by benzene is represented in the middle part of the figure. The right side of the figure illustrates the identification of [^{3}H]tryptamine recovered in the benzene layer at the end of the extraction procedure after thin layer cochromatography on silica gel plates using chloroform–methanol–concentrated NH_4OH (12:7:1 by vol) as the solvent.

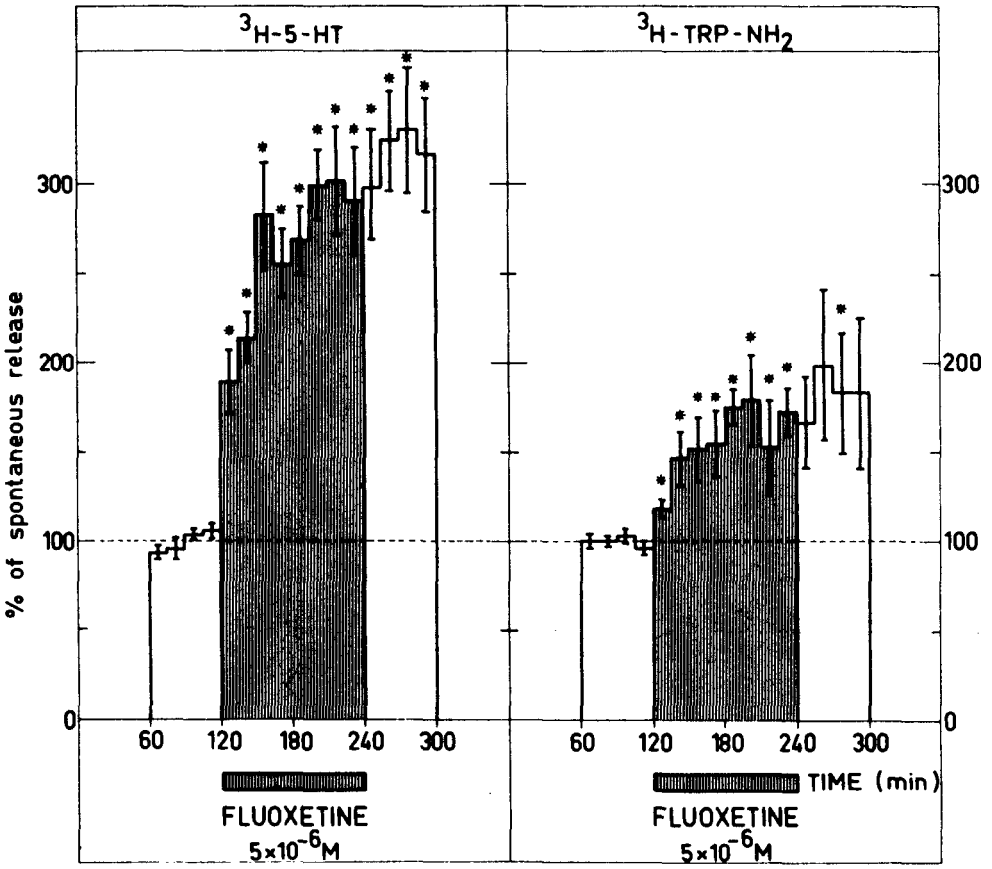

Fig. 3. Effects of fluoxetine on the spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine. Experiments were carried out as described in Fig. 1 except that fluoxetine (5×10^{-6} M), a potent inhibitor of the 5-HT uptake process, was added for 2 h in the superfusing medium 2 h after the onset of the superfusion with L-[^{3}H]tryptophan. The quantities of [^{3}H]5-HT and [^{3}H]tryptamine ([^{3}H]TRP-NH$_2$) found in each successive fraction were expressed as a percentage of the mean spontaneous release of the [^{3}H]indoleamines estimated during the 4 fractions which preceded the drug application. Results are the mean ± S.E.M. of values obtained in 6 experiments. * $P < 0.05$ when compared to the mean spontaneous release of [^{3}H]5-HT, and [^{3}H]TRP-NH$_2$ determined from the 4 superfusate fractions collected just before the treatment.

conditions, the release of [^{3}H]5-HT was immediately reduced by 60%. This effect lasted for the entire application of the calcium-free medium enriched with cobalt (10 mM). The spontaneous release of [^{3}H]tryptamine was similarly reduced, the effect being even more pronounced during the last 30 min (Fig. 4).

The introduction of TTX (9×10^{-6}M) into the artificial CSF reduced the spontaneous release of [^{3}H]5-HT by only 25% (Fig. 5). However, this effect was transient since the release of [^{3}H]5-HT returned to control levels 30 min before the end of the TTX application. In contrast, the efflux of [^{3}H]tryptamine in superfusates was not affected by the presence of TTX.

To further verify the blocking action of TTX on sodium channels, the effects of

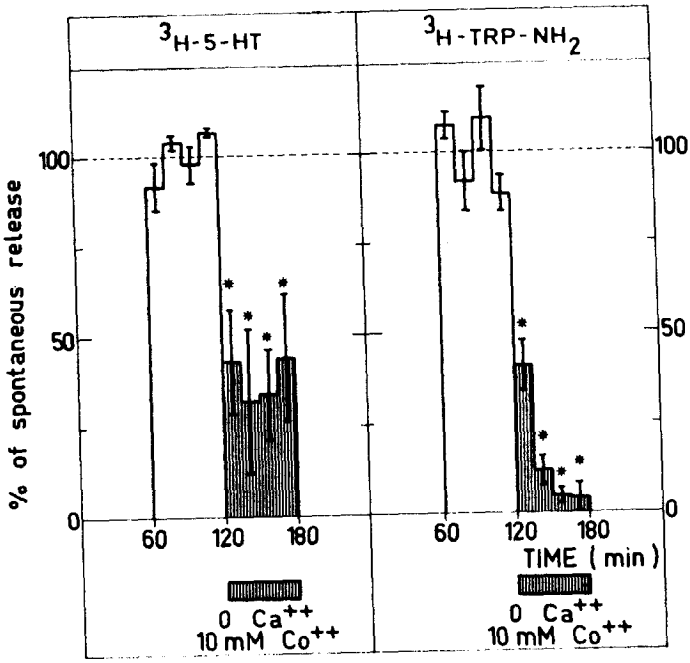

Fig. 4. Effect of a calcium-free medium containing cobalt on the spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine. Two hours after the beginning of the superfusion with L-[^{3}H]tryptophan a calcium-free medium containing cobalt chloride (10 mM) and the ^{3}H-precursor was delivered to the push-pull cannula. [^{3}H]5-HT and [^{3}H]TRP-NH$_2$ were estimated in superfusates and results were expressed as described in Fig. 3. Results are the mean ± S.E.M. of values obtained in 5 experiments. * $P < 0.05$ when compared to the mean spontaneous release of respective [^{3}H]indoleamines.

batrachotoxin (10^{-6}M) were examined in the absence and in the presence of TTX. The introduction of batrachotoxin for 30 min in the CSF induced a 600% increase in the release of [^{3}H]5-HT (Fig. 5), but was without effect on the [^{3}H]tryptamine efflux. The stimulatory effect of batrachotoxin was inhibited by TTX, since the increase in [^{3}H]5-HT release reached 50% and only appeared during the first 15 min of the batrachotoxin application (Fig. 5).

(3) Effect of potassium in the absence or in the presence of LSD on the release of [^{3}H]5-HT and [^{3}H]tryptamine

As expected, the depolarization of nerve terminals with potassium chloride (60 mM) during 30 min stimulated the release of [^{3}H]5-HT; the peak of the effect appeared during the second 15 min of potassium application (300%). Thereafter, the release of [^{3}H]5-HT rapidly returned to control levels (Fig. 6). In contrast, the potassium depolarization did not increase the efflux of [^{3}H]tryptamine. Surprisingly, a slight, but significant (30%), decrease in [^{3}H]tryptamine release was detected when the stimulating effect of potassium on [^{3}H]5-HT release was maximal (data not shown).

We have previously shown that LSD reduced the potassium-evoked release of [^{3}H]5-HT synthesized from [^{3}H]tryptophan in rat striatal slices[17]. This also occurs in

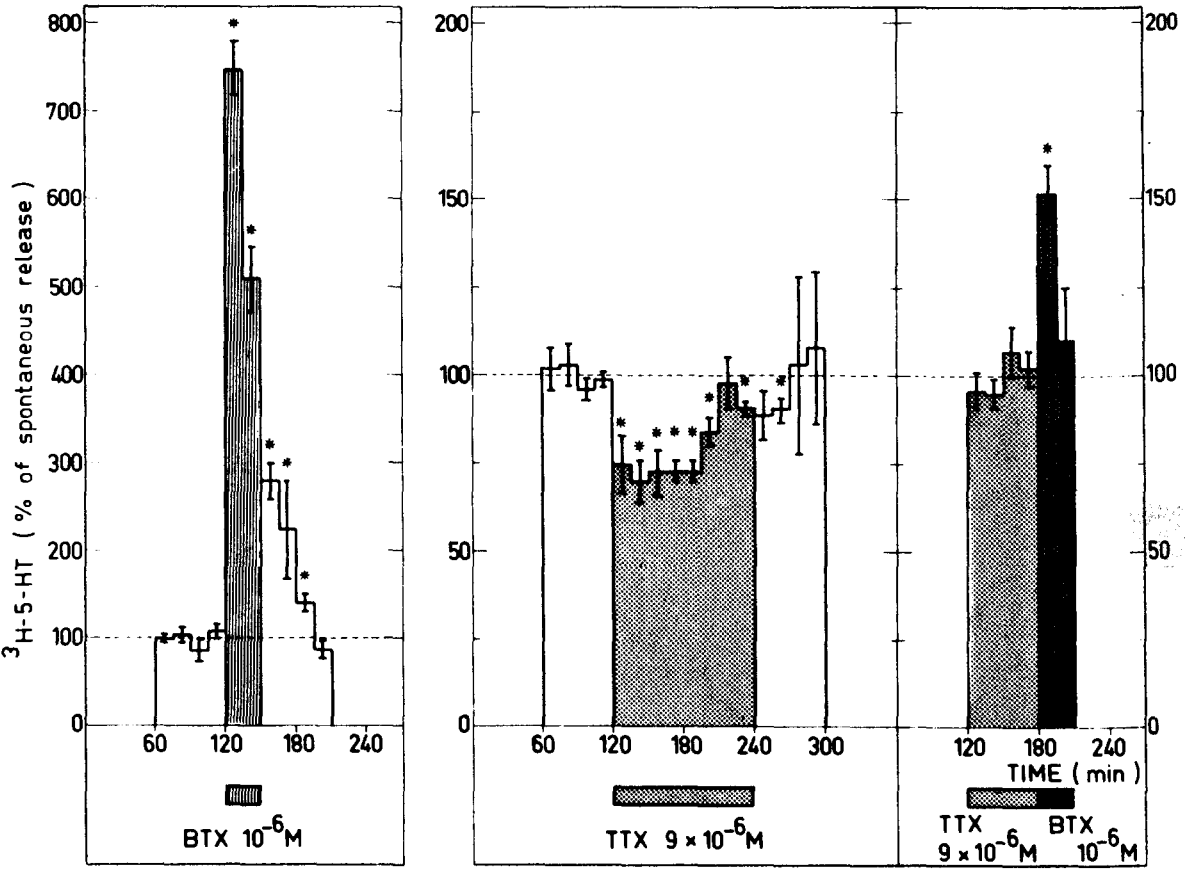

Fig. 5 Effects of tetrodotoxin and batrachotoxin on the spontaneous release of [^{3}H]5-HT. Bratachotoxin (BTX, 10^{-6} M) or tetrodotoxin (TTX, 9×10^{-6} M) were added for 30 min and 160 min respectively in the superfusing medium containing L-[^{3}H]tryptophan 2 h after the beginning of the superfusion. In other experiments (right panel) BTX was added during the last 30 min of TTX application. [^{3}H]5-HT was estimated in superfusates and results were expressed as described in Fig. 3. The effect of BTX in the presence of TTX was expressed as a percentage of the mean release of [^{3}H]5-HT during the 4 fractions which preceded BTX application. Results are the mean ± S.E.M. of values obtained in 6 experiments. * $P < 0.05$ when compared to the mean spontaneous release of [^{3}H]5-HT.

vivo since the stimulating effect of potassium (60 mM) on [^{3}H]5-HT release was inhibited by more than 50% in the presence of LSD (10^{-5}M). The 5-HT agonist did not significantly affect the spontaneous release of [^{3}H]5-HT (Fig. 6) or the efflux of [^{3}H]tryptamine.

(4) Changes in the release of [^{3}H]5-HT induced by stimulation or interruption of nerve impulse flow in serotoninergic neurons

L-Glutamic acid was used to stimulate the activity of the 5-HT neurons projecting to the caudate nucleus. For this purpose, cats were implanted with a push-pull cannula in the nucleus raphe dorsalis and another one in the caudate nucleus. L-

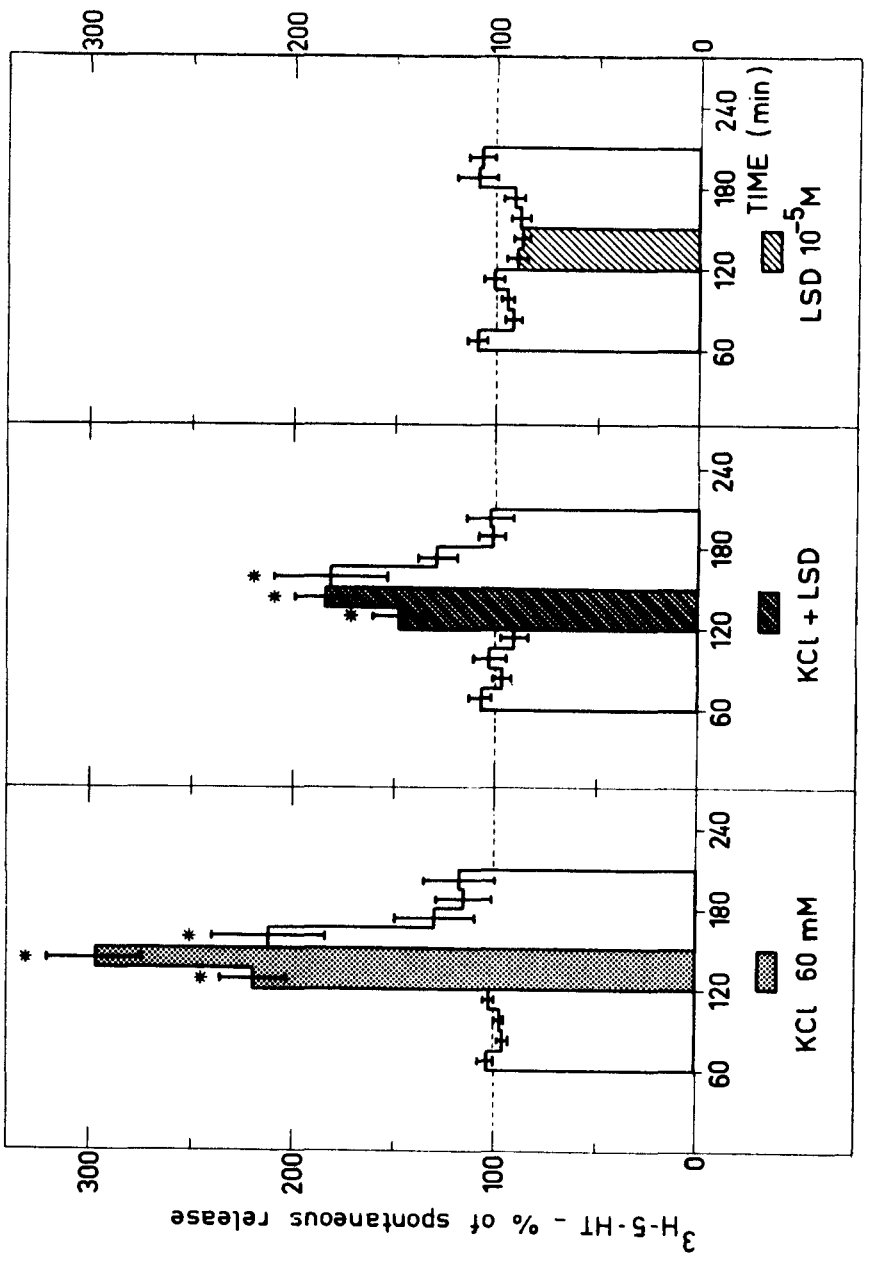

Fig. 6. Effect of potassium depolarization in the absence or in the presence of LSD on the release of [^{3}H]5-HT. Experiments were performed as described in Figs. 1 and 3. Potassium chloride (60 mM) and LSD (10^{-5} M) were added alone or simultaneously for 30 min in the superfusing medium containing [^{3}H]tryptophan, 2 h after the beginning of the experiment. Results were expressed as described in Fig. 3. They are the mean ± S.E.M. of values obtained in 5–8 experiments. * $P < 0.05$ when compared to the mean spontaneous release of [^{3}H]5-HT.

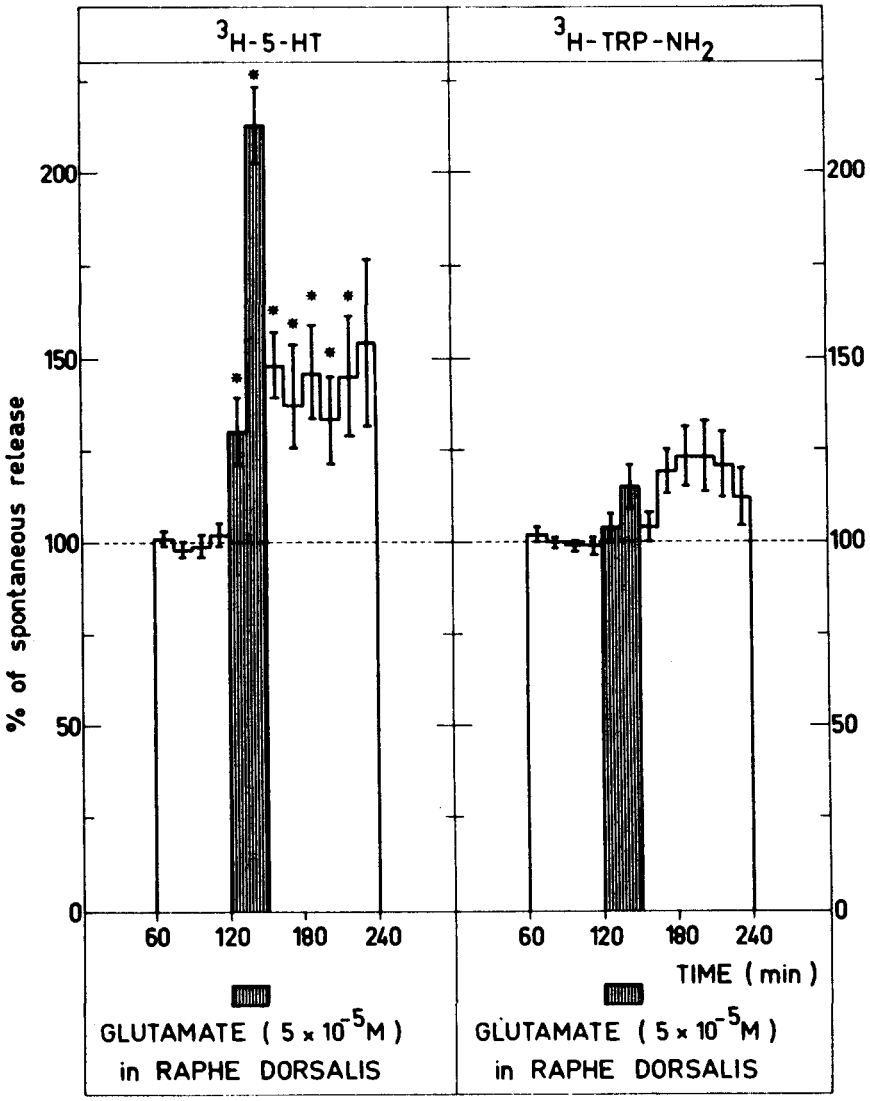

Fig. 7. Effects of the local application of L-glutamic acid in the nucleus raphe dorsalis on the release of [^{3}H]5-HT and [^{3}H]tryptamine from the caudate nucleus. Cats were implanted with a push-pull cannula into the caudate nucleus to estimate the release of [^{3}H]5-HT and [^{3}H]tryptamine continuously synthesized from L-[^{3}H]tryptophan as described in Fig. 1. The nucleus raphe dorsalis was simultaneously superfused with an artificial CSF medium using a push-pull cannula and L-glutamic acid (5×10^{-5} M) was added for 30 min only in this medium 120 min after the beginning of the experiments. Results are the mean $\pm$ S.E.M. of values obtained in 6 experiments. * $P < 0.05$ when compared to the mean spontaneous release of [^{3}H]5-HT.

Glutamic acid (5×10^{-5}M) was delivered for 30 min to the push-pull cannula superfusing the 5-HT cell bodies 2 h after the beginning of the local superfusion of the caudate nucleus with L-[^{3}H]TRP.

L-Glutamic acid stimulated the release of [^{3}H]5-HT by 50% during the first 15

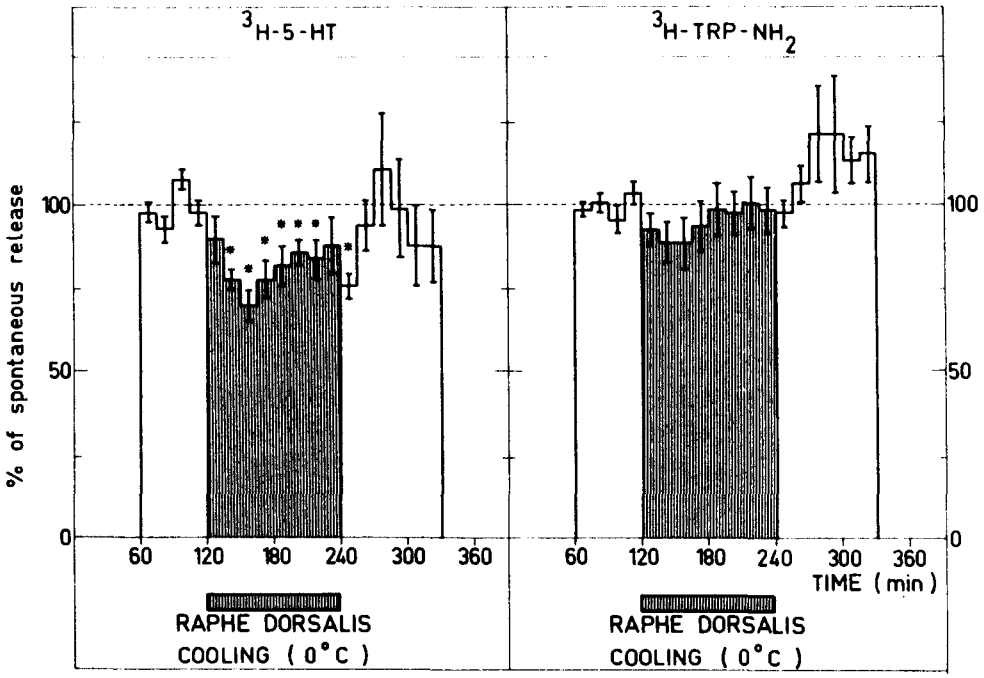

Fig. 8. Effects of the local cooling of the nucleus raphe dorsalis on the release of [^{3}H]5-HT and [^{3}H]-tryptamine from the caudate nucleus. Experiments were performed as described in Fig. 1 except that a cryoelectrode was also inserted in the nucleus raphe dorsalis. This allowed a local cooling (0 °C at the tip of the cryoelectrode) of the nucleus for 2 h. Results expressed as described previously (Fig. 3) are the mean ± S.E.M. of values obtained in 6 experiments. $^*P < 0.05$ when compared to the mean spontaneous release of [^{3}H]5-HT and [^{3}H]tryptamine.

min. The effect was much more striking in the following 15 min, since the stimulation of the ^{3}H-transmitter release reached 200% that of the spontaneous release (Fig. 7). Although less pronounced (about 140%), a persistent stimulation of [^{3}H]5-HT release was still detected for at least 1 h after the cessation of the L-glutamic acid application. The efflux of [^{3}H]tryptamine in the caudate nucleus was almost not affected during the stimulation of the 5-HT neurons with L-glutamic acid (Fig. 7).

In other experiments, a cryoelectrode was introduced into the nucleus raphe dorsalis to interrupt the activity of 5-HT neurons. The local cooling of this nucleus at 0 °C, for 2 h, induced a slight but significant reduction in [^{3}H]5-HT release; the maximal effect was 30%. The release of [^{3}H]5-HT returned to the levels of the spontaneous release shortly after the cessation of cooling. Slight perturbations in the efflux of [^{3}H]tryptamine were also seen but they were not significant (Fig. 8).

DISCUSSION

The release of [^{3}H]5-HT synthesized from [^{3}H]TRP has already been estimated in ventricular perfusates of the rat[16,19]. However in these studies, the ^{3}H-transmitter release was studied after a short period of labeling with the ^{3}H-precursor. Since newly

synthesized [^{3}H]5-HT may be preferentially involved in the release process, we have developed an isotopic method adapted for measuring the in vivo release of [^{3}H]5-HT continuously synthesized from L-[^{3}H]TRP. Moreover, a push-pull cannula was used to study the release of 5-HT from terminals of the serotoninergic pathway projecting to the caudate nucleus. This was achieved despite the limited 5-HT innervation of this structure when compared with the extensive network of dopaminergic terminals. This approach presents several advantages, as already shown in studies of our group on the in vivo release of [^{3}H]DA endogenously synthesized from [^{3}H]tyrosine in terminals[30] and dendrites[31] of the nigrostriatal dopaminergic neurons. Since tryptophan hydroxylase is selectively located in 5-HT terminals, [^{3}H]5-HT released into superfusates can be formed only in these terminals. In contrast to what may occur in studies in which unlabeled 5-HT is measured, possible interferences with 5-HT originating from platelets can be excluded when the isotopic method is used. Due to the high specific activity of [^{3}H]TRP, quantities of [^{3}H]5-HT as low as 2 pg (two times the blank value) can be precisely measured. Furthermore, the rapidity of the isolation procedure for [^{3}H]5-HT allowed the simultaneous analysis of about 100 samples, which is not the case when unlabeled 5-HT is estimated with the radioenzymatic method.

One of the main difficulties of this approach was to separate the small quantity of [^{3}H]5-HT released from the large amount of [^{3}H]TRP ($\approx$ 60 μCi/ml) contained in superfusates. Another obstacle was the presence of [^{3}H]tryptamine in the superfusates. A rapid procedure was developed to isolate selectively the [^{3}H]indoleamines from [^{3}H]TRP and then to separate [^{3}H]5-HT from [^{3}H]tryptamine. Under the conditions used, the average quantity of [^{3}H]5-HT spontaneously released (1.7 nCi per 0.5 ml/15 min) was about 6 times the blank value and the amount of [^{3}H]tryptamine in superfusates was always lower than that of [^{3}H]5-HT. Furthermore, the efflux of both [^{3}H]amines rapidly reached a steady state level which could be maintained for at least 5 h. No significant variations in the content of [^{3}H]5-HT and [^{3}H]tryptamine were seen from one 15 min fraction to another during resting state.

Variations in the release of [^{3}H]5-HT induced by changes in the activity of the 5-HT neurons could be seen. A marked and transient increase in [^{3}H]5-HT release was observed under local depolarization of the 5-HT terminals with potassium (60 mM) or batrachotoxin (10^{-6}M)[2]. Furthermore, the blockade of sodium channels with TTX (9×10^{-6}M)[28,29] abolished the batrachotoxin-evoked release of [^{3}H]5-HT. Vogt and her colleagues reported that the release of 5-HT was enhanced in ventricular perfusates of the cat during the electrical stimulation of the linearis raphe nucleus[3,4,18]. Using another approach, we also observed that the stimulation of 5-HT neurons projecting to the caudate nucleus resulted in an increased release of [^{3}H]5-HT from terminals. Indeed, the local application of L-glutamic acid in the nucleus raphe dorsalis, which has been shown to accelerate the firing rate in 5-HT neurons[1,12], stimulated the release of [^{3}H]5-HT in the caudate nucleus. A transient increase in [^{3}H]5-HT release was also detected in two experiments during the local mechanical stimulation of the 5-HT neurons obtained by slightly turning the inner tube of the push-pull cannula inserted in the nucleus raphe dorsalis.

Attempts were also made to demonstrate that the spontaneous release of [^{3}H]5-

HT was not a passive phenomenon and was also dependent on nerve activity. It was markedly reduced when the influx of calcium in 5-HT terminals was tentatively diminished by omitting calcium and adding cobalt (a blocker of calcium channels)[45] in the superfusing fluid. TTX, used at a concentration of 5×10^{-7}M, has been shown to persistently reduce by 40% the spontaneous release of [^{3}H]DA synthesized from [^{3}H]tyrosine in the caudate nucleus of the cat[30]. Although our experimental conditions were identical, the neurotoxin at this concentration was ineffective in the release of [^{3}H]5-HT. However, a slight but significant reduction (30%) in [^{3}H]5-HT release was observed when TTX was applied at 9×10^{-6}M. Furthermore, in contrast to that observed with [^{3}H]DA, the TTX inhibiting effect on [^{3}H]5-HT spontaneous release did not persist during its entire application. Such a transient effect has also been observed in our study on the release of unlabeled 5-HT from the dorsal surface of the caudate nucleus[42]. This may suggest that the sensitivity of 5-HT neurons to TTX differs from that of dopaminergic neurons. Such differences in sensitivity to TTX among nerve fibers have already been observed in other preparations (ref. 13 and see review ref. 28). Finally, a slight but prolonged reduction in the spontaneous release of [^{3}H]5-HT was seen under local cooling of the nucleus raphe dorsalis. This procedure, which should interrupt tonic nerve activity in 5-HT neurons, similarly affected the release of [^{3}H]DA in the caudate nucleus when it was applied to the substantia nigra[30]. Despite the reduction of [^{3}H]5-HT release seen in the absence of calcium, it cannot be excluded that part of [^{3}H]5-HT which is still released in the presence of TTX or during the reversible cooling of the nucleus raphe dorsalis corresponds to a simple diffusion of the [^{3}H]amine in the extraneuronal space. Alternatively, the release of [^{3}H]5-HT still detected in the presence of TTX could be attributed to spontaneous resting discharges similar to those observed at the neuromuscular junction[20]. On the other hand, the 5-HT cell bodies innervating the caudate nucleus may not all be affected by the punctual and limited cooling induced by the cyroelectrode[7,8].

Measuring the release of [^{3}H]5-HT previously formed from [^{3}H]TRP in ventricular perfusates of the rat, Gallager and Aghajanian[16] observed that the peripheral administration of LSD (75 or 150 μg/kg) decreased the rate of [^{3}H]5-HT efflux. This was attributed to a reduced firing of 5-HT neurons. LSD could also act at a presynaptic level. In fact, the inhibiting effect of LSD on the potassium-induced release of [^{3}H]5-HT observed in the present study is in agreement with previous in vitro results which have suggested the presence of presynaptic 5-HT receptors involved in the local control of the transmitter release[17].

Tryptamine which results from the decarboxylation of tryptophan is found in small amounts when compared to 5-HT in several central structures such as the spinal cord, the caudate nucleus and the hypothalamus[21,34,37]. Since both 5-HT and catecholaminergic neurons are rich in aromatic amino acid decarboxylase activity[22,35], [^{3}H]tryptamine could be equally formed from [^{3}H]TRP in their terminals. In fact, the levels of tryptamine in the caudate nucleus are slightly reduced after the degeneration of 5-HT or of dopaminergic neurons[24]. According to some authors, specific tryptaminergic neurons innervate the spinal cord and tryptamine could act as a neurotransmitter[6,25]. Our results indicate that this does not seem to be the case for [^{3}H]tryptamine

endogenously formed from [^{3}H]TRP in the cat caudate nucleus. In contrast to that observed with [^{3}H]5-HT, the efflux of [^{3}H]tryptamine was not stimulated under local depolarization with potassium or batrachotoxin or during the L-glutamic acid-induced stimulation of the 5-HT neurons although [^{3}H]tryptamine is partly formed in their terminals. Finally, no significant changes in the efflux of [^{3}H]tryptamine were observed during local application of TTX or local cooling of the raphe neurons. Since the efflux of [^{3}H]tryptamine was largely reduced by omitting calcium and adding cobalt in the superfusing fluid, it might involve a calcium dependent mechanism as already suggested in in vitro studies[38]. However, further studies will be required to clarify this point.

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