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SEROTONIN-SENSITIVE ADENYLATE CYCLASE AND [^{3}H]SEROTONIN BINDING SITES IN THE CNS OF THE RAT—I

KINETIC PARAMETERS AND PHARMACOLOGICAL PROPERTIES

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Abstract—The 5-HT receptor linked to adenylate cyclase and the high affinity binding site for [^{3}H]-5-HT were compared on the basis of their kinetic and pharmacological properties in the CNS of new born rats. Under normal assay conditions, the apparent affinity of 5-HT for its specific binding sites (K_d = 1–2 nM) was much higher than that for the receptor coupled to adenylate cyclase (K_A app = 0.5–1.0 μM). When measured under the conditions of the cyclase assay, the apparent K_d for the binding was increased to 11.9 nM, a value which is still more than 40 times lower than the K_A app characterizing the activation of adenylate cyclase by 5-HT. GTP affected both the binding of [^{3}H]-5-HT and the 5-HT-sensitive adenylate cyclase. Guanyl nucleotides appeared to be essential for the activation of adenylate cyclase by 5-HT as 5-HT was inactive in a preparation of washed membranes unless added in the presence of GTP or GppNHp. In whole homogenates, GTP increased the affinity of 5-HT for the receptor–adenylate cyclase complex (K_A app = 0.33 μM in the presence of 10 μM GTP). The specific binding of [^{3}H]-5-HT was reduced by GTP and GppNHp but not GMP or ATP. However, the range of concentrations inducing a significant effect ($\geq$ 0.10 mM GTP) was far higher than those which increased the 5-HT-induced activation of adenylate cyclase.

There was little in common between the pharmacological profiles of the two systems. A group of 5-HT agonists containing a piperazine heterocycle [1- (*m*-trifluoromethylphenyl) piperazine, quipazine and MK-212] effectively displaced [^{3}H]-5-HT from its binding sites but exerted no action on the 5-HT-sensitive cyclase, affecting neither the basal nor the 5-HT-stimulated cAMP production. Likewise, there was no correlation between the respective potencies of a series of 5-HT antagonists for inhibiting the binding of [^{3}H]-5-HT and the 5-HT-induced cAMP production. These data suggest that the 5-HT receptor linked to adenylate cyclase is not identical with that which is measured by the binding of [^{3}H]-5-HT and, thus, provide evidence for the possible existence of multiple receptors for 5-HT in the rat brain.

The existence in brain membranes of an adenylate cyclase activity which can be stimulated by serotonin (5-hydroxytryptamine, 5-HT) has been described by several authors [1–6]. As expected of an enzyme coupled with a 5-HT receptor, the 5-HT-sensitive adenylate cyclase activity can be stimulated by specific agonists [1, 5]. Conversely, known 5-HT antagonists inhibit the stimulating effect of 5-HT on adenylate cyclase activity [1, 5].

Serotonin receptors in the CNS have also been studied by measuring the binding of radioactive 5-HT to membranes. With this technique, specific high-affinity binding sites for [^{3}H]-5-HT have been identified [7–10]. Although it is difficult to prove that these binding sites correspond to functional 5-HT receptors, they do exhibit the appropriate pharmacological profile [7–10]. Furthermore, alterations in [^{3}H]-5-HT binding possibly related to some kind of denervation supersensitivity phenomenon have been observed on several occasions; thus, selective degeneration of serotoninergic terminals [9], long-term depletion of 5-HT stores produced by chronic treatment with *para*-chlorophenylalanine [11], chronic [12] and even acute [13] treatment with 5-HT receptor blocking agents significantly increased the number of [^{3}H]-5-HT binding sites in various brain areas.

The large difference between the affinities of 5-HT for its specific binding sites (K_d = 1–2 nM [9]) and for the receptor coupled with adenylate cyclase (K_A app = 0.5–1.0 μM [4]) suggests that they correspond to two distinct classes of 5-HT receptors. However, other possibilities must also be examined, including the differences in the assay conditions and in the tissue preparations used for measuring these two parameters. Thus, whereas [^{3}H]-5-HT binding was usually measured on brain membranes from adult rats, most experiments dealing with 5-HT-sensitive adenylate cyclase were performed with tissue preparations from newborn rats. This resulted from the fact that the activity of 5-HT-sensitive adenylate cyclase in cell-free extracts from adult rats is too low for reliable quantitative measurements [1, 4].

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In the present study, attempts were made to compare the kinetic characteristics and pharmacological properties of [^{3}H]-5-HT binding sites and 5-HT-sensitive adenylate cyclase under conditions as nearly identical as possible. In particular, both the adenylate cyclase activity and the [^{3}H]-5-HT specific binding were measured in preparations from newborn animals. The persistence of large differences in the properties of these two markers of receptors, together with those reported in the accompanying paper [14], stongly suggests that they do correspond to two distinct types of central 5-HT receptors.

MATERIALS AND METHODS

Chemicals. The following compounds were used: 5-hydroxytryptamine creatinine sulfate (5-HT, Merck); ATP, GTP, GMP and 5′-guanylylimidodiphosphate [Gpp (NH)p], cyclic AMP, creatine kinase and creatine phosphate were purchased from Boehringer–Mannheim; ethylene glycol bis (β-amino ethylether)-*N, N′*-tetraacetic acid (EGTA) and theophylline were from Sigma. Psychotropic drugs were: 1-(*m*-trifluoromethylphenyl) piperazine (Aldrich); quipazine (Miles); metergoline (Farmitalia); cinanserine and fluphenazine (Squibb & Sons); *d*-lysergic acid diethylamide (*d*-LSD) and pizotifen (Sandoz); spiroperidol (Janssen); methiothepin (Hoffman–LaRoche); clozapine (Dr. A. Wander, Bern); thioproperazine (Rhône–Poulenc); MK-212 [6-chloro-2-(1-piperazinyl) pyrazine] and cyproheptadine (Merck, Sharp & Dohme); α-flupenthixol (Labaz) and mianserine (Organon).

[G-^{3}H]-5-HT (generally labelled, 10.7–15.2 Ci/mmole), [α-^{32}P]ATP (sodium salt, 10–20 Ci/mmole) and cyclic [^{3}H]-AMP (ammonium salt, 25 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, U.K. [^{3}H]-5-HT was purified in the morning of each binding experiment according to the procedure of Hamon *et al.* [15].

All other compounds (salts, etc.) were the purest commercially available.

Animals. Sprague–Dawley (Charles River strain) rats were housed in a controlled environment (24°, 60% relative humidity, alternate cycles of 12hr light and 12hr darkness, food and water *ad lib.*) for at least 7 days before being used. Newborn animals were used regardless of sex while only male adults (250–350 g) were taken. Animals were decapitated, and their brains were dissected in the cold (4°). All subsequent steps in the tissue preparations (see below) were also carried out at 0–4°.

Preparation of membranes. The membranes for the binding assays were prepared as previously described [9]. Briefly, tissues were homogenized in 10 vol. (v/w) of 0.32 M sucrose using a Potter–Elvehjem apparatus fitted with a Teflon pestle. The homogenate was centrifuged at 750 *g* for 10 min and the supernatant was carefully decanted and further centrifuged at 10,000 *g* for 30 min. The resulting pellet (P2) was resuspended in 10 vol. of ice-cold water and gently stirred for 30 min. The osmotically shocked material was then centrifuged at 35,000 *g* for 20 min. The sedimented membranes were resuspended in 40 vol. of 0.05 M Tris–HCl, pH 7.4, and incubated for 10 min at 37° in order to remove endogenous 5-HT still adsorbed to them (see Ref. 9). After further centrifugation at 35,000 *g* for 20 min, the collected pellet was washed once by resuspension in 40 vol. of 0.05 M Tris–HCl, pH 7.4, and centrifugation. The final pellet was gently homogenized in 40–80 vol. of 0.05 M Tris–HCl, pH 7.4, containing 5.7 mM ascorbic acid, 10 μM pargyline and 4 mM $CaCl_2$. This suspension was directly used for the [^{3}H]-5-HT binding assay.

Measurement of [^{3}H]-5-HT *binding*. The binding of [^{3}H]-5-HT was measured according to the method of Bennett and Snyder [7] using the modifications previously described [9]. In brief, 2 ml aliquots of the final membrane suspension (corresponding to 0.15–0.96 mg protein) were incubated at 37° for 7 min in the presence of various concentrations of [^{3}H]-5-HT from 0.30 to 6.0 nM. The samples were then rapidly filtered through Whatman GF/B filters under vacuum. The filters were washed three times with 5 ml of ice-cold 0.05 M Tris–HCl, pH 7.4, containing ascorbic acid, pargyline and $CaCl_2$ (as above). After drying, they were finally vigorously stirred in 10 ml of Aquasol® for radioactivity counting by liquid scintillation spectrometry.

When increasing concentrations of unlabelled 5-HT were added to the assay mixture, the binding of [^{3}H]-5-HT to brain membranes progressively decreased to a plateau corresponding to 15–30 per cent of that in the absence of unlabelled ligand. This plateau was reached with 0.5 μM of unlabelled 5-HT. The specific [^{3}H]-5-HT binding (i.e. the displaceable component) was calculated as the difference between total binding minus that persisting in the presence of a large excess, 10 μM, of unlabelled 5-HT. In all cases, binding assays in the absence or the presence of unlabelled 5-HT were done at least in triplicates.

Under these conditions, [^{3}H]-5-HT bound to specific sites distinct from those on monoamine oxidase A and on the reuptake carrier in synaptosomal membranes (see Ref. 9).

Measurement of adenylate cyclase activity. Adenylate cyclase activity was measured as described by Enjalbert *et al.* [4]. Tissues were homogenized using a Dounce homogenizer and then filtered through a silk screen (150 μm pore diameter) before use. The final assay mixture (40 μl) contained 25 mM Tris-maleate (pH 7.2), 0.5 mM unlabelled ATP, 1 mM $MgSO_4$, 0.5 mM EGTA, 0.2 mg/ml creatine kinase, 20 mM creatine phosphate, 10 mM theophylline, 2.0 μCi [α-^{32}P]ATP, 1.0 nCi cyclic [^{3}H]AMP (added as the recovery standard) and 10 μl of a 1 : 20 homogenate. This mixture was incubated for 5 min at 30°, and the reaction was stopped by the addition of 100 μl of a solution containing 5 mM ATP, 5 mM cyclic AMP, 50 mM Tris–HCl (pH 7.4) and 1% sodium lauryl sulfate. Isolation of cyclic [^{32}P]- and [^{3}H]-AMP was accomplished using Dowex AG 50W-X8 and alumina columns as described by Salomon *et al.* [16].

Protein concentrations were determined by the method of Lowry *et al.* [17] using bovine serum albumin as the standard.

Statistical determinations were performed according to Snedecor and Cochran [18] with $P = 0.05$ chosen as the maximum level of significance.

RESULTS

Comparison of the properties of the binding sites for [^{3}H]-5-HT *in adult and newborn rats.* Whereas all binding studies using [^{3}H]-5-HT as the labelled ligand have been performed with membranes prepared from adult rats, most data on the 5-HT-sensitive adenylate cyclase were obtained with cell-free preparations from newborn rats (see introduction to paper). Therefore, the direct comparison of the characteristics of [^{3}H]-5-HT binding and 5-HT-sensitive adenylate cyclase already reported in the literature is not possible. This led us to analyse the properties of [^{3}H]-5-HT binding sites in newborn animals.

As in adult rats, [^{3}H]-5-HT was found to bind to specific sites in lysed P2 fractions from the CNS of newborns. Scatchard analysis indicated that the affinity of [^{3}H]-5-HT for binding sites was not different from one area to the other in newborn rats (Table 1 and data not shown for other brain areas). Furthermore, it was also identical with that calculated from experiments using lysed P2 fractions from adult rats (Table 1). In contrast, marked differences were noted in the concentrations of binding sites in tissues from newborn and adult rats; as shown in Table 1 for the hippocampus and the striatum, B_{max} values (per g of fresh tissue) in 1-week-old animals were about 25 per cent of those found in adults. Further details concerning the ontogenetic changes of [^{3}H]-5-HT binding in other brain areas are provided in the accompanying paper [14].

The displacement by various drugs of [^{3}H]-5-HT from its binding sites in membranes from adult or newborn forebrain also suggested that the same site was measured in both age groups. As seen in Fig. 1, there was no difference in the logit–log displacement curves of [^{3}H]-5-HT binding for the 5-HT agonist, quipazine, or for the antagonist, spiroperidol. Similar conclusions were drawn using other drugs such as fluphenazine, methiothepin and metergoline (data not shown).

Effect of the assay conditions for the measurement of adenylate cyclase activity on the binding of [^{3}H]-5-HT. Another complicating factor in the comparison of the kinetic properties of the binding sites of [^{3}H]-5-HT and the 5-HT-sensitive adenylate cyclase is the differences in the media in which the measurements are carried out. As indicated in Materials and Methods, binding is usually measured using washed membranes suspended in a rather simple medium, while adenylate cyclase is assayed in a whole homogenate in the presence of a variety of added chemicals. To see if these differences in composition could explain the differences in the measured kinetic values, the effects of each component of the cyclase milieu on the binding of [^{3}H]-5-HT was examined

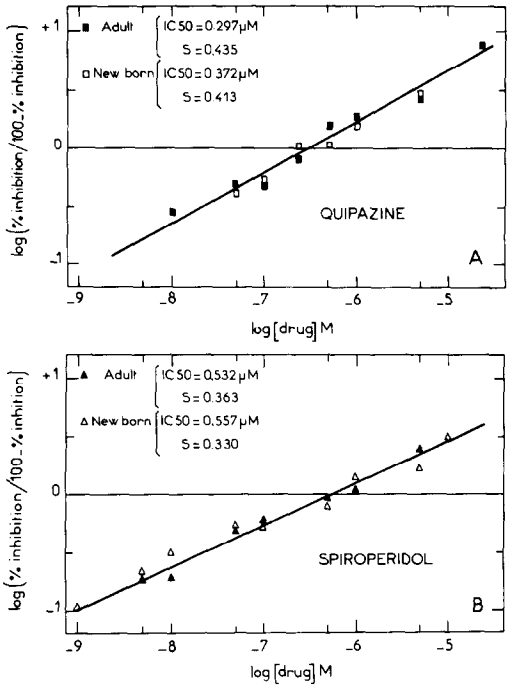

Fig. 1. Logit–log plots of the inhibition of [^{3}H]-5-HT binding by quipazine (A) or spiroperidol (B) in newborn and adult rats. The binding of [^{3}H]-5-HT was measured in lysed P2 fractions from whole forebrains of newborn (1-day-old) or adult rats in the presence of 1.5 nM of the labelled ligand and various concentrations of quipazine or spiroperidol. The IC_{50} (the concentration of added drug required to inhibit by 50 per cent the binding of [^{3}H]-5-HT) was calculated from the logit–log plots using linear regression analysis, as was the slope(s) of the curve. Each point represents the mean of triplicate determinations. Full symbols: adult rats; empty symbols: newborn rats.

Table 1. Kinetic parameters of [^{3}H]-5-HT specific binding in the striatum and the hippocampus of newborn and adult rats*

	Striatum		Hippocampus	
	Newborn	Adult	Newborn	Adult
K_d	1.80 ± 0.15	1.61 ± 0.08	1.81 ± 0.25	1.82 ± 0.10
B_{max}	1.90 ± 0.28 †	7.28 ± 1.02	2.18 ± 0.13 †	8.56 ± 1.23

* Lysed P2 membranes were prepared from newborn (1-week-old) or adult (3-month-old) rats as described in Materials and Methods. Binding assays in triplicates were performed with six different concentrations of [^{3}H]-5-HT ranging between 0.61 and 5.55 nM. The dissociation constant (K_d) and the total number of binding sites (B_{max}) for [^{3}H]-5-HT were determined from Scatchard plots. For adult as well as for newborn rats, these plots gave only one straight line in agreement with previous findings [9]. K_d is expressed in nM, B_{max} in pmoles of [^{3}H]-5-HT bound to membranes prepared from 1 g of fresh tissue. Each value is the mean ± S.E.M. of 5–18 individual experiments.

† $P < 0.05$ when compared to values from adult animals.

Table 2. Effects of the adenylate cyclase incubation medium on the binding of [^{3}H]-5-HT to membranes from colliculi of newborn rats*

Addition	Specifically bound [^{3}H]-5-HT
None	159.9 ± 5.0
10 mM Theophylline	163.9 ± 2.5
20 mM Creatine phosphate	135.3 ± 3.3
Creatine kinase (4 μg/ml)	162.1 ± 5.5
75 mM Sucrose	161.9 ± 4.8
0.5 mM ATP	142.5 ± 12.9
1.0 mM $MgSO_4$	190.9 ± 5.8
0.5 mM ATP + 1.0 mM $MgSO_4$	187.4 ± 10.9
0.5 mM EGTA	160.9 ± 4.8
0.5 mM ATP + 1.0 mM $MgSO_4$ + 0.5 mM EGTA	164.6 ± 9.3
Complete cyclase medium	139.2 ± 8.0

* Lysed P2 fractions were prepared from colliculi of 6-day-old rats in the standard fashion (see Materials and Methods) except that the final resuspension was carried out in 25 mM Tris-maleate, pH 7.2. The specific binding of [^{3}H]-5-HT was measured in the presence of 2 nM of the labelled ligand and various compounds as indicated in the left column. Given concentrations are those reached in the final binding assay mixtures. They are equal to those used for the measurement of adenylate cyclase activity. Specifically bound [^{3}H]-5-HT, expressed as fmoles/mg protein, is the mean ± S.D. of triplicate determinations for each condition.

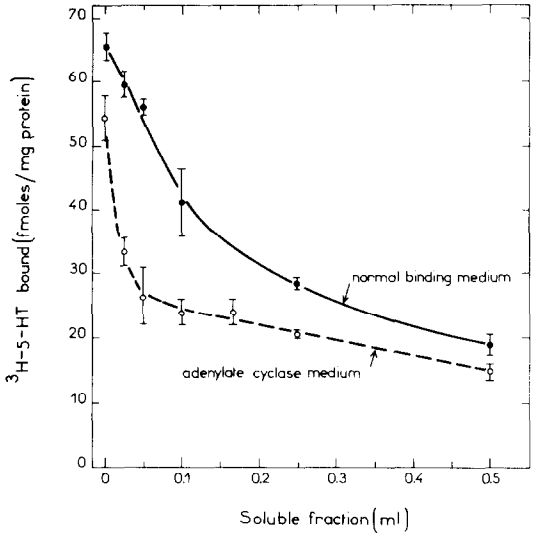

Fig. 2. Effect of the addition of the soluble tissue fraction on the binding of [^{3}H]-5-HT. The specific binding of [^{3}H]-5-HT was carried out at a concentration of 1.40 nM of the labelled ligand. Membranes were prepared from the forebrains of 1-day-old rats in the standard fashion (see Materials and Methods) except that for the final step, a fraction of the membranes was resuspended in the medium used for the adenylate cyclase assay rather than the normal binding medium. For the preparation of the soluble fraction, the forebrains were homogenized in 20 vol. of 50 mM Tris–HCl, pH 7.4, using a Polytron tissue disrupter, and the homogenate was centrifuged at 40,000 *g* for 20 min. The supernatant was decanted and labelled as the 'soluble fraction'. A 0.5 ml portion of this fraction diluted to 2 ml for the binding assay was equivalent to the concentration of the soluble fraction present in the cyclase assay.

using membranes from colliculi of newborn rats. This structure was chosen because it has often been used in studies concerning 5-HT-sensitive adenylate cyclase [1, 4, 5]. As shown in Table 2, slight changes were produced by creatine phosphate (−15 per cent) and $MgSO_4$ (+19 per cent). The $MgSO_4$-induced increase in [^{3}H]-5-HT binding persisted in the presence of ATP but was completely prevented by EGTA (Table 2). When all the components of the cyclase medium were included, the specific binding of [^{3}H]-5-HT was reduced by ~ 13 per cent (Table 2). Kinetic analysis revealed that this effect was due to a slight decrease in the apparent affinity of [^{3}H]-5-HT for its binding site (a 28 per cent increase in the K_d value was detected when the binding was measured in the complete cyclase medium; see Table 3).

Because the adenylate cyclase activity was measured in a whole homogenate which contains soluble cell components, the influence of the addition of this soluble fraction on the binding of [^{3}H]-5-HT was also examined. For these experiments, forebrains from newborn rats were used in order to obtain enough membranes and soluble materials. As shown in Fig. 2, replacing the normal binding medium (0.05 M Tris–HCl, 5.7 mM ascorbic acid, 10 μM pargyline, 4 mM $CaCl_2$, final pH 7.4) by the complete cyclase medium (see Table 2) resulted in a slight reduction in [^{3}H]-5-HT binding to forebrain membranes (−17 per cent) quite comparable to that observed with collicular preparations (Table 2). The addition of increasing amounts of the soluble fraction progressively decreased the binding of [^{3}H]-5-HT measured either under 'normal' or 'cyclase assay' conditions (Fig. 2). At a concentration of the soluble fraction which equalled that found in the adenylate cyclase assay and in the presence of the cyclase medium, specific [^{3}H]-5-HT binding was only 23 per cent of that

Table 3. Kinetic parameters of [^{3}H]-5-HT binding to forebrain membranes from newborn rats under various assay conditions*

Assay conditions	[^{3}H]-5-HT binding K_d (nM)	[^{3}H]-5-HT binding B_{max} (fmoles/mg protein)
− Normal binding medium	1.97	165
+ Soluble fraction (0.5 ml)	9.21	158
− Cyclase assay medium	2.52	171
+ Soluble fraction (0.5 ml)	11.89	157

* Forebrain membranes and the 'soluble fraction' were prepared as described in the legend to Fig. 2. [^{3}H]-5-HT binding was measured with 0.45–3.60 nM of the labelled ligand under normal or cyclase assay conditions and in the presence or the absence of 0.5 ml (per 2 ml final volume) of the soluble fraction. Scatchard plots of [^{3}H]-5-HT binding gave only one straight line under all conditons. K_d and B_{max} were calculated by linear regression analysis of these plots. Each value is the mean of two independent experiments.

occurring under normal binding conditions (Fig.2). Kinetic analysis revealed that this reduction was associated with a marked decrease in the affinity of [^{3}H]-5-HT for its binding site (Table 3). No change in the total number of binding sites (B_{max}) was detected under these conditions (Table 3). Even with this reduction in the affinity of [^{3}H]-5-HT for its binding site, the K_d (11.89 nM, Table 3) was still at least 40 times lower than the K_A app (0.5–1.0 μM) of the 5-HT-sensitive adenylate cyclase.

Effects of GTP on the binding of [^{3}H]-5-HT *and on the activation of adenylate cyclase by* 5-HT. GTP has been shown to play an important role in the activation of a number of adenylate cyclase systems, its presence appearing to be essential for optimal coupling between the enzyme and its receptor (see Ref. 19 for a review). Indeed, when adenylate cyclase activity was measured in a whole homogenate from colliculi of newborn rats, the addition of GTP significantly increased the stimulating effect of 5-HT. Previous studies [20] have shown that the maximal effect was reached with 10 μM GTP. Similar findings were obtained using a lysed P2 fraction from colliculi of newborn rats. Whereas 5-HT alone did not exert any significant action on the basal adenylate cyclase activity, in the presence of 10 μM of either GTP or GppNHp, 1–100 μM of the indoleamine produced slight ($\leq$ 25 per cent) but significant increases in the enzymic activity (Table 4). In addition, data in Table 4 also indicate that 10 μM GTP or GppNHp alone stimulated the adenylate cyclase activity in washed membranes whereas no effect was detected with whole homogenates from colliculi of newborn rats [20]. With a whole homogenate of colliculi from newborn rats as the source of 5-HT-sensitive adenylate cyclase, kinetic analysis revealed that the K_A app of 5-HT was reduced from 0.56 to 0.33 μM by the addition of 10 μM GTP to the assay mixture (Fig.3).

GTP was also found to affect the binding of [^{3}H]-5-HT. In this case, the addition of the nucleotide caused a decrease in the specific binding of the ligand to membranes prepared from the forebrains of newborn rats (Table 5). However, GTP was markedly less potent in affecting the binding than the cyclase, having no significant effect at 10 μM; even at a concentration of 1 mM, the nucleotide only inhibited [^{3}H]-5-HT binding by 47 per cent (Table 5). This effect was relatively specific since GppNHp, but neither GMP nor ATP, also induced a reduction in [^{3}H]-5-HT binding (Table 5).

Pharmacological properties of [^{3}H]-5-HT *binding sites and the receptor coupled with the* 5-HT-*sensitive adenylate cyclase.* Although all known 5-HT antagonists displaced [^{3}H]-5-HT specifically bound to membranes from the forebrains of newborn rats,

Table 4. Effects of GTP and GppNHp on 5-HT-sensitive adenylate cyclase activity in washed membranes from colliculi of newborn rats*

(5-HT)	5-HT alone	Cyclic AMP production (pmoles/min/mg protein) + 10^{-5} M GTP	(Δ)	+ 10^{-5} M GppNHp	(Δ)
0	2.61 ± 0.10	5.25 ± 0.01		4.05 ± 0.19	
10^{-7} M	2.27 ± 0.07	—		—	
10^{-6} M	2.48 ± 0.05	5.42 ± 0.03 ‡	(0.17)	4.67 ± 0.07 †	(0.62)
10^{-5} M	2.46 ± 0.05	—		—	
10^{-4} M	2.48 ± 0.16	6.19 ± 0.07 ‡	(0.94)	5.07 ± 0.15 †	(1.02)

* A lysed P2 fraction from colliculi of 1-day-old rats was prepared as described in Materials and Methods and finally suspended in 2 mM Tris-maleate, pH 7.2, containing 2 mM EGTA and 300 mM sucrose as whole homogenates for the measurement of adenylate cyclase activity [4]. Adenylate cyclase activity was then measured in the presence of various concentrations of 5-HT with or without 10 μM GTP or 10 μM GppNHp. Results are expressed as means ± S.E.M. of triplicate determinations in two independent experiments. Δ is equal to the difference between basal cyclic AMP formation and that occurring in the presence of 5-HT.

† $P \leq 0.05$, ‡ $P \leq 0.01$ when compared to corresponding values in the absence of added 5-HT.

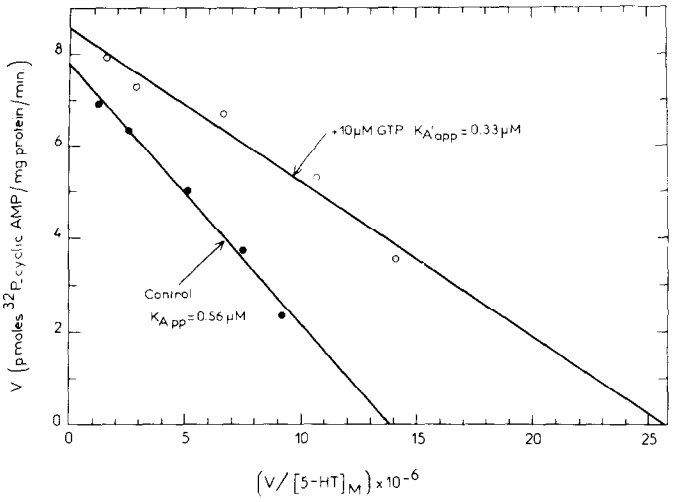

Fig. 3. Hofstee plots of activity of 5-HT-sensitive cyclase as a function of 5-HT concentration with or without 10 μM GTP. Adenylate cyclase was measured in a collicular homogenate from 1-day-old rats in the presence of 0.25–5 μM 5-HT. V corresponds to the production of [^{32}P]cyclic AMP due to 5-HT and is expressed as pmoles/mg protein and per min. Each point is the mean of triplicate determinations. The concentrations of 5-HT producing half maximal stimulation of the adenylate cyclase activity (K_A app and K'_A app) were calculated by linear regression analysis of the plots.

large differences were observed with respect to their potencies. For instance, the efficacy of metergoline for displacing bound [^{3}H]-5-HT was more than 100 times greater than that of mianserine (Table 6). It was thus possible to classify 11 putative antagonists according to their relative potencies. As shown in Table 6, this order did not apply to their blocking action on 5-HT-sensitive adenylate cyclase as the least active on [^{3}H]-5-HT binding, i.e. cyproheptadine was in fact more potent than metergoline for inhibiting the enzyme (Table 6). As illustrated by the large differences in R value (R = ratio of IC_{50} for the adenylate cyclase to IC_{50} for [^{3}H]-5-HT binding) from one drug to the other, no correlation was found between the efficacies of a given antagonist for displacing bound [^{3}H]-5-HT and for inhibiting 5-HT-sensitive adenylate cyclase activity (Table 6).

5-HT agonists of the piperazine series were also quite potent for displacing [^{3}H]-5-HT bound to forebrain membranes from newborn rats (Table 6). However, none of these drugs (from 0.1 μM to 0.1 mM) were found to affect 5-HT-sensitive adenylate cyclase activity in collicular homogenates from newborn animals. The basal adenylate cyclase activity was also unaffected except with quipazine which induced a slight reduction (−23 per cent) at the highest concentration used (0.1 mM).

DISCUSSION

Previous studies on the 5-HT-sensitive adenylate cyclase in newborn rats [4, 5] and the specific high affinity binding of [^{3}H]-5-HT in adults [9] revealed marked discrepancies between the characteristics of these two markers of 5-HT receptors in the CNS. In particular, the affinity of 5-HT for binding sites (K_d = 1–2 nM) appeared to be almost three orders of magnitude higher than for the receptor coupled to the adenylate cyclase (K_A app = 0.5–1.0 μM). Although this difference could mean that the 5-HT-

Table 5. Effects of nucleotides on the binding of [^{3}H]-5-HT*

Concentration of nucleotide	Binding of [^{3}H]-5-HT in the presence of GTP	GppNHp	GMP	ATP
10 μM	97.7 ± 2.6	92.9 ± 1.1	96.8 ± 2.6	—
100 μM	87.6 ± 0.7	78.2 ± 2.4	93.1 ± 1.9	94.9 ± 0.7
1 mM	52.8 ± 6.6	68.9 ± 1.8	90.9 ± 1.9	83.6 ± 0.6

* The specific binding of [^{3}H]-5-HT was measured in the presence of 1.0 nM of the labelled ligand. Membranes were prepared from the forebrains of 6-day-old rats in the following way: the forebrains were homogenized in 40 vol. of 50 mM Tris–HCl, pH 7.4, using a Polytron tissue disrupter and were then centrifuged at 40,000 g for 20 min. The crude membranes were washed by resuspension in the buffer, recentrifuged, and then resuspended and incubated at 37° for 10 min to eliminate endogenous 5-HT (see Ref. 9). After the incubation, the membranes were washed twice as above and finally resuspended in 50 vol. of 50 mM Tris–HCl, pH 7.4, for use in the binding assays. Each value represents the mean ± S.E.M. of triplicate determinations from two separate experiments and is expressed as per cent of control binding measured in the absence of added nucleotide.

Table 6. Effects of various drugs on the specific binding of [^{3}H]-5-HT and the 5-HT-sensitive adenylate cyclase activity*

Drugs	IC_{50} [^{3}H]-5-HT binding (nM)	IC_{50} 5-HT-sensitive adenylate cyclase (μM)	R
Antagonists			
Metergoline	5.8	18.1	3121
Cinanserine	236	21.5	91
Pizotifen	287	68.3	238
Clozapine	353	5.6	16
Methiothepin	463	11.9	26
Spiroperidol	500	3.8	8
Mianserine	647	27.6	43
α-Flupenthixol	964	10.3	11
Fluphenazine	1950	11.5	6
Thioproperazine	2366	5.8	2
Cyproheptadine	6000	11.5	2
Agonists			
1-(*m*-trifluoromethylphenyl)-piperazine	117	NE †	—
Quipazine	365	NE	—
MK-212	2050	NE	—

* The specific binding of [^{3}H]-5-HT was measured using a lysed P2 fraction from forebrains of 1-day-old rats. The concentration of the labelled ligand was 1.45 nM. The 5-HT-sensitive adenylate cyclase activity was measured in collicular homogenates from 1-day-old rats in the presence of 10 μM 5-HT. IC_{50} values (concentrations required for reducing by half [^{3}H]-5-HT binding or 5-HT-sensitive adenylate cyclase activity) were determined from logit–log plots (see Fig. 1). Each value is the mean of two to three independent experiments. R is the ratio of IC_{50} for adenylate cyclase to that for [^{3}H]-5-HT binding.

† NE: no effect in the range 0.1 μM–0.1 mM.

sensitive adenylate cyclase and the [^{3}H]-5-HT binding sites correspond to distinct classes of 5-HT receptors, other explanations could be put forward. Thus, the same class of receptors might well undergo profound alterations during ontogenetic development, exhibiting a higher affinity in adults than during the early life period. In addition, respective assay conditions for the measurement of [^{3}H]-5-HT binding and 5-HT-sensitive adenylate cyclase might influence differently the same receptor. Finally, the same receptor could exist under active (coupled with adenylate cyclase) and desensitized (uncoupled with the enzyme) forms exhibiting different affinities for 5-HT. These various hypotheses were examined in the present study.

Analysis of [^{3}H]-5-HT binding in membrane preparations from newborn rats revealed that high affinity binding sites also exist with the same characteristics as in adult rats. In particular, the K_d value and the ability of competing drugs to displace [^{3}H]-5-HT from these sites were identical in newborns and in adults. Although the accurate determination of K_A app of 5-HT-sensitive adenylate cyclase could not be made with brain homogenates from adult rats (the stimulation by 5-HT was too low [1, 4, 14]), it has to be emphasized that the range of efficient 5-HT concentrations (0.5–50 μM) for stimulating this particular enzyme is similar in preparations from adults or newborns [14]. This led to the conclusion that the affinity of 5-HT for the receptor coupled to adenylate cyclase is very likely unaltered during ontogenetic development. Therefore, the large difference between K_d (for [^{3}H]-5-HT binding) and K_A app (for 5-HT-sensitive adenylate cyclase) could not result from developmental changes.

Addition of the chemicals used in the cyclase assay to the standard membrane preparation for the binding assay only resulted in a 10–15 per cent decrease in the binding of [^{3}H]-5-HT. A larger reduction was seen when the binding was carried out in the presence of the cyclase medium with the addition of the soluble tissue fraction (Fig. 2) which is normally present in the measurement of adenylate cyclase activity but which is absent from the washed membranes of the binding assay. Under these conditions, the affinity of 5-HT for its binding sites was decreased 6–8 times when compared to that found with optimal conditions. This probably resulted from the addition of endogenous inhibitors contained in the soluble extracts, notably 5-HT (see Ref. 9) and GTP (this paper). The K_D value characterizing [^{3}H]-5-HT binding under adenylate cyclase assay conditions, i.e. 11.9 nM, was still more than 40 times less than the K_A app of the 5-HT-sensitive adenylate cyclase. Therefore, the respective assay conditions could not completely explain the large difference between the affinities of 5-HT for the specific binding sites and the 5-HT-sensitive adenylate cyclase.

If the [^{3}H]-5-HT binding site were indeed identical to the 5-HT receptor linked to adenylate cyclase, the discrepancy between K_d and K_A app might be explained by a 'low degree' of coupling between the receptor and the enzyme. Because GTP apparently regulates the degree of coupling of adenylate cyclase

to its receptors (for a review see Ref. 19), its effects on 5-HT-sensitive adenylate cyclase and [^{3}H]-5-HT binding sites were measured. GTP was found to increase the cyclic AMP production induced by 5-HT with a shift of K_A app from 0.56 to 0.33 μM. In fact, GTP or its stable analog, GppNHp, appeared to be an absolute requirement for the activity of 5-HT-sensitive adenylate cyclase because 5-HT was inactive in a washed membrane preparation in the absence of these compounds (Table 5). This is in agreement with other reports showing that GTP increases the 5-HT-stimulated cyclic AMP formation in mammalian brain tissues [6, 20], neuroblastoma–brain hybrid cells [21, 22] and membranes from the liver fluke [23].

Although GTP was also found to affect the binding of [^{3}H]-5-HT in membranes from newborn animals, it was less potent than in the 5-HT-sensitive adenylate cyclase system. At 10 μM, the optimal concentration for increasing 5-HT-stimulated cAMP production, GTP had no effect on the binding, and raising the concentration to 1 mM only resulted in a 47 per cent decrease in the specific binding of [^{3}H]-5-HT. This discrepancy between the ranges of efficient GTP concentrations with respect to the enzyme and the [^{3}H]-5-HT binding might suggest that the nucleotide affects these two systems by completely different mechanisms. Indeed, Marchais and Bockaert (personal communication) recently observed that GTP could affect the binding of ligands to sites independent of any adenylate cyclase. Therefore, the GTP effect on [^{3}H]-5-HT binding does not itself allow the conclusion that these binding sites were associated to adenylate cyclase. Furthermore, under optimal 'coupling conditions', i.e. with 10 μM GTP, the K_A app of the 5-HT-sensitive adenylate cyclase was still almost 30 times higher than the K_d of [^{3}H]-5-HT binding under the adenylate cyclase assay conditions.

All these observations strongly suggest that [^{3}H]-5-HT did not label the 5-HT receptor coupled with adenylate cyclase. This conclusion is strengthened by analysing the respective pharmacological properties of [^{3}H]-5-HT binding and 5-HT-sensitive adenylate cyclase. Thus, whereas some antagonists (cyproheptadine, thioproperazine) were approximately equipotent for inhibiting [^{3}H]-5-HT binding and 5-HT-sensitive adenylate cyclase activity, others (pizotifen, metergoline) were much more potent against the binding than against the 5-HT-sensitive enzyme. Studies on the effects of agonists further emphasized these discrepancies because, apart from the indole-agonists (bufotenine, 5-methoxy-*N*, *N*-dimethyltryptamine) which both activated the 5-HT-sensitive adenylate cyclase [1, 5] and displaced bound [^{3}H]-5-HT [7, 8], those of the piperazine group (quipazine and derivatives, [24–26]) affected only [^{3}H]-5-HT binding. This suggests a considerable difference between the structural properties of the [^{3}H]-5-HT binding sites and the 5-HT receptor linked to adenylate cyclase.

Recent studies on the 5-HT-dependent myoclonus in the guinea pig [27] suggested that a correlation might exist between the efficiencies of various drugs in this behavioral test and their potencies for activating or blocking the 5-HT-sensitive adenylate cyclase [5]. In contrast, no correlation apparently existed between the behavioral effects and the actions of various drugs on the specific high affinity binding of [^{3}H]-5-HT ([9], this paper). In particular, quipazine and other piperazine derivatives which efficiently displaced [^{3}H]-5-HT from specific binding sites were very poorly active for evoking occasional myoclonic jerking in the guinea pig [27]. However, further studies will be necessary to compare more precisely the pharmacological properties of the 5-HT-sensitive adenylate cyclase and of the [^{3}H]-5-HT high affinity binding with the potencies of various drugs for inducing or blocking 5-HT-dependent behaviours.

In conclusion, the present study offers evidence for two types of 5-HT receptors in rat brain: the first one, which is sensitive to μM concentrations of 5-HT, is linked to an adenylate cyclase whereas the second one, characterized by a high affinity for 5-HT, is apparently not linked to such an enzyme. Recently, Fillion *et al.* [28] reported the stimulation of adenylate cyclase activity with nM concentrations of 5-HT in rat striatal membranes, thus suggesting that the high affinity binding site for 5-HT might also be coupled to this enzyme. Attempts to confirm their findings were completely negative [20] and no satisfactory explanation can be given for this discrepancy at present. Whatever the membrane component (cyclase, ionophore. . .) that is associated with the high affinity binding site for 5-HT, the present data strongly suggest that the concept of multiplicity of 5-HT receptors already proposed for the periphery [29], cultured neuroblastoma hybrid cells [22] and ganglionic nervous system of molluscs [30] can be extended to the CNS in the rat.

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