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Characteristics of Central 5-HT Receptors and Their Adaptive Changes following Intracerebral 5,7-Dihydroxytryptamine Administration in the Rat

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SUMMARY

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Lysed synaptosomal (P_2) fractions from adult rat brain were extensively washed and incubated in such a way that most bound 5-HT was eliminated. These membranes contained a high affinity (K_d = 1.6 nM) binding site for [^{3}H]5-HT that exhibited the expected properties of a specific receptor of 5-HT. In particular, known agonists and antagonists of 5-HT in the central nervous system inhibited the binding of [^{3}H]5-HT to synaptosomal membranes with IC_{50} values ranging between 5 nM (metergoline) and 2.18 μM (MK-212). The slopes of logit-log competitive inhibition plots of [^{3}H]5-HT binding by various drugs including an agonist (quipazine) and several antagonists (methiothepin, mianserine) were less than 1.0 suggesting possible negative cooperativity. This did not occur with 5-HT itself since the Hill coefficient of [^{3}H]5-HT binding was not significantly different than one. Chemical lesioning of serotoninergic neurons by the intracerebral injection of 5,7-dihydroxytryptamine (5,7-HT) resulted in a significant increase in the number of high affinity binding sites for [^{3}H]5-HT in the hippocampus (+39%) but not in the striatum 18 days after the injection. This change was first detected on the 8th day after 5,7-HT treatment (+25%) when 5-HT levels in the hippocampus were decreased by 80% as compared with normal adult values. Neither the affinity nor the Hill coefficient of [^{3}H]5-HT binding, nor the characteristics of the binding sites for [^{3}H]dihydroalprenolol (a *beta*-noradrenergic antagonist) and [^{3}H]haloperidol (a DA antagonist) in the hippocampus and the striatum, respectively, were altered by 5,7-HT treatment. These findings are discussed in relation to the reported behavioral evidence for supersensitivity toward 5-HT agonists in 5,7-HT-treated animals.

INTRODUCTION

Several methods have been developed to study the effects of serotonin (5-HT) on presumed postsynaptic specific receptors in the central nervous system. Some of these have been based on the measurement of 5-HT-dependent spinal reflexes (1, 2), on the observation of typical 5-HT-dependent behaviors (3-7) and on the recording of the firing of cells innervated by 5-HT containing neurons (8). More recently, two biochemical tests have been proposed to directly investigate the properties of central

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5-HT receptors; they consist of measuring the responses of a specific 5-HT-sensitive adenylate cyclase (9, 10) and studying the characteristics of high affinity binding sites for 5-HT in synaptic membranes using appropriate ligands (11–14). This last method has been shown to be a powerful tool for characterizing the 5-HT receptor. However, with this approach, some striking discrepancies have been reported in the literature with regard to the properties of the receptor. For example, Bennett and Snyder (12) described only one high affinity binding site in rat brain having a K_d of 8–15 nM, while Fillion *et al.* (13) mentioned the existence of two high affinity binding sites, with the one that exhibited the higher affinity for 5-HT (K_d = 1–3 nM) being specific for membrane preparations enriched in postsynaptic elements.

In many systems that employ monoamines as neurotransmitters, the phenomenon of "denervation supersensitivity" has been described, i.e., when the receptor is denied access to its transmitter it becomes "supersensitive." In the central nervous system this reaction has been shown for both the noradrenergic (15, 16) and dopaminergic (17) systems, where it is expressed as an increase in the density or absolute number of specific receptors. Behavioral studies have shown that the destruction of central 5-HT-containing neurons results in an increased sensitivity to 5-HT and its agonists (2, 5, 6, 18–20). However, binding studies have failed to demonstrate any definite changes in receptor properties under these conditions (12, 13). Bennett and Snyder (12) did report a 2-fold decrease in the K_d for the binding of [^{3}H]5-HT after lesions had been produced in the serotoninergic systems, but this was attributed to lower levels of endogenous 5-HT present in the membrane preparation from the animals with lesions rather than to an effect on the receptors.

Because of the clear evidence pointing to a supersensitivity of behavioral responses to 5-HT after destruction of 5-HT neurons and the biochemical evidence for the development of supersensitivity by other monoamine receptors, it was decided to reinvestigate the problem of the properties of the 5-HT receptor after destruction of serotoninergic neurons. The present report describes the examination of this problem and provides further data concerning the characteristics of the 5-HT receptor in the central nervous system of the rat.

MATERIALS AND METHODS

Chemicals

Indole derivatives. The following compounds were obtained: Iprindole (Wyeth); harmaline (Sigma Chemical Company); harmalol (Sigma Chemical Company); harmine (Fluka); tabernanthine and vincamine (Institut de Chimie des Substances Naturelles, CNRS; Gif sur Yvette); 5,7-dihydroxytryptamine (5,7-HT, Regis).

Serotonin agonists and antagonists. Compounds obtained were: serotonin creatinine sulfate (Merck); quipazine (Miles); MK-212 (6-chloro-2-[1-piperazinyl]pyrazine, Merck, Sharp & Dohme); metergoline (Farmitalia); methysergide (Sandoz); methiothepin (Hoffmann-La Roche); cinanserine (Squibb & Sons); pizotifen (Sandoz); mianserine (Organon).

Other compounds. Other compounds purchased included dopamine (Calbiochem); noradrenaline (Calbiochem); clozapine (Sandoz); fluphenazine (Squibb & Sons); (+)-butaclamol (Ayerst); (−)-alprenolol (Ciba-Geigy); fenfluramine (Servier); pargyline (Abbott); reserpine (Ciba-Geigy); fluoxetine (Lilly110140, Eli Lilly and Co.); desmethylimipramine (Ciba-Geigy); morphine (Rhône-Poulenc); ticlopidine (Institut de Chimie des Substances naturelles, CNRS, Gif sur Yvette); $CaCl_2$ (Prolabo); $MgCl_2$ (Merck); adenosine-5′-triphosphate (ATP, Boehringer Mannheim).

Labeled ligands. [^{3}H]5-HT (generally labeled, 12–15.2 Ci/mmol, Radiochemical Centre, Amersham) was purified before each experiment by ion-exchange chromatography (21). (—)-[^{3}H]dihydroalprenolol (58.5 Ci/mmol, NEN), G-[^{3}H]haloperidol (13.15 Ci/mmol, Radiochemical Centre, Amersham) and S-adenosyl-L-[methyl-^{3}H]methionine (5 Ci/mmol, Radiochemical Centre, Amersham) were used directly for respective binding and radioenzymatic assays.

Animals. Male Sprague-Dawley rats

(250–350 g) were used. The animals were housed in a control environment (24°, 60% relative humidity, alternate cycles of 12 hr light and 12 hr darkness, food and water *ad libitum*) for at least 10 days before any experiment. The chemical destruction of ascending serotoninergic neurons was accomplished in rats under halothane anesthesia, according to a modification of the method described by Björklund *et al.* (22). In order to improve the specificity of 5,7-HT against serotoninergic neurons, desmethylimipramine (25 mg/kg i.p.) was administered 45 min before the local intracerebral injection of the neurotoxic agent (8 μg of 5,7-HT in 4 μl of isotonic NaCl containing 0.01% ascorbic acid). This latter injection was carried out over a 5 min period using a stainless steel cannula (outer diameter = 0.21 mm; inner diameter = 0.13 mm) acutely implanted at the following coordinates: A = 2.6 mm, L = 0.4 mm, H = 2.2 mm (23). The injection of 5,7-HT resulted in extensive and reproducible losses of 5-HT from the right cerebral hemisphere anterior to the site of injection (see RESULTS). 5-HT was also depleted from the left cerebral hemisphere but less reproducibly. Therefore, only structures from the right hemisphere were used to determine the characteristics of the 5-HT receptor after lesions of serotoninergic neurons were produced.

Tissue preparation. Rats were killed by decapitation and the brains were dissected at a cold temperature (4°). All subsequent operations were also carried out at cold temperatures (0–4°). After dissection, the tissues (generally 3–4 forebrains or 12–18 pooled areas such as the striatum or the hippocampus from control or 5,7-HT-treated rats) were homogenized in 10 volumes of 0.32 M sucrose, using a Potter-Elvehjem homogenizer 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 (P_2) was resuspended in ice-cold water (10 volumes of original wet weight) and maintained for 30 min at 4° with periodic agitation to allow lysis of synaptosomal components. After lysis the samples were centrifuged at 35,000 × *g* for 20 min. The supernatant was discarded and the pellet was resuspended in 0.05 M Tris-HCl, pH 7.4 (40 volumes of original weight). This was incubated for 10 min at 37°, after which it was centrifuged at 35,000 × *g* for 20 min. In some experiments (see RESULTS), the incubation at 37° was omitted or an incubation at 0° was substituted to illustrate its effect on endogenous 5-HT remaining in the preparation. The pellet was washed once by resuspension in 0.05 M Tris-HCl, pH 7.4, and then centrifuged at 35,000 × *g* for 20 min. The sedimented material (P_f) was resuspended in 0.05 M Tris-HCl, pH 7.4 (40–90 volumes of original weight), which under standard conditions contained ascorbic acid (5.7 mM), pargyline (10 μM) and $CaCl_2$ (4 mM). This final resuspension was used for the [^{3}H]5-HT binding assay.

Binding assays. The method described by Bennett and Snyder (12) was used with slight adaptations to measure the high affinity binding of [^{3}H]5-HT. Aliquots (2 ml) of the resuspended tissue (P_f) described above were placed in glass tubes, and after the addition of [^{3}H]5-HT and unlabeled ligands the tubes were incubated at 37° for 7 min. The samples were then rapidly filtered through Whatman GF/B filters under vacuum. The incubation tubes and filters were rinsed twice with 5 ml aliquots of the ice-cold Tris buffer used for the final resuspension of the tissue (see above). The filters were then rinsed for a third time with a 5 ml aliquot of the buffer, after which they were placed in 10 ml of PCS (Amersham Searle Corp.) or Lumagel (Lumac Systems A.G.). Radioactivity was then determined by liquid scintillation spectrometry. Nonspecific binding was defined as the radioactivity which remained on the filter with membrane samples incubated in the presence of 10 μM unlabeled 5-HT and specific binding as the difference between the radioactivity of a given sample and the nonspecific binding. Under the conditions used, specific binding, which varied linearly as a function of protein concentration in the sample (0.15–2 mg/ml), represented 80–85% of the total binding. The incubation time chosen, i.e., 7 min, was sufficient to reach binding equilibrium without any significant

degradation of [^{3}H]5-HT (12).

The binding of [^{3}H]dihydroalprenolol was carried out according to the method of Lucas and Bockaert (24). The tissue was prepared as described above, except that the final pellet (P_f) was resuspended in 0.05 M Tris-HCl, pH 7.8 (32 volumes of original weight, approximately 0.65 mg protein/ml) containing EDTA (5 mM), ascorbic acid (1.1 μM) and pargyline (10 μM). Binding assays were performed on 0.2 ml of this final suspension incubated with 0.45 nM–12 nM [^{3}H]dihydroalprenolol for 10 min at 37°. Samples were then filtered through Whatman GF/B filters under vacuum. The tubes and filters were each washed twice with 1.0 ml aliquots of ice cold Tris buffer, and the filters were washed an additional time with 5 ml of the buffer. Radioactivity trapped by the filters was determined as described above. Nonspecific binding was defined as the radioactivity remaining on the filter in the presence of 1 μM (−)-alprenolol.

The presence of dopamine (DA) receptors was determined by the binding of [^{3}H]haloperidol (25). The final tissue pellet (P_f) was resuspended in 0.05 M Tris-HCl pH 7.4 (80 volumes of original weight, approximately 0.3 mg of protein/ml) containing ascorbic acid (5.7 mM), pargyline (10 μM), NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM). Aliquots (2 ml) of this final suspension were incubated with 0.90 nM–4.7 nM [^{3}H]haloperidol for 10 min at 37°. Filtration of the samples and determination of radioactivity were performed as described above for the binding of [^{3}H]5-HT. Nonspecific binding was considered as the radioactivity that remained on the filter in the presence of 0.1 μM (+)-butaclamol.

Measurement of monoamines. Endogenous 5-HT was measured in various areas of the central nervous system using a radioenzymatic assay consisting of the conversion of 5-HT to [^{3}H]melatonin with S-adenosyl-L-[methyl-^{3}H]methionine as the [^{3}H]methyl donor (26). The 5-HT bound to membrane material at various steps of the "tissue preparation" was extracted at 4° as follows: perchloric acid was added to the membrane suspension to a final concentration of 0.4 N to remove molecules noncovalently bound to membrane proteins. Precipitated material was discarded by centrifugation and the supernatant was adjusted to pH 7.6 by the addition of 4 N KOH and 0.3 M KH_2PO_4/K_2HPO_4, pH 7.6. Potassium perchlorate was removed by centrifugation and 5-HT in the clear supernatant was selectively adsorbed on a Sephadex G10 column (27). 5-HT was eluted with 0.5 N formic acid and finally converted into [^{3}H]melatonin, as described above. This labeled compound was chromatographed on silicagel plates and the radioactivity of the melatonin spot finally measured by liquid scintillation counting (27).

Dopamine was measured by a radioenzymatic microassay (28). The spectrofluorimetric method of Laverty and Taylor (29) was used for the determination of norepinephrine levels.

Proteins were measured using bovine serum albumin as the standard (30).

Statistics were performed according to Snedecor and Cochran (31). When p was greater than 0.05 (by a two-tailed Student's t-test), the difference was considered to be nonsignificant.

RESULTS

Importance of the removal of endogenous 5-HT from the membrane preparation for the study of the properties of the [^{3}H]5-HT high affinity binding site. As described in the MATERIALS AND METHODS section, the procedure used to prepare the membrane material was not very different from that described by Bennett and Snyder (12). Since these authors had mentioned that their tissue preparation could contain enough residual endogenous 5-HT to affect the value of the apparent K_d for [^{3}H]5-HT, it was decided to search for a method of preparing the membranes which would maximally remove endogenous 5-HT. This was essential for a valid comparison of the properties of the 5-HT receptors from control animals with those from animals whose serotoninergic systems had been destroyed.

Addition of an incubation at 37° for 10 min to the procedure of preparing the membranes resulted in a significant increase in the binding of [^{3}H]5-HT (Table 1). The efficacy of this step did not appear to depend solely on the dissociation of 5-HT

TABLE 1

Effect of preincubation of a lysed P_2 fraction on [^{3}H]5-HT binding

A lysed P_2 fraction was prepared from the whole forebrain, as described in MATERIALS AND METHODS. The final pellet was suspended in 40 vol. of 0.05 M Tris-HCl, pH 7.4, and directly used for binding assays or incubated for 10 min at 0° or 37° with or without 10 μM pargyline. The incubated preparations were centrifuged at 35,000 × *g* for 20 min, and the pellets were resuspended in 40 vol. of 0.05 M Tris-HCl, pH 7.4, for subsequent binding. In one case (D), the suspension of incubated lysed P_2 membranes was incubated again at 37° for 10 min and finally collected by centrifugation. [^{3}H]5-HT binding was assayed with 2.63 nM labeled ligand. Each value is the mean ± S.E.M. of quadruplicate determinations of fmoles of [^{3}H]5-HT bound per mg protein.

Supplementary step	Bound ^{3}H-5-HT	(%)
None	40.9 ± 1.9	(100)
A. 10 min 0°	59.2 ± 1.2	(145)
B. 10 min 37° + pargyline	61.2 ± 1.2	(150)
C. 10 min 37°	78.2 ± 4.0*	(191)
D. 2 × 10 min 37°	78.5 ± 3.5*	(192)

* $p < 0.05$ when compared to values corresponding to preparations incubated at 0° or at 37° in the presence of 10 μM pargyline.

from the membranes, which occurs more rapidly at 37° than in the cold (12). It seemed that the destruction by monoamine oxidase of 5-HT dissociated from membranes played an important role since the addition of pargyline, an inhibitor of this enzyme, diminished the effectiveness of this step. The effect of the incubation was maximal at 10 min as exposure of the membranes to a second 10 min period of incubation did not further augment the binding of [^{3}H]5-HT (Table 1).

Scatchard analysis of the binding of [^{3}H]5-HT to membranes incubated or not incubated at 37° (Fig. 1) revealed no difference in the maximal binding of the two preparations but an approximately 4-fold difference in the apparent affinity of the receptor for [^{3}H]5-HT. The direct measurement of endogenous 5-HT still bound to membrane material at various steps of the "tissue preparation" (see MATERIALS AND METHODS) revealed that the competitive inhibitor of [^{3}H]5-HT binding eliminated by a 10 min incubation period at 37° was very probably 5-HT itself. As shown in Table 2, the estimated concentration of endogenous 5-HT still present in the final suspension used for [^{3}H]5-HT binding assays, i.e., 0.06 nM, was less than 4% of the K_d value of the binding site for [^{3}H]5-HT (mean, 1.6 nM). This explained why a second incubation period of 10 min at 37° did not further increase the binding of [^{3}H]5-HT to synaptosomal membranes (Table 1).

Properties of the high affinity binding site for [^{3}H]5-HT. Since the membrane material used was not purified extensively preliminary experiments were performed to characterize the high affinity binding site for [^{3}H]5-HT. In contrast to what occurs with synaptic vesicles (32), neither ATP, Mg^{2+}, nor their combination induced any increase in [^{3}H]5-HT binding (Table 3). Indeed, a significant reduction of this binding was observed after the addition of ATP, with or without Mg^{2+} (Table 3). As previ-

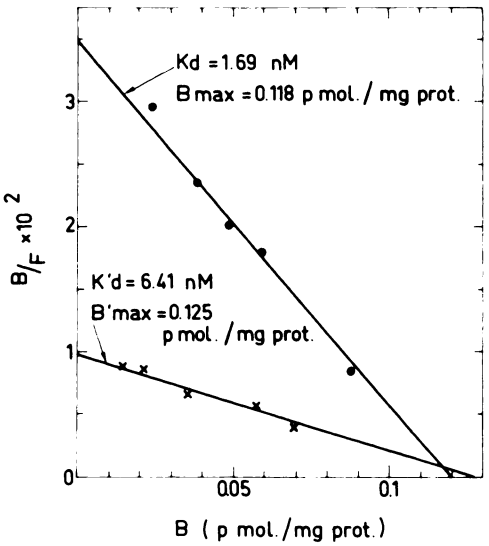

FIG. 1. *Scatchard plots of [^{3}H]5-HT specifically bound to lysed P_2 membranes before and after incubation at 37° for 10 min.*

A lysed P_2 fraction from the whole forebrain of adult rats was used (see Table 1). ^{3}H-5-HT binding was measured as described in MATERIALS AND METHODS, with the concentration of the labeled ligand varying from 0.41 nM to 7.4 nM. Each point is the mean of quadruplicate determinations. B represents the amount of [^{3}H]5-HT specifically bound in picomoles per mg of membrane protein. F represents the amount of free [^{3}H]5-HT in each sample. ●, P_2 fraction preincubated at 37° for 10 min; ×, unincubated P_2 fraction.

TABLE 2

Endogenous 5-HT content in various preparations from the rat forebrain

Various fractions of the adult rat forebrain were prepared, as described in MATERIALS AND METHODS. The values of protein (in mg) and 5-HT (in ng) correspond to their total amounts in fractions obtained with 1 g of rat forebrain (i.e., 10 ml of the 0.32 M sucrose homogenate). Each value is the mean ± S.E.M. of 4 independent determinations. Figures in the right column correspond to the calculated concentration of 5-HT still present in the final binding assay mixture (obtained by diluting the lysed P_2 fractions to 40 ml when starting with 1 g of fresh tissue).

Preparation (10 ml)	Protein	5-HT	
		In the preparation	In the binding assay mixture
	mg	*ng*	*nM*
Homogenate	144.2 ± 1.2	341.2 ± 16.3	—
P_2 Fraction	44.1 ± 1.5	111.5 ± 4.2	—
Lysed P_2 fraction + 10 min at 0°	21.3 ± 0.8	17.5 ± 0.6	2.48
Lysed P_2 fraction + 10 min at 37°	20.9 ± 0.2	0.47 ± 0.02	0.06

TABLE 3

Effects of ATP-Mg^{2+} with or without Ca^{2+} on [^{3}H]5-HT high affinity binding

An incubated lysed P_2 fraction from the whole forebrain was used. Each compound was added to the medium just before the assay. [^{3}H]5-HT binding was measured with 1.25 nM of the labeled ligand. Each value represents fmoles of specifically bound [^{3}H]5-HT per mg of membrane protein (mean ± S.E.M.) of quadruplicate determinations.

Addition	Bound [^{3}H]5-HT	%
—	32.6 ± 1.2	(100)
0.5 mM ATP	24.4 ± 0.7	(75)
2 mM ATP	11.1 ± 0.6*	(34)
2 mM Mg^{2+}	31.0 ± 0.7	(95)
10 mM Mg^{2+}	30.0 ± 1.3	(92)
2 mM ATP-Mg^{2+}	17.4 ± 0.7*	(53)
2 mM ATP-10 mM Mg^{2+}	18.5 ± 0.8*	(57)
4 mM Ca^{2+}	43.4 ± 1.7*	(133)
4 mM Ca^{2+} + 2 mM ATP-Mg^{2+}	18.9 ± 0.4*	(58)

* $p < 0.05$ when compared to values corresponding to no addition.

ously described (12), the presence of 4 mM $CaCl_2$ significantly increased the amount of [^{3}H]5-HT bound to membranes (Table 3).

An examination of various agents considered to be antagonists or agonists of 5-HT showed that they were able to inhibit the binding of ^{3}H-5-HT under the conditions used (Table 4). The inhibition curve of [^{3}H]5-HT binding by 5-HT (Fig. 2A) indicated a Hill coefficient very close to 1 (at least with 5-HT concentrations ranging between 0.5 nM and 0.1 μM). In contrast, the logit-log competitive inhibition plot of ^{3}H-5-HT binding by quipazine, a 5-HT agonist (4), gave a slope significantly less than 1 (Table 4, Fig. 2A). Comparison of the IC_{50} values clearly indicated that the two agonists presently studied, quipazine and MK-212, were much less efficient than 5-HT in preventing the binding of [^{3}H]5-HT to its high affinity binding site.

Among 5-HT antagonists, metergoline and methysergide were the two drugs with the greatest ability to prevent the binding of [^{3}H]5-HT, metergoline being almost as potent as 5-HT (Fig. 2B, Table 4). As already noted with 5-HT agonists, the slope of the logit-log inhibition plot of [^{3}H]5-HT binding by antagonists was either close to 1 (metergoline, methysergide, cinanserine, pizotifen) or significantly less than 1 (methiothepin, mianserine, see Table 4 and Fig. 2B). Moreover, the determination of the type of inhibition using Lineweaver-Burk and Dixon plots revealed that all compounds did not behave as competitive inhibitors (Table 4). Therefore, the determination of the K_I from the IC_{50} value ($K_I = IC_{50}/1 + ([^3H]5\text{-}HT/K_d)$ is not appropriate for all compounds inhibiting the high affinity binding of [^{3}H]5-HT.

Among other substances tested on [^{3}H]5-HT binding, only clozapine and to a lesser extent fluphenazine displaced bound [^{3}H]5-HT from its high affinity site in synaptosomal membranes (Table 4). The very poor

TABLE 4

Characteristics of inhibition of [^{3}H]5-HT high affinity binding by various drugs

For each drug the IC_{50} and the m values (slopes of the logit-log inhibition plots, see Fig. 2) are the means of at least two independent determinations. When indicated, the number of experiments is given in brackets. For each determination, 5–6 different concentrations of a given drug were used. The type of inhibition and the K_I were determined using Lineweaver-Burk and Dixon plots.

Drug	IC_{50} (nM)	K_I (nM)		m	
Agonists					
5-HT	3.9	1.60 + 0.13 competitive	(3)	0.96 ± 0.05	(4)
Quipazine	342	233 ± 52 competitive	(4)	0.45 ± 0.05	(4)
MK-212	2,180	–		0.86	
Antagonists					
Metergoline	5.0	3.06 ± 0.41 competitive	(3)	1.05	
Methysergide	28.2	–		0.91	
Methiothepin	372	308 ± 67 noncompetitive	(3)	0.62 ± 0.05	(3)
Cinanserine	236	–		0.99	
Pizotifen	287	–		0.92	
Mianserine	794	–		0.68 ± 0.05	(3)
Indoles					
Iprindole	12,880	–		0.83	
Harmaline	24,000	–		0.47	
Harmalol	29,500	–		0.58	
Harmine, Tabernanthine, Vincamine		<20% at 100 μM			
Other compounds					
Clozapine	371	–		1.01	
Fluphenazine	2,510	–		0.69 ± 0.05	(3)
Fluoxetine	18,190	–		1.1	
Dopamine	19,500	–		1.05	
Norepinephrine	100,000	–		1.08	
Morphine, Fenfluramine, Triclopidine, Reserpine		<20% at 10 μM			

efficacy of catecholamines, fluoxetine (a potent and specific inhibitor of 5-HT uptake [33]) and reserpine (Table 4) to inhibit the high affinity binding of [^{3}H]5-HT to synaptosomal membranes further confirms the specificity of this [^{3}H]5-HT binding site.

Studies of the regional distribution of the high-affinity binding of [^{3}H]5-HT indicated that it was denser in hippocampus, striatum, colliculi and hypothalamus, four areas which contain relatively high levels of endogenous 5-HT. However, there was no significant correlation (correlation coefficient = 0.334) between the capacity to bind [^{3}H]5-HT and the endogenous 5-HT levels among the 8 regions containing the 5-HT terminals that were examined (Table 5). The correlation was even poorer when areas containing 5-HT cell bodies were also included (correlation coefficient = 0.105, Table 5). Kinetic analyses of [^{3}H]5-HT binding revealed that, at least for the striatum, the hippocampus, the cerebral cortex and the lateral brain-stem, the differences noted above only concerned the number of binding sites and not the affinity for [^{3}H]5-HT (K_d : 1.4–2.1 nM).

Effects of the destruction of serotoninergic neurons on the binding of [^{3}H-]5-HT. In view of the regional distribution of 5-HT receptors (Table 5), the hippocampus and striatum were the two structures chosen for the study of the effects of the destruction of 5-HT neurons.

Destruction of the ascending serotoninergic pathways by the intracerebral injection of 5,7-HT resulted in an 80–90% depletion of 5-HT in both the striatum and hip-

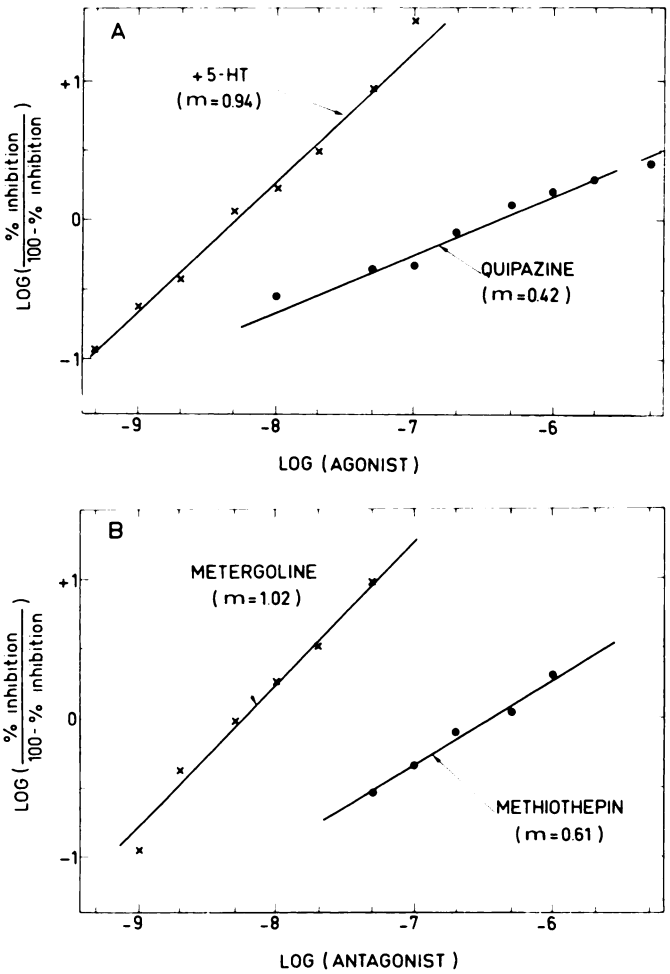

FIG. 2. *Logit-log inhibition plots of [^{3}H]5-HT binding by two agonists, 5-HT and quipazine (A) and two antagonists, metergoline and methiothepin (B)*

(A) With 1.65 nM [^{3}H]5-HT but in the absence of drug, the amount of [^{3}H]5-HT specifically bound to the incubated lysed P_2 fraction from the whole forebrain was equal to 51.6 ± 1.4 fmoles per mg of protein. Each point is the mean of quadruplicate determinations. *m* is the slope of the plot. (B) The same protocol was followed except that 5-HT agonists were replaced by 5-HT antagonists.

pocampus 18 days after the drug treatment (Table 6). At this time there was no detectable difference in the affinity of the receptor for [^{3}H]5-HT between control animals and animals with lesions in either structure (Fig. 3, Table 6). However, when the concentration of receptors (B_{max}) was calculated using Scatchard analysis, a significant increase of approximately 40% in the number of receptor sites was found in the hippocampus after the destruction of serotoninergic neurons (Table 6, Fig. 3). There also appeared to be a tendency toward an increase in the receptor concentration in the striatum, although this change (+25%) was not statistically significant. Studies of the inhibitory effects of 5-HT and quipazine on ^{3}H-5-HT binding in the hippocampus and the striatum demonstrated that neither the respective IC_{50} values nor the slopes of logit-log inhibition plots (see Fig. 2A and Table 4) were altered in 5,7-HT treated animals when compared to controls.

The binding of (—) [^{3}H]dihydroalprenolol to *beta* noradrenergic receptors in the hippocampus and the binding of [^{3}H]halo-

TABLE 5

Regional distribution of the high affinity binding of [^{3}H]5-HT in the central nervous system of the adult rat

Binding assays were performed with incubated, lysed P_2 fractions from each structure. Each value is the mean ± S.E.M. of quadruplicate determinations of femtomoles of specifically bound [^{3}H]5-HT per 1 mg protein in the presence of 1.42 nM labeled ligand. Endogenous 5-HT is expressed as μg/g tissue wet weight. Linear regression analysis of the quantity of bound [^{3}H]5-HT versus endogenous 5-HT for each structure (except for those containing serotoninergic cell bodies) gave a correlation coefficient of 0.334.

Structure	Bound [^{3}H]5-HT	Relative Binding (%)	Endogenous 5-HT
Hippocampus	80.6 ± 5.0	(100)	0.45 ± 0.04
Striatum	61.2 ± 3.1	(69)	0.51 ± 0.03
Colliculi	53.3 ± 3.0	(63)	0.79 ± 0.06
Hypothalamus	46.9 ± 2.5	(58)	1.04 ± 0.04
Cerebral cortex	44.2 ± 2.3	(55)	0.28 ± 0.03
Raphe	38.0 ± 1.6	(45)	1.79 ± 0.04
Thalamus	36.1 ± 0.5	(43)	0.68 ± 0.07
Lateral brain stem	23.1 ± 2.1	(27)	0.76 ± 0.06
Spinal cord	15.7 ± 0.7	(19)	0.52 ± 0.02
Cerebellum	5.9 ± 0.5	(7)	0.08 ± 0.01

peridol to dopaminergic receptors in the striatum were measured to determine if the phenomenon observed was specific for 5-HT receptors or a generalized change in receptor density. As shown in Table 6, no change in either of these types of binding was detected after the administration of 5,7-HT. This was consistent with the relatively small decreases in striatal dopamine levels (Control: 8.9 ± 0.3 μg/g; 5,7-HT: 5.9 ± 0.3 μg/g; 34%, $p < 0.001$). Forebrain norepinephrine levels (control: 0.31 ± 0.01 μg/g; 5,7-HT: 0.28 ± 0.01 μg/g; 10%, $p < 0.05$) found in the right side on the eighteenth day following this treatment.

An examination of the time course of the decline of 5-HT and the appearance of receptor "supersensitivity" in the hippocampus revealed that 4 days after the 5,7-HT injection, 5-HT levels had already fallen to 32% of control values but that no change in the receptor concentration could be seen (Fig. 4). By the eighth day, 5-HT had fallen to about 20% of control levels and at this time a significant increase (+25%) in receptor density could be measured. Between the eighth and eighteenth days after the destruction of serotoninergic neurons, 5-HT

TABLE 6

Effect of 5,7-HT administration on 5-HT levels, [^{3}H]5-HT binding, [^{3}H]dihydroalprenolol binding and [^{3}H]haloperidol binding in the hippocampus and the striatum of the rat

5,7-dihydroxytryptamine was injected as described in MATERIALS AND METHODS 18 days before killing the animals. 5-HT levels (in μg/g of fresh tissue) and K_d and B_{max} of the [^{3}H]5-HT high affinity binding are the means ± SEM of 5 determinations corresponding to 5 independent experiments as described in the legend to Fig. 3. K_d and B_{max} of the high affinity binding of [^{3}H]dihydroalprenolol ([^{3}H]DHAL) and [^{3}H]haloperidol ([^{3}H]HAL) were obtained in 1 experiment. In all cases, K_d is expressed in nM and B_{max} in fmoles of the labeled ligand bound per mg of membrane protein.

	Hippocampus		Striatum	
	Control	5,7-HT	Control	5,7-HT
5-HT (μg/g)	0.44 ± 0.03	0.06 ± 0.01*	0.48 ± 0.03	0.09 ± 0.02*
[^{3}H]5-HT binding				
K_d	1.73 ± 0.11	2.02 ± 0.26	1.57 ± 0.08	1.59 ± 0.13
B_{max}	225 ± 26	312 ± 25*	155 ± 15	193 ± 15
[^{3}H]DHAL binding				
K_d	2.05	2.50	—	—
B_{max}	59.9	62.3	—	—
[^{3}H]HAL Binding				
K_d	—	—	1.35	1.22
B_{max}	—	—	563	559

* $p < 0.05$ when compared to respective control values.

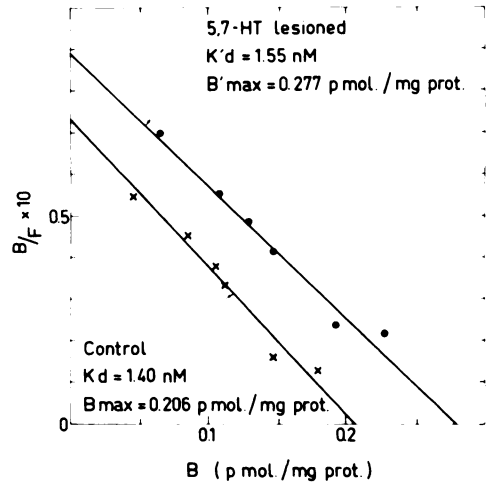

FIG. 3. *Scatchard plots of specifically bound [^{3}H]5-HT to a lysed P_2 fraction from the hippocampus of control or 5,7-HT pretreated rats*

The intracerebral administration of 5,7-HT was performed 18 days before sacrifice as described in MATERIALS AND METHODS. The P_f fractions obtained from pooled right hippocampi from 14 treated rats (●–●) and from hippocampi of both sides from 8 control rats (×–×) were prepared as described in MATERIALS AND METHODS. Specific binding of [^{3}H]5-HT was measured with 0.45, 0.90, 1.35, 1.80, 4.50, and 5.40 nM of the labeled ligand. Each point is the mean of quadruplicate determinations (see the legend to Fig. 1 for the meaning of B and F).

levels changed little further reaching about 13% of control values while the binding of [^{3}H]5-HT increased to a value 39% above the controls (Fig. 4).

DISCUSSION

In the present report, the binding of [^{3}H]5-HT has been used to further describe the characteristics of the high affinity 5-HT receptor. Using a lysed P_2 fraction of rat brain homogenates, it was found that limited washing of this membrane preparation in the cold allowed enough endogenous 5-HT to remain to affect the apparent affinity of the receptor for [^{3}H]5-HT (Tables 1 and 2, Fig. 1). This residual 5-HT could be removed by incubating the membrane preparation at 37° for 10 min; the K_d for 5-HT changed from about 6–12 nM in unincubated preparations to about 1.4–2.0 nM in incubated preparations. These results may explain some of the discrepancies between previously reported values for the K_d of the high affinity binding of [^{3}H]5-HT. For example, using a relatively crude membrane preparation, Bennett and Snyder (12) reported a K_d ranging from 8–15 nM for the binding of [^{3}H]5-HT in rat brain, while Fillion et al. (13), using a more elaborate procedure that would have allowed a more complete removal of endogenous 5-HT, found a value of 1–3 nM. In addition, using less purified preparations, the latter authors also described a binding site with an affinity for 5-HT (K_d = 10–30 nM) in the range of that reported by Bennett and Snyder (12) and of that presently obtained using nonincubated preparations. Therefore, rather than suggesting the existence of at least two high affinity binding sites (13), the various reported K_d values might simply result from the persistence in the membrane preparations of more or less endogenous 5-HT competitively inhibiting the binding of exogenous [^{3}H]5-HT.

Although the lysed P_2 fraction used was not a highly purified membrane prepara-

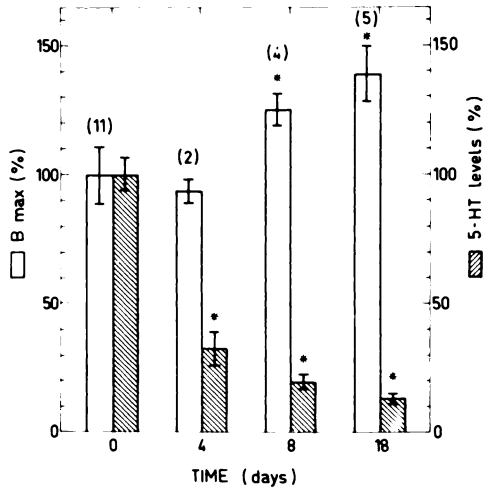

FIG. 4. *Time course of changes in 5-HT levels and the number of high affinity binding sites for [^{3}H]5-HT in the right hippocampus of 5,7-dihydroxytryptamine treated rats*

Each bar represents the percent of respective control values. In control rats 5-HT levels were equal to 0.44 ± 0.03 μg/g of fresh tissue (mean ± S.E.M. of 5 independent determinations). The B_{max} value corresponding to 100 was equal to 225 ± 26 fmoles of [^{3}H]5-HT bound per mg of membrane protein. The number of experiments (see legend to Fig. 3) performed at each time is indicated in brackets. *$p < 0.05$ when compared to respective control values.

tion, several observations suggested that [^{3}H]5-HT was bound to a postsynaptic site. First, [^{3}H]5-HT cannot be bound to monoamine oxidase since the affinity of this enzyme for 5-HT is quite low (K_m = 0.15 mM) and pargyline was added in the binding assay mixture to completely inhibit the catabolism of [^{3}H]5-HT. Since fluoxetine, a potent and specific inhibitor of the 5-HT reuptake process (33), was a very poor inhibitor of the high affinity binding of ^{3}H-5-HT, the presynaptic uptake mechanism is not significantly involved in this binding. Finally, the high affinity binding of [^{3}H]5-HT is not associated with synaptic vesicles since ATP-Mg^{2+}, which stimulates the uptake of [^{3}H]5-HT into these organelles (32), was inhibitory. In addition, Ca^{2+}, which induces the release of vesicular neurotransmitters (34), increased significantly the high affinity binding of [^{3}H]5-HT (Table 3, [12]). These results were confirmed by the fact that reserpine failed to interfere with this binding. In contrast, known agonists (particularly quipazine) and antagonists of 5-HT were found to affect the binding of [^{3}H]5-HT with relatively high potencies, suggesting that this binding site exhibits the properties of a specific receptor of 5-HT. Furthermore, chemical lesions of serotoninergic neurons with 5,7-HT did not result in any loss of high affinity binding sites, indicating that the 5-HT receptor studied was located postsynaptically.

As indicated by its regional distribution, the high affinity binding site for [^{3}H]5-HT occurred preferentially in 5-HT rich areas, although the correlation between [^{3}H]5-HT binding and 5-HT levels in the central nervous system of the adult rat was rather poor. Similar findings have already been reported for various ligands of postsynaptic receptors by Snyder (35), who proposes that the geometry of pre- and post-synaptic elements varying from one area to the other might explain these apparent discrepancies.

Previous studies comparing the binding of [^{3}H]5-HT and [^{3}H]LSD have led to the conclusion that the 5-HT receptor might exist in two forms, one favoring the binding of serotoninergic agonists and the other that of antagonists (12, 13). Similar agonist-antagonist receptor forms have been proposed for other monoamine receptor systems. For example, U'Prichard *et al.* (36) found that there appeared to be agonist and antagonist forms of the alpha-noradrenergic receptor. These workers postulated a noninterconvertible two-state model for the *alpha* noradrenergic receptor since the slopes of logit-log plots of inhibition of the binding of a labeled agonist or antagonist by either agonists or antagonists were always equal to 1.0. In the dopaminergic system of the caudate nucleus, the situation appears to be different (37). Using the same logit-log representation, displacement of a labeled agonist by agonists or the inhibition of the binding of a labeled antagonist by antagonists gave slopes of 1.0, but for the displacement of a labeled agonist by antagonists, or vice versa, they were less than 1.0. This was explained by proposing that the dopaminergic receptor exists in two states, with restricted interconvertability. For the high affinity [^{3}H]5-HT receptor, some drugs (agonists *and* antagonists) inhibited the binding of [^{3}H]5-HT with slopes of 1.0 while others gave values less than 1.0 (Table 3). In the case of inhibition of [^{3}H]5-HT binding by cold 5-HT, the logit-log representation, which is equivalent to the Hill plot, indicated that the Hill coefficient (i.e., the slope) was equal to 1.0. Therefore, some drugs, but not 5-HT, may induce negative cooperativity with regard to their inhibition of the binding of [^{3}H]5-HT. However, the interpretation of the slope of the logit-log inhibition plot is difficult and other phenomena (heterogeneity of binding sites, etc.) may also produce slopes of less than 1. How the present findings fit into the concept of agonist and antagonist-favoring forms of the 5-HT receptor will require further work.

While previous studies (12, 13) failed to show the development of denervation supersensitivity of 5-HT receptors in the brain of the rat, the present study shows that in the hippocampus, at least, there is an increase (39%) in the number of 5-HT-receptors after the destruction of serotoninergic neurons, with no change in their apparent affinity for [^{3}H]5-HT. These data are comparable with previous reports of supersensitivity developed by other mono-

amine receptors For example, Creese *et al.* (17) found a 40% increase in dopamine receptors in the rat striatum after destruction of the dopaminergic nigro-striatal pathway, and Sporn *et al.* (15) reported a 31% increase in *beta* adrenergic receptors in rat cerebral cortex after destruction of catecholamine-containing neurons. In neither of these systems was there a change in the affinity of the receptor for its ligand. It is not clear why previous studies were unable to demonstrate the development of 5-HT receptor supersensitivity. In one case (13), the lesions of the 5-HT system produced about a 66% reduction in 5-HT terminals as determined by the decrease of [^{3}H]5-HT uptake into synaptosomes. It is possible, therefore, that the surviving neurons produced enough transmitter to prevent the increase in receptors. Likewise, in the present work, when there was only a 68% decrease in 5-HT levels (4 days after the injection of 5,7-HT), no measurable change in the quantity of 5-HT receptors was found. In another case no change was detected in the number of receptors in rat forebrain, even though there was an 83% decrease in the uptake of [^{3}H]5-HT 2 weeks after the production of the lesions (12). However, in our study an increase in the number of binding sites could be detected in the hippocampus as early as 8 days after the intracerebral injection of 5,7-HT, a time when 5-HT levels were depleted by 80%. This apparent discrepancy might be explained if denervation supersensitivity of 5-HT receptors does not occur in all structures innervated by serotoninergic neurons and that by using the total forebrain (12), supersensitivity produced in selected structures was masked. Indeed, the data presented here suggest that there can be regional differences since a significant increase in receptors was found in the hippocampus but not in the striatum (Table 5). The reason for the differences between the hippocampus and striatum with regard to the response of the 5-HT receptors to destruction of serotoninergic neurons has not yet been determined. However, it might be related to the differences in the types of serotoninergic innervation of these two structures. For example, the striatum receives most of its serotoninergic innervation from the nucleus dorsalis raphe while the innervation of the hippocampus originates mainly from the nucleus medianus raphe (38). Since the firing rate of units in the nucleus medianus is higher than that in the nucleus dorsalis (see references 39 and 40, 41), the destruction of these two systems might result in a greater response of postsynaptic elements in those structures innervated by the more active of the two serotoninergic pathways.

Under the conditions used it was seen that an increase in the number of receptors could be detected only by the eighth day after the intracerebral injection of 5,7-HT. This time course differs from that observed for the development of behavioral supersensitivity after similar lesions, since it was detected as early as 24 hours after 5,7-HT treatment (6, 18). This discrepancy might result from the existence of amplifying mechanisms which enhance the 5-HT induced response in postsynaptic elements in the absence of any detectable increase in postsynaptic receptors. Indeed, it has already been demonstrated in pinealocytes that supersensitivity involved not only the *beta* adrenergic receptors but also intracellular enzymes (42). Therefore, the supersensitivity of 5-HT receptors might be only one component of the biochemical changes which lead to enhanced behavioral responses to 5-HT agonists.

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