Brain Res. 346, 250-262 (1985). N°2 DES 2294

i) 250-262
Elsevier

BRE 11095

Multiple High Affinity Binding Sites for 5-Hydroxytryptamine: a New Class of Sites Distinct from 5-HT_1 and S_2

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(Accepted January 21st, 1985)

Key words: binding site — serotonin — 5-hydroxytryptamine — synaptosomal membrane — receptor — brain — enkephalin — α-melatonin stimulating hormone

Two different classes of binding sites probably related to serotonergic receptors have already been reported: 5-HT_1 binding sites recognize [^{3}H]5-hydroxytryptamine with a high affinity (K_d = 3 nM) and S_2 binding sites recognize [^{3}H]spiroperidol and [^{3}H]ketanserine. An additional population of sites has been observed in crude membrane preparations or fractions enriched with synaptosomal membranes obtained from rat brain cortex. This population was observed as a single class of sites in a synaptosomal fraction (L fraction — according to Laduron (1977)). It corresponded to a dissociation constant K_d = 13–15 nM, and B_{max} = 0.80 ± 0.15 pmol/mg protein. Displacement experiments showed that it recognized preferentially the 5-HT structure (bufotenin, 5-MeO-tryptamine). Tryptamine was a weak displacer and 5,7-dihydroxytryptamine totally inefficient. Neither 8-OH-DPAT, nor quipazine had any effect.

Methiothepin, cinanserin and cyproheptadine displaced 5-HT from these sites whereas ergot derivatives did not. Contrary to 5-HT_1 binding, this recently observed binding was not altered by GTP; α-MSH reduced the corresponding B_{max} whereas Leu-enkephalin did not. The degenerative lesion of the serotonergic fibers led to a slight increase in the B_{max} of the binding without altering the K_d which means that corresponding sites are not located on serotonergic fibers and might be postsynaptically located.

INTRODUCTION

Today our knowledge of cerebral serotonin receptors in vertebrates is still incomplete and controversial. Two different classes of binding sites have already been reported. The first one, called 5-HT_1 was observed several years ago. It has a dissociation constant of 3 nM[5,9]. Its existence has since been confirmed by numerous laboratories[15]. A second binding activity, called S_2, was reported by Peroutka and Snyder[37] using [^{3}H]spiroperidol as a ligand and by Leysen and Laduron[25] using [^{3}H]ketanserine. This second site binds serotonergic antagonists as well as histaminergic and adrenergic antagonists with high affinity, whereas 5-hydroxytryptamine (5-HT) itself has relatively low affinity for the site. However, correlations between the abilities of drugs to inhibit [^{3}H]ketanserine binding and serotonergic behavior suggest that S_2 is presumably related to a serotonergic receptor system[25].

Recently, a new putative 5-HT agonist, 8-hydroxy-2-(di-N,N-*n*-propylamino)tetralin (8-OH-DPAT) was reported by Golzan et al.[16] to label pre- and postsynaptic binding sites. Middlemiss[30], however, did not find any presynaptic effect for this drug. Pedigo et al.[35] observed the existence of heterogeneities in the displacement curves of [^{3}H]5-HT by various drugs; they suggested that subtypes (5-HT_1A and 5-HT_1B) of the 5-HT_1 site corresponded to the phenomenon.

In our laboratory[10], experimental evidence has been presented which points to the existence of a second class of binding sites for [^{3}H]5-HT. This site has a slightly lower affinity for 5-HT (K_d = 13–15 nM) than that corresponding to the 5-HT_1 site. Similar binding activity was also observed by Shih and Young[41], Segawa et al.[42] and more recently by van den Berg et al.[45]. The hypothesis that it could repre-

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sent another conformational state of the 5-HT_1 site has been put forward[13].

In this paper, experimental arguments show that this binding site is distinct from the 5-HT_1 site and suggest that it may represent a particular population of binding sites possibly related to a serotonin receptor system.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Iffa-Credo) (180–230 g) were housed in controlled conditions (22 °C; light cycle 12 h alternated with darkness 12 h — food and water available ad libitum). They were kept for at least a week before any treatment began.

The selective lesion of the serotonergic neurons was carried out on 14-day-old male rats by injecting intracerebroventricularly 5,7-dihydroxytryptamine (5,7-DHT) (0.5 μg/g of animal weight in a total volume of 3 μl of sterile physiological saline). The coordinates of the tip of the cannula at the injection point were: A = 2.5 mm, B = 2.5 mm, C = 3 mm from the lambda point as described by König and Klippel[2]. Control rats received physiological saline under similar experimental conditions. All rats received desmethylimipramine (25 mg/kg i.p.) 30 min prior to the intraventricular injection. The animals were sacrificed 6 days after the injection.

Preparation of the membranes

Crude membranes. These rats were sacrificed by decapitation and the brains rapidly dissected out in the cold (4 °C). Cortical tissue was rinsed with physiological saline at 0 °C and homogenized in 20 vols. (v/w) of 5 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 units/liter aprotinine, using an Ultraturrax homogenizer. The homogenate was centrifuged at 20,000 *g* for 15 min and the resulting pellet resuspended in the same buffer incubated at 37 °C for 5 min and recentrifuged at 20,000 *g* for 15 min. The resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4) containing, 1 mM EDTA, 2 mM PMSF and 5 units/liter aprotinin — to reach a concentration of 400–600 μg equivalent protein per ml. The binding of [^{3}H]5-HT was tested with 300 μl aliquots of the suspension.

Subcellular fractionation

(a) Fraction enriched with synaptosomal membranes

The synaptosomal membranes were prepared according to the method described by Cotman and Matthews[7]. Briefly, brain tissue was homogenized in a Potter Elvejhem apparatus with 30 vols. of 0.32 M sucrose in 5 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 2 mM PMSF and 5 units/liter aprotinin and centrifuged at 600 *g* for 10 min to eliminate cellular debris and nuclei; the resulting supernatant was centrifuged at 20,000 *g* for 15 min. The resulting pellet was resuspended in a small volume of the same buffer, layered on a discontinuous density gradient (Ficoll 7% and 13% dissolved in sucrose 10%) and centrifuged at 63,000 *g* for 90 min at 2–4 °C.

Synaptosomes were collected at the interface of the Ficoll layers and lyzed in 5 mM Tris-HCl pH 8.2 containing 1 mM EDTA, 2 mM PMSF and 5 units/liter aprotinin (0–2 °C for 1 h). The lyzate was centrifuged (20,000 *g* for 20 min). The resulting pellet, resuspended in 50 mM Tris-HCl buffer containing EDTA, PMSF and aprotinin, constituted the fraction enriched with synaptosomal membranes.

(b) Synaptosomal L and M fractions

These fractions were essentially obtained according to Laduron's description[22]. Briefly, brain tissue was homogenized in 5 vols. of ice-cold 0.25 M sucrose with a Teflon–glass homogenizer and the homogenate centrifuged at 600 *g* for 10 min. The pellet was homogenized and centrifuged. The resulting supernatant, added to the first supernatant was centrifuged at 10,000 *g* for 15 min and the resulting supernatant resuspended and recentrifuged. The pellet obtained from this centrifugation was the M fraction. The supernatants from M fraction were pooled and centrifuged at 20,000 *g* for 15 min and the resulting pellet was the L fraction.

The whole process was conducted in the presence of 2 mM EDTA, 1 mM PMSF and 5 units/liter aprotinine.

Binding assays

Membrane fractions were suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM PMSF and 5 units/liter aprotinin. Aliquots (300–500 μg protein for crude membranes, 100–250 μg protein for subcel-

lular membrane fractions) were incubated at 37 °C for 5 min and washed by centrifugation (20,000 *g* for 10 min). After resuspension in the same buffer, they were incubated at 22 °C for 20 min in the presence of increasing concentrations (generally 2–40 nM) of tritiated 5-hydroxytryptamine. Bound and free radioactivities were separated by a rapid vacuum filtration technique as previously described[8] and similar to that reported by Bennett and Snyder[5]. The reversible binding was measured in the presence of an excess of non-radioactive 5-HT (50 μM). A series of experiments has been performed in the presence of 0.50 μM non-radioactive 5-HT without qualitative changes.

The analysis of the binding curves was performed by Scatchard representation and by a non-linear regression analysis of the direct binding curve with a computerized program developed by Vindimian et al.[46]. This program was especially useful in the case of a non-linear Scatchard representation of the curve. In that case indeed, the binding corresponds either to the existence of multiple classes of binding types obeying the Clark equation or to a single population of sites obeying a Hill equation with a Hill nH < 1 coefficient, characteristic of a cooperative phenomenon. The limitations of the significance of the Scatchard representation have been demonstrated in the case of linear graphs corresponding to a single class of sites[19,20]. They are even more conspicuous in the case of non-linear graph. Thus, it is difficult to estimate the parameters of the binding with precision from a non-linear Scatchard plot despite the various methods proposed in the litterature[8,40]. Therefore, it seemed preferable to complete this estimation with that obtained from the computerized program proposed by Vindimian et al.[46]. In a few words, the program is based on a non-linear regression analysis of the experimental curve expressing B as a F function. This makes it possible to compare the experimental data to theoretical values calculated from the equations of Clark[6], Hill[18] or Adair[1] which mathematically express different types of interactions between ligands and macromolecules. The best-fit of theoretical values versus experimental ones obtained for a given equation determine the corresponding parameters of the interaction. In the reported experiments, when only one site was concerned, the binding parameters calculated with the Scatchard representation were not significantly different from those obtained from the computer program; for clarity's sake, the latter values have not been illustrated.

Regional distribution of 5-HT binding sites in the presence of GTP

The binding of [^{3}H]5-HT was measured in the presence of GTP (0.1 mM) in brain tissue homogenates using concentrations of the tritiated amine ranging from 1 to 40 nM.

It was determined in cortex, striatum, hippocampus and raphe. The parameters of the binding were calculated from the linear Scatchard plot of the corresponding curves.

Solubilization of 5-HT binding sites

Membranes isolated from rat brain cortex were incubated for 30 min at 4 °C in 50 mM Tris-HCl buffer pH 7.4 containing 1 mM PMSF and 5 units/liter aprotinin and 1% sodium cholate. The incubate was centrifuged at 120,000 *g* for 60 min and the resulting supernatant used as solubilized material.

The binding of [^{3}H]5-HT to the solubilized material was determined as usual except for proteins which were precipitated with 50% (final concn.) ammonium sulfate prior to the filtration step.

Protein measurements

Proteins were measured according to the method described by Lowry et al.[22].

Drugs

Drugs were obtained from commercial sources.

Tritiated 5-HT was obtained from NEN. The specific radioactivity was 26.8 Ci/mmol. Thin-layer chromatography on silica gel using *n*-butanol 25, acetic acid 4, water 10 was performed to test the purity of the radioligand; the radioactivity migrated together with 5-HT; contaminants represented a negligible proportion of the activity (1–3%).

RESULTS

Binding of 5-HT to cerebral tissue preparations

(a) Binding of [^{3}H]5-HT to synaptosomal membranes prepared according to Cotman and Matthews[7]

Cortical membrane preparations were obtained as

described in Materials and Methods. The incubation of synaptosomal membranes in the presence of increasing concentrations of [^{3}H]5-HT (from 1 to 35 nM) yielded a binding curve illustrating the existence of two different classes of sites. The Scatchard graph of the corresponding equation was curvilinear. The non-linear regression analysis using the computerized program[46] determined the best fit of the experimental curves with that corresponding to a Clark equation describing two classes of binding sites having the following parameters: $K_d = 3 \pm 1.5$ nM and $B_{max} = 0.11 \pm 0.09$ pmol/mg prot. for a first population of sites; and $K_d = 20 \pm 5.6$ nM and $B_{max} = 0.205 \pm 0.13$ pmol/mg prot. for a second population of sites (Fig. 1).

(b) Binding of [^{3}H]5-HT to synaptosomal fractions L and M prepared according to Laduron[22]

The M fraction was incubated in the same conditions as in the previous paragraph. The binding observed in the concentration range of [^{3}H]5-HT (1–30 nM) pointed to the existence of a single class of sites corresponding to the high affinity binding observed in the synaptosomal membranes.

The Scatchard representation of the binding curve made it possible to determine the parameters of the binding: $K_d = 4.5 \pm 0.6$ nM, $B_{max} = 0.27 \pm 0.04$ pmol/mg prot.

The L fraction similarly treated also contained a single population of sites as shown by the linear Scatchard representation of the binding curve. The obtained parameters were: $K_d = 17.8 \pm 3$ nM, $B_{max} = 0.86 \pm 0.14$ pmol/mg protein (Fig. 3). The ratio specific to total binding at saturation was $35.92 \pm 0.88\%$ in M fraction and $31.5 \pm 2.50\%$ in L fraction.

Regional distribution

The binding of [^{3}H]5-HT measured in the presence of GTP (0.1 mM) was determined in cerebral cortex, hippocampus striatum and raphe area — in the rat brain. Indeed, GTP very markedly decreases the 5-HT$_1$ binding without altering this second type of binding (cf. Fig. 7).

The affinity constants measured in cortex, striatum

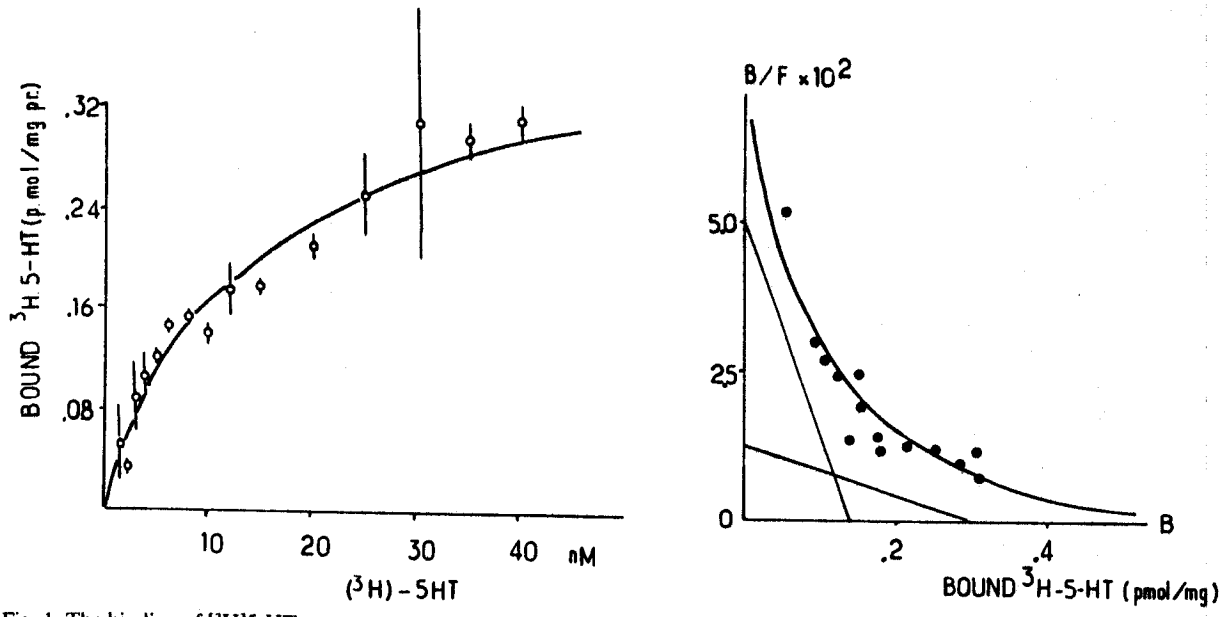

Fig. 1. The binding of [^{3}H]5-HT to synaptosomal membranes prepared from rat brain cortex according to the technique described by Cotman and Matthews[7]. The binding assays were performed as described in Materials and Methods with 14 concentrations of [^{3}H]5-HT ranging from 2 to 35 nM. Each point is the mean of triplicate determinations. The experiment was reproduced 10 times with similar results. In this experiment the estimation of the binding parameters by computerized non-linear regression analysis revealed the existence of two types of binding having the following parameters: $K_{d_1} = 3.6$ nM and $B_{max} = 0.18$ pmol/mg prot. and $K_{d_2} = 24.3$ nM and $B_{max} = 0.295$ pmol/mg prot. The Scatchard plot of the saturation curve expresses B/F (pmol/mg protein/nM) as a function of B (pmol/mg protein).

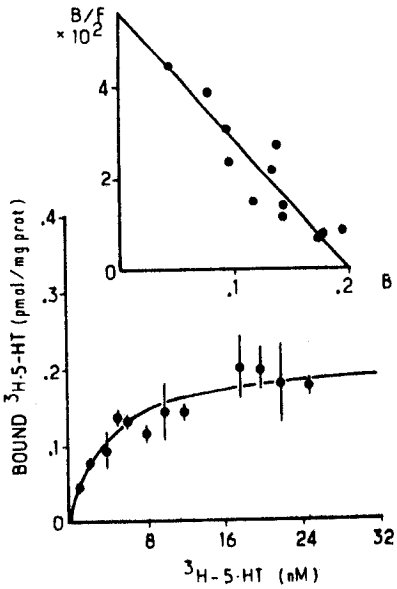

Fig. 2. The binding of [³H]5-HT to M fraction prepared from rat brain cortex according to Laduron[22]. The binding assays were performed as described in Materials and Methods with 12 concentrations of [³H]5-HT ranging from 2 to 25 nM. The ratio specific to total binding was 35–40% at saturation. Each point is the mean of triplicates. The bars express the standard deviation. The experiment was reproduced 5 times with similar results (see text). The Scatchard plot of the binding curve expresses the binding of [³H]5-HT in pmol/mg protein in abscissae and bound over free radioactivity in ordinate (pmol/mg/nM). The slope and the intercept with the abscissae were calculated by linear regression analysis. In that experiment, parameters corresponding to the binding were K_d = 3.9 nM, B_{max} = 0.20 pmol/mg protein.

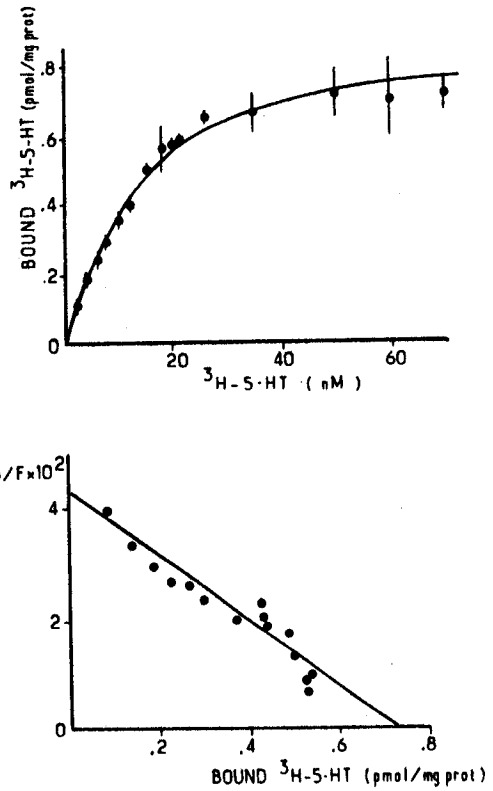

Fig. 3. The binding of [³H]5-HT to L fraction prepared from rat brain cortex according to the technique described by Laduron[22]. The binding assays were performed as described in Materials and Methods with at least 12 concentrations of [³H]5-HT ranging from 2 to 65 nM. Each point is the mean of triplicates; the bars express the standard deviation. The experiment was reproduced 5 times with very similar results (see text). The Scatchard plot of the binding curve made it possible to determine the binding parameters; in this experiment K_d = 16.9 nM and B_{max} = 0.720 pmol/mg protein.

and hippocampus were very similar and slightly lower in raphe; the B_{max} values for each area showed a heterogeneous distribution with the highest level in striatum, the lowest in raphe, the cortex and hippocampus corresponding to intermediate values (Table I).

TABLE I

5-HT$_2$ binding to different brain regions in rat brain

The binding of [³H]5-HT (concentration range 1–40 nM) was measured in the different brain areas using tissue homogenate as described in Materials and Methods. GTP (0.1 mM) was added 5 min before the end of the incubation. The K_d and B_{max} values were obtained from Scatchard plots of the saturation curves. The latter curves were linear.

Area	K_d *(nM)*	B_{max} *(pmol/mg rat)*	*n*
Striatum	18.5 ± 1.9	1.42 ± 0.13	3
Cortex	20.2 ± 0.1	0.87 ± 0.03	3
Hippocampus	19.3 ± 2.1	0.58 ± 0.02	3
Raphe	13.8 ± 0.2	0.25 ± 0.05	3

Solubilization of the binding sites

The synaptosomal fraction prepared according to Cotman and Matthews[7] was solubilized as described in Materials and Methods. The binding of [³H]5-HT to that preparation corresponded to the existence of two binding populations as indicated by the curvilinear Scatchard representation; the non-linear regression analysis of the data made it possible to estimate the parameters: K_d = 2.58 ± 0.45 nM; K_d' = 30.2 ± 7.4 nM and B_{max} = 0.057 ± 0.024 pmol/mg prot.; B_{max}' = 0.11 ± 0.04 pmol/mg prot. (n = 5) (Fig. 4).

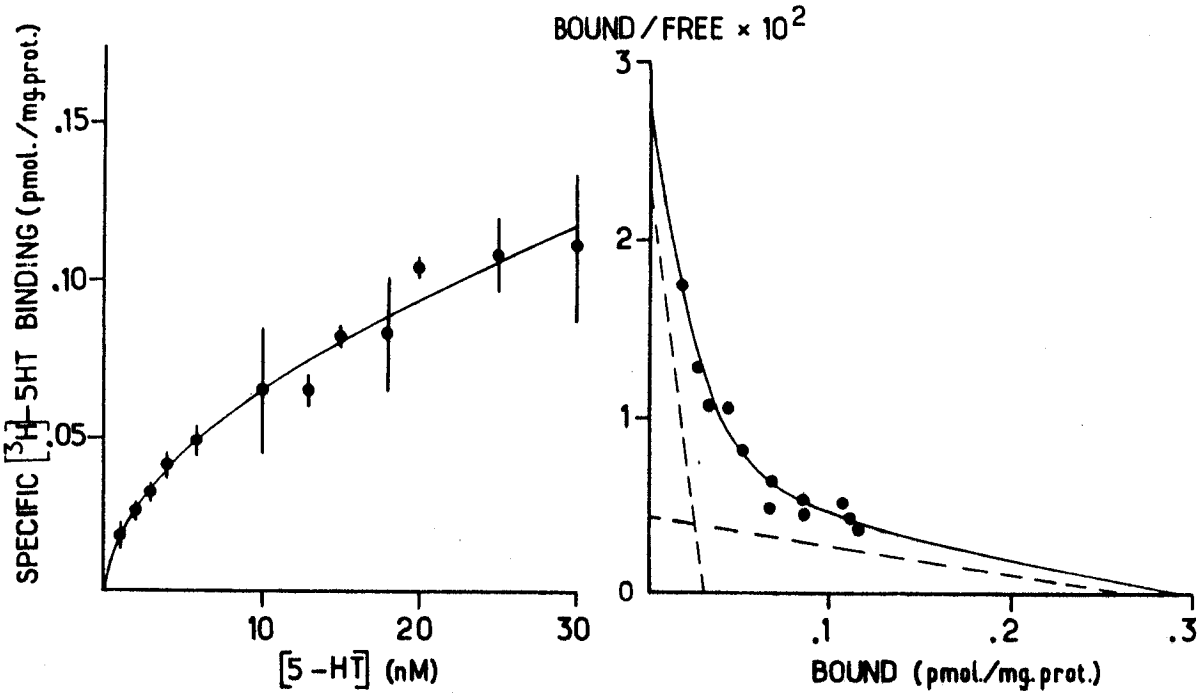

Fig. 4. The binding of [³H]5-HT to synaptosomal membranes from rat brain cortex solubilized with sodium cholate. The binding assays were performed as described in Materials and Methods with 12 concentrations of [³H]5-HT ranging from 2 to 30 nM. Each value represents the mean of triplicates ± S.D. The experiment was reproduced 5 times. The Scatchard representation of the binding curve is curvilinear. The computerized non-linear regression analysis of the binding curve reveals the existence of two populations of binding sites having the following parameters: K_{d_1} = 1.28 nM, B_{max_1} = 0.03 pmol/mg prot. K_{d_2} = 58 nM, B_{max_2} = 0.25 pmol/mg prot.

The binding of [³H]5-HT (1–40 nM) to solubilized L fractions and M fractions was determined by the same procedure as that used for solubilized synaptosomal membranes. Solubilized M fraction bound [³H]5-HT to a single population of sites corresponding to K_d = 2.6 ± 0.2; B_{max} = 0.75 ± 0.05 pmol/mg prot. (not shown) whereas solubilized the L fraction bound the amine to a different population of sites characterized by K_d = 8–10 nM, B_{max} = 1.5 pmol/mg prot. (Fig. 5).

Displacement experiments

Various drugs have been tested for their capacity to interfere with the binding of [³H]5-HT in synaptosomal L fraction. Membranes were incubated with 20 nM [³H]5-HT in the presence of increasing concentrations of the drug studied (14 concentrations from 1 nM to 1 mM) and the binding of the amine determined as usual.

Among serotonergic agonists, 5-HT itself was the most efficient displacer of the tritiated amine. The totality of the binding was inhibited at 10^{-6} M and 85–90% was already displaced at 10^{-6} M, a concentration which is only 5 times higher than the concentration of radioactive 5-HT.

Among 5-HT derivatives, 5-OH-DMT (bufotenin) was from 5 to 8 times less efficient than 5-HT itself; the methoxy derivative (5-MeOT) was almost equipotent with 5-HT whereas mono- and dimethylated MeO-derivatives were 2 and 8 times less efficient respectively; N-NOMT displaced [³H]5-HT with an IC_{50} = 600 nM and tryptamine at 10 μM reduced the binding by only 50%. Quipazine, 5,7-DHT and 8-OH-DPAT were totally unable to displace the amine.

Among serotonergic antagonists, methiothepin was the most efficient displacer, cyproheptadine and cinanserin being 10 and 20 times less efficient, respectively; ergot derivatives such as methysergide, metergoline and LSD did not displace 5-HT from these sites. Unrelated substances did not show any significant efficiency (Fig. 6) (Table II).

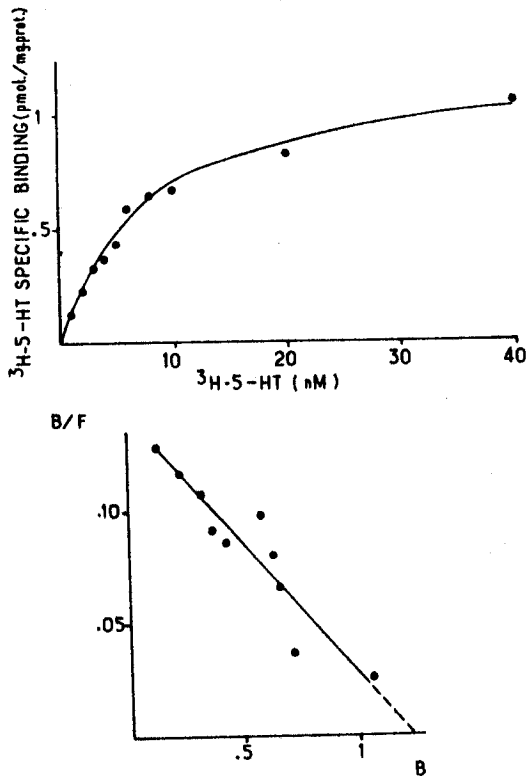

Fig. 5. The binding of [^{3}H]5-HT to solubilized L fraction prepared from rat brain cortex according to Laduron[22]. The binding assays were performed as described in Materials and Methods with 10 concentrations of [^{3}H]5-HT ranging from 2 to 40 nM. Each point is the mean of duplicates. The experiment was reproduced twice. The Scatchard plot of the binding curve was linear and corresponded to a single class of site having the following parameters: K_d = 8.2 nM, B_{max} = 1.2 pmol/mg prot. It expressed B/F (pmol/mg/nM) as a function of B (pmol/mg).

Effect of GTP

The effect of 5'-guanosine triphosphate (GTP) has been determined on the binding of [^{3}H]5-HT to L fraction. The concentration used in these assays was 0.1 mM. No significant change of the 5-HT$_3$ binding curve has been observed (Fig. 7a). In comparison, GTP markedly inhibited the 5-HT$_1$ binding when measured in the same conditions (Fig. 7b).

Effect of α-melatonin stimulating hormone (α-MSH) and Leu-enkephalin

The binding of [^{3}H]5-HT measured in synaptosomal L fraction in the presence of α-MSH (0.1 μM) is reduced when compared with the control; the B_{max} diminishes without significant modification of the K_d (Fig. 8). On the contrary, Leu-enkephalin at the same concentration does not change this binding (not shown).

Binding of [^{3}H]5-HT to synaptosomal membranes after degeneration of serotonergic neurons

The degeneration of serotonergic neurons was induced in young rats by stereotaxic intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT), a neurotoxic drug acting upon serotonergic neurons preferentially. Desmethylimipramine (25 mg/kg) was injected i.p. 30 min prior to the administration of 5,7-DHT to protect the catecholaminergic neuronal system from the effect of the neurotoxin. Under these conditions, the serotonergic uptake measured in synaptosomes prepared from the cerebral tissue of treated animals was reduced by 80% when compared with that measured in parallel with control animals

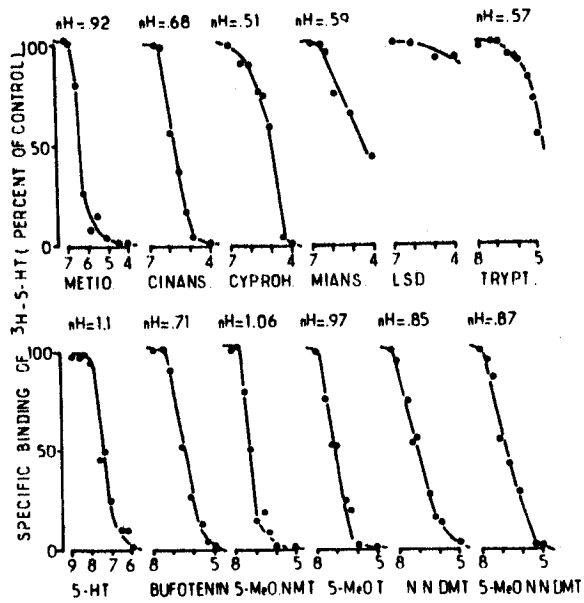

Fig. 6. The effect of various compounds on the specific binding of [^{3}H]5-HT to L fraction isolated from rat brain cortex. The L fraction was prepared as described in Materials and Methods. The specific binding of [^{3}H]5-HT (55% of the total binding) was determined at 20 nM in the presence of various concentrations of each tested compound: 5-hydroxytryptamine (5-HT), 5-hydroxy-N,N-dimethyltryptamine (bufotenin), 5-methoxy-N-methyltryptamine (5-MeO.N.MT), 5-methoxytryptamine (5-MeO.T), N,N-dimethyltryptamine (N.N.DMT), 5-methoxy-N,N-dimethyltryptamine (5-MeO.N.N.DMT), tryptamine (Trypt.), methiothepin (Metio.), cyproheptadine (cyproh.), cinanserin (cinans.), mianserin (mians.), lysergic acid diethylamide (LSD). nH expresses the Hill coefficient; it was calculated from the Hill plot of the inhibition curve.

TABLE II

The effect of various drugs on the specific binding of [³H]5-HT to synaptosomal L fraction isolated from rat brain cortex

The L fraction was prepared as described by Laduron[22] and the binding of [³H]5-HT was determined at 20 nM in the absence (control) or presence of the tested compound. The IC_{50} was calculated by the linear regression analysis of logit-log inhibition plot. Each value is the mean of (n) separate experiments. When n = 2, the two IC_{50}s did not differ by more than 20%.

Drug	*IC_{50} (nM)*	*nH*
Agonists		
5-HT	45	1.1 (6)
5-OH-N,N-DMT	220	0.71 (3)
5-MeO-tryptamine	40–80	0.97 (2)
5-MeO-N-MT	75	1.06 (2)
5-MeO-N,N-DMT	300	0.87 (2)
N,N-DMT	600	0.85 (3)
Tryptamine	>100,000	0.57 (3)
Antagonists		
Methiothepine	350 ± 120	0.92 (3)
Cinanserine	3000 ± 280	0.68 (3)
Cyproheptadine	7500 ± 650	0.51 (3)
Mianserin	60,000	0.59 (2)
LSD	>100,000	(3)
Metergoline	>100,000	(3)
Methysergide	>100,000	(3)
Spiroperidol	>100,000	(3)
Compounds not related to 5-HT		
Haloperidol	>100,000	(2)
Chlorimipramine	>100,000	(3)
Naloxone	>100,000	(2)
Phenoxybenzamine	>100,000	(2)

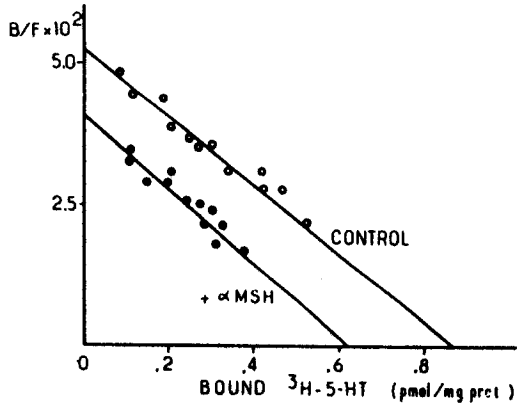

Fig. 8. The effect of α-melatonin stimulating hormone. The binding of [³H]5-HT to synaptosomal L fraction was determined as described in Materials and Methods. The binding assays were performed in the absence (control) or in the presence of 0.1 μM α-MSH. In these experiments, the incubation medium was that described in Materials and Methods; moreover, bestatine (1 μM) was added as protease inhibitor. Controls performed in the presence of bestatine were not significantly modified. In this experiment, the parameters of the binding of [³H]5-HT were as follows: K_d = 16.3 nM B_{max} = 0.84 pmol/mg protein for the control; K_d = 15.1 nM and B_{max} = 0.62 pmol/mg protein for the α-MSH-treated membranes. The experiment was reproduced three times. The Scatchard plot of the saturation curve expresses B/F (pmol/mg prot./nM) as a function of B (pmol/mg/protein).

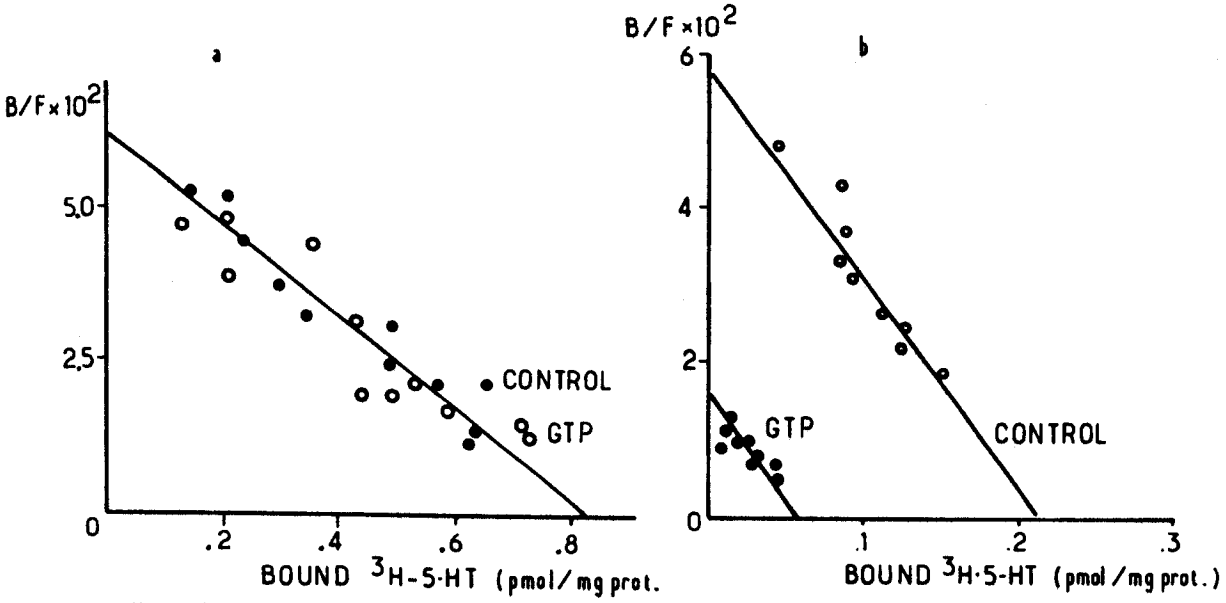

Fig. 7. a: the effect of 5'-guanosine triphosphate (GTP) on the binding of [³H]5-HT to synaptosomal L fraction isolated from rat brain cortex. The L fraction was prepared according to Laduron[22]. The binding assays were performed in the absence (control) or in the presence of GTP (0.1 mM) as described in Materials and Methods. In this experiment, the binding parameters were K_d = 13.6 nM and B_{max} = 0.820 pmol/mg prot. for the control curve. These parameters were not significantly modified in the presence of GTP (n = 3). GTP was added to the incubation medium 5 min before the end of the incubation duration. b: the M fraction was tested under the same experimental conditions. Control corresponded to K_d = 3.6 nM and B_{max} = 0.21 pmol/mg protein. In the presence of GTP (0.1 mM) K_d = 3.7 nM and B_{max} = 0.036 pmol/mg protein.

receiving NaCl by a stereotaxic injection. The considerable decrease of the 5-HT uptake was regarded as an indication of the occurrence of the degeneration of serotonergic fibers.

The binding of [^{3}H]5-HT determined in crude membrane fraction prepared from treated animals was measured in the presence of GTP since the nucleotide reduced the binding of [^{3}H]5-HT at the 5-HT$_1$ site very markedly[14,27], whereas it did not modify that corresponding to the lower affinity binding. Under these conditions, the degeneration of serotonergic fibers by 5,7-DHT did not reduce the binding activity when compared with the control; on the contrary, it increased it slightly (15–30%) without modification of the corresponding dissociation constant (Fig. 9).

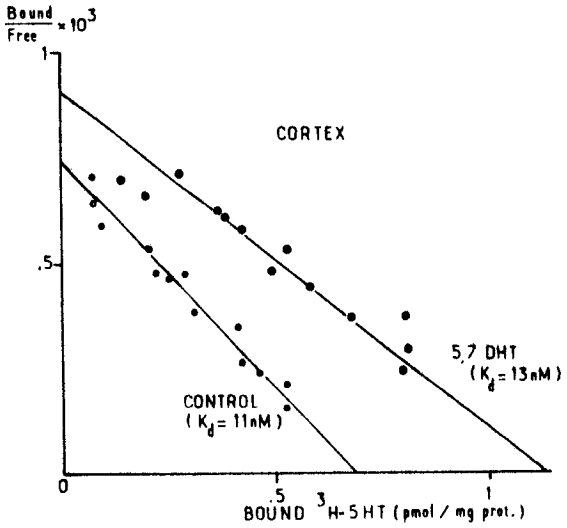

Fig. 9. The binding of [^{3}H]5-HT to rat brain cortex homogenate following 5,7-DHT lesion. Fourteen-day-old rats received 5,7-DHT (0.5 mg/kg) intraventricularly; control rats received the same volume of saline solution. Six days after the injection, the animals were sacrificed and their cerebral cortex dissected out and homogenized with an Ultra-Turrax (20,000 rpm for 2 min) at 2–4 °C. The homogenates were incubated at 37 °C for 5 min and washed by centrifugation (20,000 *g* for 10 min) and resuspended in 40 vols. of Tris-HCl buffer 50 mM, pH 7.4. The binding assays of [^{3}H]5-HT were performed with 12–14 concentrations of [^{3}H]5-HT ranging from 2 to 40 nM in the presence of GTP (0.1 mM). The Scatchard plot of the binding curves corresponds to the following parameters: for control rats K_d = 11 nM and B_{max} = 0.7; and for treated animals K_d = 13 nM and B_{max} = 1.20 pmol/mg protein. It expresses B/F (pmol/mg prot./nM) as a function of B (pmol/mg prot.).

DISCUSSION

Several types of 5-HT receptors seem to be present in cerebral tissue; some of them being coupled with adenylate cyclase[11,24,34] and other types possibly unrelated to the enzyme activity[17,44]. Several bits of experimental evidence also suggest the multiplicity of sites which are able to recognize the 5-HT structure[10,31,32,42,43,45]. For the present, the existence of two main types of sites has been established: 5-HT$_1$ having a high affinity for 5-HT (see a review by Fillion[14]) and S_2 which recognizes spiroperidol[38] and ketanserin[25]. The results submitted in this paper put forward the existence of a new class of sites which are able to recognize the 5-HT structure with a high affinity.

The binding of [^{3}H]5-HT measured in crude membrane preparations or in fractions enriched with synaptosomal membranes obtained according to the technique of Cotman and Matthews[7] corresponds to complex binding curves. Their representation by a Scatchard plot is not linear but upward concave. Their analysis by non-linear regression calculations[46] shows that the binding curve fits optimally with the equation expressing the existence of two populations of sites obeying a Clark equation corresponding to the dissociation constants: K_d = 3 nM and K_d close to 15 nM, respectively. The first constant is similar to that of the 5-HT$_1$ sites, the second one corresponds to that previously reported by some authors[10,42,43,45]. It is apparently distinct from that described by Monroe and Smith[38] in rat spinal cord tissue corresponding to a dissociation constant K_d = 57.8 nM, and unobserved in cortex by these authors. The ratio of specific to total binding at saturation increases from 25% in crude membranes to 30–40% in the fraction enriched with synaptic membranes and favors the hypothesis that the two sites which are present in that synaptosomal fraction are related to neuronal elements.

The technique described by Laduron[22] separates the L and M subfractions contained in the synaptosomal membrane fraction. The measurement of the binding of [^{3}H]5-HT in these fractions illustrates the separation of the two binding types: the M fraction actually contains a single population of sites obeying the Clark equation; this is strongly supported by the conclusions drawn from the non-linear regression analysis of the direct binding curve and also by the

linearity of the Scatchard plot of the curve. The parameters calculated by computer-assisted analysis are K_d = 4.9 nM and B_{max} = 0.29 pmol/mg protein; they are very similar to those obtained from Scatchard plot (K_d = 4.5 nM, B_{max} = 0.27 pmol/mg protein). The two analyses show that M fraction contains a single class of sites which merely represents the 5-HT_1 population.

The L fraction isolated in parallel with the M fraction binds [^{3}H]5-HT according to a Clark equation and a linear Scatchard plot; the binding parameters of the single class of sites observed in L fraction are K_d = 17.8 nM, B_{max} = 0.82 pmol/mg protein. The dissociation constant is very similar to one of those observed in synaptosomal fractions and approximately corresponds to it. N and P fractions obtained in parallel with Laduron's technique[22] represent the rest of the subfractions which constitute the initial homogenate; the N fraction does not bind [^{3}H]5-HT whereas the P fraction contains a slight percentage of a binding which has an affinity constant K_d = 12 nM and is insensitive to GTP (results not shown). The latter binding in the P fraction presumably corresponds to a contamination by the L fraction.

Therefore, these results show that the two types of binding observed in synaptosomal fractions are separable and can be resolved as two distinct populations of sites characteristic of L and M fractions, respectively.

Other authors[5,33] previously used the subcellular fractionation technique described by Laduron et al.[22]. They did not report the existence of different sites; a possible explanation for this discrepancy is that their experiments were performed using a single concentration of [^{3}H]5-HT which did not permit the determination of the parameters of the binding and to distinguish different classes of sites.

It should also be noted that the homogenization step in the present assays seems crucial; it requires a low speed and a small clearance of the pestle with regard to the homogenizer. The non-observance of these conditions leads to a poor subcellular separation, the two sites being then mostly in L and P fractions.

Provisionally and to facilitate the discussion, it has been suggested to call the second population of synaptosomal sites '5-HT_3'. Indeed, this site recognizes [^{3}H]5-HT with a high affinity and is presumably distinct from the 5-HT_1 site. It can neither be called 5-HT_2 for it could be mistaken for the S_2 site, nor S_3 site as S type sites do not recognize 5-HT with a high affinity, e.g. S_2 binds spiroperidol or ketanserin[26,38]. The duality of the binding of [^{3}H]5-HT is also found in solubilized membrane preparations, i.e. detergent-solubilized synaptosomal membrane fraction binds the amine with two dissociation constants (K_d = 2.58 ± 0.45 nM; K'_d = 30.2 ± 7.4 nM). This result agrees with the findings of Van der Berg et al.[45] which demonstrate the existence of two distinct bindings in solubilized crude membrane prepared from bovine cerebral cortex with two dissociation constants: K_{d_1} = = 1.3 nM and K_{d_2} = 22 nM. Interestingly enough, the two types of bindings are separated in the solubilized M fraction (K_d = 2.5 nM) and L fraction (K_d = 8–10 nM). The solubilization of the membranes induces a considerable environmental change of the binding site and may explain the slight alteration in the dissociation constant observed in solubilizate. Despite this drastic change in phospholipid environment, the duality of the binding is maintained supporting the hypothesis that 5-HT_1 and 5-HT_3 sites are related to different entities.

The chromatographic analysis of the solubilized protein responsible for the two affinities will answer more definitely to that question. At present, experiments are being undertaken to study this particular point.

Displacement experiments have been performed in conditions which made it possible to interpret the results optimally as they have been carried out with the L fraction which contains the 5-HT_3 site as a single population of binding sites for [^{3}H]5-HT. Under these conditions the displacing effects of the different compounds which have been studied indeed only concern the 5-HT_3 sites; 5-HT_1 binding does not interfere with the results as it would in a crude membrane fraction. The serotonergic structure appears necessary to displace the binding of [^{3}H]5-HT to these sites since 5-hydroxy- and 5-methoxy-derivatives can inhibit the binding of the amine. It is interesting to note that unsubstituted or mono-methylated amino-derivatives displace the amine with a Hill coefficient close to unity whereas di-methylated compounds, either substituted in the 5-position (5-OH-N, N-DMT and 5-MeO-N,N-DMT) or not (N,N-DMT), are less efficient and correspond to a Hill coefficient

close to 0.7–0.8. This may indicate that 5-hydroxy- or 5-methoxy-amines, are able to inhibit the binding of [^{3}H]5-HT to the 5-HT$_3$ site in a competitive way whereas dimethyl compounds act differently. Among other agonists, 8-OH-DPAT does not seem to play any significant role at the 5-HT$_3$ site and therefore possibly represents a specific marker of the 5-HT$_1$ site[16]. Golzan et al.[16] suggested that 8-OH-DPAT could also label the autoreceptor. On the other hand, quipazine, which was supposed to label the autoreceptor sites too[28] does not affect the 5-HT$_3$ site. From these observations it is inferred that the 5-HT$_3$ site may not be related to the autoreceptor. The serotonergic nature of the site is indicated by the fact that methiothepin totally inhibits the binding of 5-HT at concentrations which are 10–13 times higher than that which produce the same effect at 5-HT$_1$ sites; moreover, cyproheptadine and cinanserin are effective at higher concentrations similar to those which act upon the 5-HT$_1$ site. On the contrary, ergot derivatives which constitute a particular class of anti-serotonergic drugs are totally inefficient. This result tends to show that the 5-HT$_3$ site is not involved in the effect of hallucinogenic drugs. The latter compounds most likely interact with the binding of 5-HT at the 5-HT$_1$ site[5,10].

The lack of effect of spiroperidol and ergot derivatives clearly confirms that the 5-HT$_3$ site is distinct from the S$_2$ site since the latter recognizes spiroperidol as well as ergot derivatives with nanomolar affinity. The 5-HT$_3$ site is not even related to the uptake system since fluoxetine, an efficient blocker of the 5-HT uptake, does not modify the binding of 5-HT at the 5-HT$_3$ site.

Since GTP was shown to inhibit the binding of 5-HT to 5-HT$_1$ sites[12,27,36], it seemed interesting to examine the effect of the nucleotides on the binding to the 5-HT$_3$ sites. First of all, it was confirmed that under the experimental conditions GTP decreased the binding of [^{3}H]5-HT to 5-HT$_1$ sites. This effect was shown on the M fraction which merely contains the 5-HT$_1$ sites: GTP at 0.1 mM reduces the B_{max} of the observed binding by 75%. It should be noted that GTP is added shortly before the end of the incubation period to avoid its degradation. Under the same experimental conditions, GTP does not induce any significant change at the 5-HT$_3$ site. Therefore, the binding of 5-HT at the 5-HT$_3$ site appears different from that at the 5-HT$_1$ site; moreover, this lack of effect of GTP may indicate that the 5-HT$_3$ site is presumably unrelated to an adenylate cyclase system sensitive to 5-HT; generally, the binding of the hormone to a site which is related to the enzyme activity is actually considerably altered by GTP[39].

It has been demonstrated that the binding of [^{3}H]5-HT is slightly modified by substance P[45] and more recently, Rostene et al.[41] have shown that VIP very markedly increases the binding of [^{3}H]5-HT to 5-HT$_1$ sites specifically, in the dorsal hippocampus. In the presence of bestatine which does not modify control values, α-MSH reduces the binding of [^{3}H]5-HT to 5-HT$_3$ sites in the cortex. This effect has as yet not been observed with Leu-enkephalin, which, on the contrary, reduces the binding at the 5-HT$_1$ site[29]. This effect suggests a possible interaction of α-MSH at the 5-HT$_3$ site. Although a certain selectivity of the α-MSH towards the 5-HT$_3$ binding represents an interesting phenomenon, experiments will have to be substantially completed, in other areas of the brain particularly, to succeed in relating this effect to any physiological significance. Anyhow, this present observation shows that the various peptides which possibly interact with the cerebral serotonergic system, may act through processes which implicate different serotonergic sites.

Under conditions which induce the degeneration of the serotonergic fibers as the dramatic decrease in the 5-HT uptake capacity of the tissue may show, the binding of 5-HT at the 5-HT$_3$ site does not decrease, but increases slightly. As a consequence, it is most unlikely that the 5-HT$_3$ site will appear to be responsible for the 5-HT uptake or to be located in serotonergic neurons. Thus, it could be a non-neuronal element, possibly glial or a non-serotonergic neuronal constituent. The first hypothesis is improbable for glial cell primary cultures do not bind 5-HT with that affinity (Beaudoin and Netter, unpublished experiments). The second hypothesis may point to a post-synaptic location of the site. More work is needed to elucidate this particular point. In conclusion, the 5-HT$_3$ binding site presents interesting features: it recognizes [^{3}H]5-HT with a high affinity, and is saturable thus expressing the existence of a limited number of sites. This number is different with regard to the area of the brain which is considered. The 5-HT$_3$ site, solubilized by detergent, seems to correspond to

a protein structure distinct from that representing the 5-HT_1 site.

Competitive studies show that the 5-HT_3 site specifically binds the 5-HT structure as well as that of some anti-serotonergic drugs. However, the fact that ergot derivatives are not recognized indicates that it is distinct from other known serotonergic sites. Interestingly enough, α-MSH interacts with the binding of [^{3}H]5-HT; investigations will have to be done to ascertain the physiological implications this effect may have. Serotonergic neurons are likely involved in the process of regulation of the site in adult life as well as during the postnatal development; this process has already been reported to occur during the synaptogenesis in the brain.

Therefore, on the basis of these results, it is not yet possible to ascribe a physiological role as a receptor to the 5-HT_3 site; however, it is also difficult to ignore it. Experiments are currently being undertaken to clarify the properties of this particular binding site for [^{3}H]5-HT.

REFERENCES

1 Adair, G. S., The hemoglobine system. The oxygen dissociation curve of hemoglobine, *J. Biol. Chem.*, 63 (1925) 529.

2 Agnati, L., Fuxe, K., Ben Fenati, F., Zini, I. and Hökfelt, T., On the functional role of coexistence of 5HT and substance P in bulbospinal 5HT neurones. Substance P reduces affinity and increases density of [^{3}H]5HT binding sites, *Acta Physiol. Scand.*, 117 (1983) 299–301.

3 Battaglia, G. and Titeler, M., Direct binding evidence for multiples states of S_2 receptors, *Abstracts 12th Annual Meeting, Minneapolis, Oct. 31 Nov. '82*, no. 76.1 (1982) 276.

4 Bauguen-Fayolle, C. and Fillion, G., Postnatal development of [^{3}H]5-Hydroxytryptamine binding sites in rat brain cortex, *Biogenic Amines*, 1 (1984) 143–155.

5 Bennett, J. P. and Snyder, S. H., Serotonin and lysergic acid diethylamide binding in rat brain membranes: relationship to postsynaptic serotonin receptors, *Mol. Pharmacol.*, 12 (1976) 373–389.

6 Clark, A. J., *The Mode of Action of Drugs on Cells*, E. Arnold, London, 1933.

7 Cotman, C. W. and Matthews, D. A., Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterization, *Biochem. Biophys. Acta*, 249 (1971) 380–394.

8 De Meyts, P. and Roth, J., Cooperativity in ligand binding: a new graphic analysis, *Biochem. Biophys. Res. Commun.*, 66 (1975) 1118.

9 Fillion, G., Fillion, M. P., Spirakis, C., Bahers, J. M. and Jacob, J., 5-Hydroxytryptamine binding to synaptic membranes from rat brain, *Life Sci.*, 18 (1976) 65–74.

10 Fillion, G., Rousselle, J. C., Fillion, M. P., Beaudoin, D., Goiny, M., Deniau, J. M. and Jacob, J., High affinity binding of [^{3}H]5-hydroxytryptamine to brain synaptosomal membranes; comparison with [^{3}H]lysergic acid diethylamide binding, *Mol. Pharmacol.*, 14 (1978) 50–59.

11 Fillion, G., Rousselle, J. C., Beaudoin, D., Pradelles, P., Goiny, M., Dray, F. and Jacob, J., Serotonin sensitive adenylate cyclase in horse brain synaptosomal membranes, *Life Sci.*, 24 (1979) 1813–1822.

12 Fillion, G. and Fillion, M. P., Transitional states of the neuronal serotoninergic sites, *Eur. J. Pharmacol.*, 65 (1980) 109–112.

13 Fillion, G., Fillion, M. P. and Rousselle, J. C., Regulation of the serotonergic receptor activity in synaptosomal membranes, *Neurosci. Lett.*, Suppl. 5 (1980) 69.

14 Fillion, G., Fillion, M. P. and Rousselle, J. C., Augmentation de l'affinité du récepteur 5HT et diminution de l'activité adénylate cyclase sensible à la 5HT par exposition prolongée à la 5HT, *J. Physiol. (Paris)*, 77 (1981) 363–368.

15 Fillion, G., 5-Hydroxytryptamine receptors in brain. In L. Iversen, S. D. Iversen and S. H. Snyder (Eds.), *Handbook of Psychopharmacology, Vol. 18*, Plenum Publishing corporation, 1982, pp. 96–117.

16 Gozlan, H., El Mestikawy, S., Pichat, L., Glowinski, S. and Hamon, M., Identification of presynaptic serotonin autoreceptors using a new ligand: (^{3}H)PAT., *Nature (Lond.)*, 305 (1983) 140–142.

17 Hamon, M., Nelson, D. L., Herbet, A. and Glowinski, J., Multiple receptors for serotonin in the rat brain. In G. Pepeu, M. J. Kuhar and S. J. Enna (Eds.), *Receptors for Neurotransmitters and Peptides Hormones*, Raven Press, New York, 1980, pp. 223–233.

18 Hill, A. V., *J. Physiol. (Lond.)*, 40 (1910) 190.

19 Klotz, I. M., Number of receptor sites from Scatchard graphs: fact and fantasies, *Science*, 217 (1982) 1247–1249.

20 Klotz, I. M., Ligand–receptor interactions: what we can and cannot learn from binding measurements, *Trends Pharmacol. Sci.*, (1983) 253–255.

21 König, J. F. R. and Klippel, R. A., The rat brain. In J. F. R. König and R. A. Klippel (Eds.), *A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem*, R. E. Krieger, New York, 1967.

22 Laduron, P. M., Tissue fractionation in neurobiochemistry an analytic tool or a source of artefacts, *Int. Rev. Neurobiol.*, 20 (1977) 251–263.

23 Lowry, O. H., Rosebrough, N., Farr, L. and Randall, R., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193 (1951) 265–275.

24 Levitzky, A., GTP–receptor interrelationships in adenylate cyclase systems. Theoretical considerations, *Biochim. Biophys. Acta*, 628 (1980) 419–424.

25 Leysen, J. E. and Laduron, P. M., A serotonergic component of neuroleptic receptors, *Arch. Int. Pharmacodyn.*, 230 (1977) 337–339.

26 Leysen, J. E., Awouters, F., Kennis, L., Laduron, P. M., Vandenberk, J. and Janssen, P. A. J., Receptor binding profile of R 41468, a novel antagonist at 5HT_2 receptors,

Life Sci., 28 (1981) 1015–1022.
27 Mallat, M. and Hamon, M., Ca^{++}–guanine nucleotide interactions in brain membranes. I — Modulation of central 5-hydroxytryptamine receptors in the rat , *J. Neurochem.*, 38 (1982) 151–161.
28 Martin, L. L. and Sanders-Bush, E., The 5HT autoreceptor: antagonism by quipazine, *Neuropharmacology*, 21 (1982) 445–450.
29 Menant, I. and Fillion, G., Modulation d'affinité des liaisons de types S_1 et S_3 de la [^{3}H]5HT par l'α-MSH et la leu enképhaline, *J. Pharmacol. (Paris)*, 15 (1984) 105.
30 Middlemiss, D. N. and Fozard, J. R., 8-hydroxy 2(D_1-*n*-propylamino)tetralin discriminates between subtypes of the $5HT_1$ recognition site, *Eur. J. Pharmacol.*, 90 (1983) 151–153 (Abstr.).
31 Monroe, P. J. and Smith, D. J., Characterization of multiple (^{3}H)5HT binding sites in rat spinal cord tissue, *J. Neurochem.*, 41 (1983) 349–355.
32 Nelson, D. L., Herbert, A., Bourgoin, S., Glowinski, J. and Hamon, M., Characteristics of central 5HT receptors and their adaptative changes following intracerebral 5,7-dihydroxytryptamine administration in the rat, *Mol. Pharmacol.*, 14 (1978) 983–995.
33 Nelson, D. L. Herbet, A., Enjalbert, A., Bochaert, J. and Hamon, M., Serotonin-sensitive adenylate cyclase and [^{3}H]serotonin binding sites in the CNS of the rat, *Biochem. Pharmacol.*, 29 (1980) 2455–2463.
34 Pagel, J., Christian, S. T., Quayle, E. S. and Monti, J. A., A serotonin-sensitive adenylate cyclase in mature rat brain synaptic membranes, *Life Sci.*, 19 (1976) 810–824.
35 Pedigo, N. N., Yamamura, H. I. and Nelson, D. L., Discrimination of multiple (^{3}H)5HT binding sites by the neuroleptic spiperone in rat brain, *J. Neurochem.*, 36 (1981) 220–226.
36 Peroutka, S. J., Lebovitz, R. M. and Snyder, S. H., Serotonin receptor binding sites affected differentially by guanine nucleotides, *Mol. Pharmacol.*, 16 (1979) 700–708.
37 Peroutka, S. and Snyder, S. H., Multiple serotonin receptors: differential binding of [^{3}H]5-hydroxytryptamine, [^{3}H]lysergic acid diethylamide, and [^{3}H]spiroperidol, *Mol. Pharmacol.*, 16 (1979) 687–699.
38 Peroutka, S. J., Lebovitz, R. M. and Snyder, S. H., Two distinct central serotonin receptors with different physiological functions, *Science*, 212 (1981) 827–829.
39 Rodbell, M., Krans, H. M. J., Pohl, S. L. and Birnbaumer, L., The glucagon sensitive adenylate cyclase system in plasma membranes of the rat liver, *J. Biol. Chem.*, 246 (1971) 1872–1876.
40 Rosenthal, H. E., A graphical method for the determination and presentation of binding parameters in a complex system, *Ann. Biochem.*, 20 (1967) 525.
41 Rostene, W. H., Modulation by vasoactive intestinal peptide (VIP) of serotonin receptors in membranes from rat hippocampus, *J. Neurosci.*, 3 (1983) 2414–2419.
42 Segawa, T., Mizuta, T. and Nomura, Y., Modifications of central 5-hydroxytryptamine binding sites in synaptic membranes from rat brain after long-term administration of tricyclic antidepressants, *Eur. J. Pharmacol.*, 58 (1979) 75–83.
43 Shih, J. C. and Young, H., The alteration of serotonin binding sites in aged human brain, *Life Sci.*, 23 (1978) 1441–1448.
44 Snyder, S. H. and Goodman, R. R., Multiple neurotransmitter receptors, *J. Neurochem.*, 35 (1980) 5–13.
45 Van den Berg, S. R., Allgren, R. L., Todd, R. D. and Ciaranello, R. D., Solubilization and characterization of high affinity [^{3}H]serotonin binding sites from bovine cortical membranes, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 3508–3512.
46 Vindimian, E., Robaut, C. and Fillion, G., A method for cooperative or non-cooperative binding studies using nonlinear regression analysis on a microcomputer, *J. Appl. Biochem.*, 5 (1983) 261–268.