Life Sciences Vol. 18, pp. 65-74
Printed in the U.S.A.

Pergamon Press

5-HYDROXYTRYPTAMINE BINDING TO SYNAPTIC MEMBRANES FROM RAT BRAIN

Gilles Fillion, Marie-Paule Fillion, Christina Spirakis, Jean-Marie Bahers and Joseph Jacob

Department of Pharmacology, Institut Pasteur
25, rue du Docteur Roux, 75015 Paris, France

(Received in final form December 2, 1975)

Summary

The ^{3}H-5HT binding capacity of rat brain synaptic membranes prepared by density gradient centrifugation has been investigated using a rapid ultrafiltration technique. A saturable, high affinity (K_D = 1.10^{-9} M), 5HT displaceable binding has been found. It is thermosensitive, temperature dependent and pH dependent. 5HT and related tryptamines are the most effective displacers of bound ^{3}H-5HT, whereas compounds which are not structurally related to 5HT (chlorpromazine, imipramine, cyproheptadine and cinanserine) and other neurotransmitters (noradrenaline, dopamine) are ineffective. The distribution of 5HT-specific binding sites in the brain is related to serotonergic input. We conclude that these 5HT binding sites might possibly represent 5HT receptor sites.

Using isolated nerve endings from rat brain, Marchbanks (1) described for 5-hydroxytryptamine (5HT) a low affinity binding ubiquitous in the brain, a medium affinity binding with a K_D of 5.10^{-4}M probably associated with monoamine oxidases and a high affinity binding with a K_D of 2.10^{-6} M. Fiszer and de Robertis (2) confirmed the existence of such high affinity binding ($3.3.10^{-6}$ M) using nerve ending membranes isolated from hypothalamus, basal ganglia and grey areas of the mesencephalon of cat brain. This year, Snyder and Bennett (3) found on rat brain a high affinity binding for 5HT (K_D = 8 nM), the amine being displaced by d.LSD.

Using a rapid ultrafiltration technique, we describe here a specific, saturable, high affinity binding of 5HT to synaptic membranes isolated from rat brain.

Methods and Materials

Preparation of the membranes

Sprague-Dawley rats (200 to 250 g) were sacrified by decapitation; the brain was quickly removed and the cerebellum discarded. Routinely, membranes were prepared according to a slightly modified version of the technique of Cotman and Matthews (4). Homogenates were prepared in ice-cold sucrose (0.32 M, 20 % W/V), using a Potter-Elvejhem apparatus, centrifuged at 1000xg for 5 minutes to remove cell debris, nuclei etc. and the resulting supernatant then centrifuged at 17,500xg for 10 minutes. The pellet was then resuspended in 0.32 M sucrose and 5 ml fractions

layered onto a discontinuous Ficoll density gradient (13.5 % and 7.5 % Ficoll dissolved in 0.32 M sucrose). After centrifugation (IEC B 60 ultracentrifuge) for 90 minutes at 90,000xg, a synaptosomal fraction was collected at the interface between the Ficoll layers, diluted with 80 ml of 0.32 M sucrose and centrifuged at 100,000xg for 20 minutes. The pellet obtained was lysed by suspension in 80 ml of Tris-HCl buffer (0.005 M, pH 8.2) for one hour at 0°C. After centrifugation (30,000 xg, 20 minutes), the pellet was resuspended in Tris-HCl buffer (pH 7.4, 0,05 M) and 5 ml fractions were layered onto a 25 ml discontinuous sucrose density gradient (35 % 5 ml ; 32,5 % 10 ml and 25 % 10 ml) and centrifuged (90,000xg, 2 hours). Synaptic membranes were collected onto 32.5 % sucrose, resuspended in Tris-HCl buffer and collected after centrifugation (90,000xg 30 minutes). If not used immediately, they were kept overnight at 0°C under nitrogen.

Electron microscopic examination confirmed that the preparations consisted mostly of synaptic plasma membranes which were not contaminated with mitochondria ; among those synaptic membrane profiles, many have an attached synaptic thickening. In a few profiles some synaptic vesicles still adhere to the membrane.

Preparation of crude mitochondrial fraction

In some assays, binding was studied using slightly purified tissue homogenates, known as "crude mitochondrial fraction", prepared according to Whittaker (5). Such fractions were prepared from various regions of the brain dissected on ice immediately after sacrifice (cortex, cerebellum, raphe, hippocampus, striatum, diencephalon) and from a peripheral organ (kidney) according to Glowinski and Iversen (6).

Binding assays

Two ml fractions, corresponding to 200 to 500 µg of protein were generally used. Incubation with ^{3}H-5HT was routinely carried out in Tris-HCl buffer 0.05 M pH 7.4 at 0°C for 25 minutes but other temperatures and incubation times were also used. Zero degree centigrade was chosen as the standard incubation temperature to minimise the degradative enzymatic activity in the incubation medium. When antagonists were assayed, they were generally added 20 minutes after the radioactive amine and incubation was continued for an additional 20 minutes (or for a longer time in kinetic experiments). In some assays the antagonists were added at varying times before the ^{3}H-5HT.

Separation of the bound and free radioactivity was performed using a slightly modified version of the technique of Pert and Snyder (7), which consists of a rapid filtration of the incubate through a Millipore® filter (0.45 µ) under vacuum, the filter being rinsed three times with a total volume of 15 ml of ice-cold Tris-HCl buffer pH 7.4. This volume was chosen after some preliminary studies had shown that a minimum of 10 ml was necessary to achieve a low and reproduceable level of residual activity on the filter ; increasing the volume to 20 ml did not reduce it further. The washing time did not exceed 30 seconds, and was usually only 20 seconds.

Radioactivity was measured by liquid scintillation spectro-

metry, using 10 ml of Bray solution (naphthalene, 50 g, 2,5 diphenyloxazole 4 g, p=bis (2-5-phenyloxazoyl) benzene 0.2 g, methanol 100 ml, ethylene glycol 20 ml and dioxan up to 1 litre) and an SL 32 Intertechnique spectrometer. The counting efficiency was 25 to 26 %.

Proteins were measured by the Lowry method (8), using serum albumin as standard.

Binding assays were performed in triplicate, each with a corresponding blank identical to the sample except that the tissue fraction was replaced by an equal volume of Tris-HCl buffer.

Chromatography

Thin layer chromatography assays were performed on silica gel using different solvent systems : chloroform - methanol - acetic acid (80 - 15 - 2 and 70 - 30 - 0,5) and butanol - acetic acid - water (13 - 3 - 5).

"Free" and "bound" radioactivity were tested after incubation of membranes in usual conditions in presence or absence of ascorbic acid ; free radioactivity was separated by filtration, specifically bound radioactivity was thereafter displaced from membranes by non radioactive 5HT (10^{-5} M). Those samples were run together with controls as 5HT alone, 5HT in presence of ascorbic acid, 5 hydroxyindole acetic acid (5HiAA) and bufotenine.

^{3}H-5HT (specific activity : 17.3 Ci/mmole in the first batch, 16 Ci/mmole in the second) was obtained from the Radiochemical Centre, Amersham, England and LSD (racemic mixture) from Hoffman-La Roche, Switzerland.

All other drugs were obtained from the usual commercial sources.

Results

Binding of ^{3}H-5HT

The total radioactivity bound to synaptosomal membranes increased as the concentration of tritiated amine was increased (5.10^{-10} to 5.10^{-7} M). A Scatchard (9) plot indicates the existence of two distinct types of binding sites (fig. 1).

The high affinity binding corresponds to a dissociation constant of $1.5 \pm 0.7\ 10^{-9}$ M (8 assays). Binding was completed within 20 minutes at 0°C and was stable for at least 60 minutes (5 assays) (fig. 2) whereas at 37°C, the equilibrium was reached within 5 to 10 minutes. When high concentrations (5.10^{-6} M ; 1.10^{-5} M) of non-radioactive 5HT were added to the incubation medium at zero degree 3 minutes after the tritiated amine (3.10^{-9} M), total binding diminished by 60 to 80 % (fig. 2). Higher concentrations of non-radioactive 5HT (up to 10^{-4} M) did not further reduce the binding. Reversible binding (calculated by substracting the ^{3}H-5HT binding in the presence of a high concentration of non-radioactive 5HT from the total binding) increased with increasing ^{3}H-5HT concentrations and reached saturation with

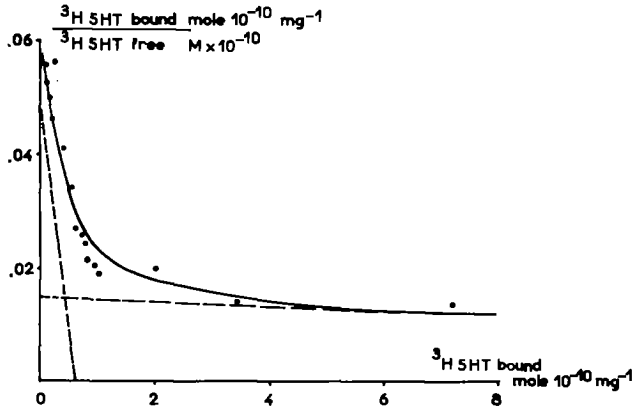

Fig. 1

Scatchard plot of ^{3}H-5HT Binding to Rat Brain Synaptic Membranes Membranes were treated as described in Methods and Material. All values are means of triplicate determinations.

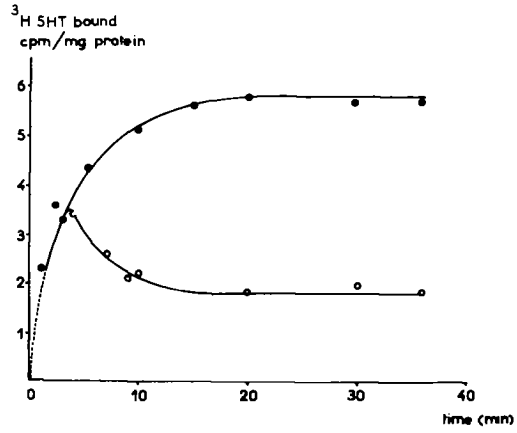

Fig. 2

Kinetics of 5HT Binding to Synaptic Membranes
- Incubation temperature : 0°C, ^{3}H-5HT concentration : 3.10^{-9} M.
- The upper curve (o—o) shows total ^{3}H-5HT binding
- The lower curve (o—o) shows the displacement of bound ^{3}H-5HT after the addition of non-radioactive 5HT (5.10^{-6} M) 3 minutes after the radioactive 5HT. Each point is the mean of triplicates.

a half-maximum close to 1.10^{-9} M (fig. 3) corresponding to the K_D previously obtained from the Scatchard plot for the high affinity component of the total binding. It is termed "5HT specific binding" for reasons which will be discussed later. On the other hand, 5HT non-displaceable binding is non-saturable as it increased linearly with the concentration of radioactive 5HT. Specific binding corresponds to 0.4 to 0.5 picomole of ^{3}H-5HT per mg protein.

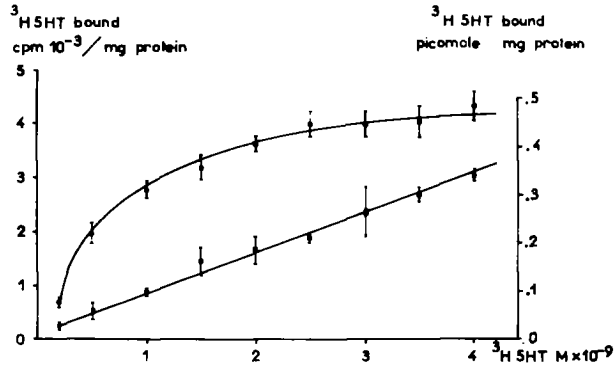

Fig. 3

Binding of ^{3}H-5HT to Synaptic Membranes from Rat Brain
Total binding corresponds to two components :
- a saturable, displaceable by non-radioactive 5HT (5.10^{-6} M) (●—●)
- a non-saturable, and non displaceable component (■ - - ■)
Each point represents the mean of three to six experiments each in triplicate. Vertical bars show the standard errors on the means.

A similar high affinity, saturable binding is observed with brain homogenates, with a K_D value of 1.5 ± 0.5 10^{-9} M (5 assays); however, this represents only 20 % of the total 5HT binding and corresponds to the fixation of 0.05 to 0.1 picomole of 5HT per mg protein.

Characterization of the bound radioactivity

Thin layer chromatography indicated that the bound radioactivity migrated for its majority (85 to 90 %) as 5HT in the different solvent systems used (Rf being respectively 47 and 45 in the first system, 62 and 63 in the second). Free radioactivity also migrated with the same Rf as 5HT (i.e. in the three solvent systems used, Rf for 5HT were respectively 18, 45 and 63 and corresponding Rf for free radioactivity 22, 46 and 63). The results were not affected by ascorbic acid. 5HiAA and bufotenine had quite different Rf (in the different systems they were respectively 74 - 72 - 80 and 53 - 61 - 48).

Stability of the binding

Specific binding to synaptic membranes increased linearly with the amount of protein within the dose range studied (0.5 to 1.5 mg per fraction). It is thermosensitive, being totally or almost totally (95 %) inhibited by pretreatment of the preparation at 90°C for 15 minutes prior 20 minutes incubation at 0°C. It is temperature-dependent ; it increases from 0 to 30°C (calculated Q_{10} is 2.1 - 3 assays), is stable between 30 and 40°C and decreases markedly at 50°C. With homogenates, temperature dependence is not the same, since specific binding increases only from 0 to 10°C, then decreases between 10 and 50°C. Specific binding to synaptic membranes is pH dependent ; it is optimal between pH 7.4 and 7.7, decreasing below pH 7.2 and above pH 8; non-specific binding increases progressively with pH (pH 6 to pH 9).

Effects of various drugs on the specific binding

The abilities of various drugs to decrease 5HT binding are illustrated in Table I. All tryptamines or structurally related compounds (LSD and dihydroergotamine) are effective in displacing bound ^{3}H-5HT. 5HT itself is the most efficient, with an ID_{50} of 1.10^{-8} M. Methoxytryptamine, tryptamine, dimethyltryptamine are 5 to 50 times less potent; d-LSD is about 40 times less efficient,

Table I

Effect of Drugs on ^{3}H-5HT Binding to Synaptic Membranes

Drugs	DI 50 %
5HT	1.10^{-8} M
Methoxytryptamine	4.10^{-8}
Tryptamine	2.10^{-7}
Dimethyltryptamine	5.10^{-7}
L S D	4.10^{-7}
Dihydroergotamine	10^{-6}
inactive at 10^{-6} M	
Cinanserine	Imipramine
5 hydroxy indole acetic acid	Chlorpromazine
inactive at 10^{-5} M	
Noradrenaline	Octopamine
Dopamine	Tetrahydroisoquinoline

3 to 6 concentrations of each drug were added for 20 minutes to synaptic membrane preparations (triplicates) preincubated for 20 minutes with ^{3}H-5HT (4.10^{-9} M).
Percentage inhibition of 5HT-specific binding relative to control were plotted on log-probit paper as a function of drug concentration.

the ID_{50} being 4.10^{-7} M. Surprisingly, low concentrations of LSD (10^{-8} M to 10^{-7} M) increase ^{3}H-5HT binding by 20 to 30 %. Other biogenic amines, such as noradrenaline, dopamine and octopamine or dopamine condensation metabolites (the tetrahydroisoquinoline :

salsolinol) are also inactive at 10^{-5} M and substances known to interfere with aminergic systems - imipramine, chlorpromazine or antiserotonin (cinanserine) are totally ineffective at 10^{-6} M (table I).

Regional distribution of specific sites

Binding has been assayed on crude mitochondrial fractions originating from various regions of the brain or from a peripheral organ (kidney).

The results show that the distribution is not random but varies with the area of the brain. The highest binding values were obtained for striatum and the values decreased in the following order : hippocampus, diencephalon, cortex, raphe, cerebellum, kidney (fig. 4).

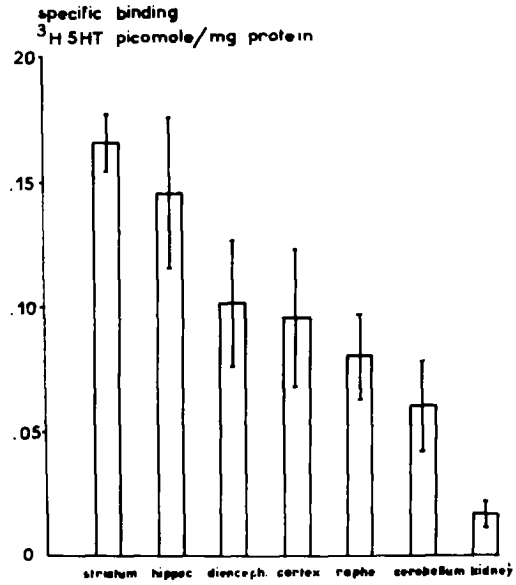

Fig. 4

Distribution of ^{3}H-5HT Specific Binding in Rat Brain
5HT-specific binding capacity of homogenates (crude mitochondrial fraction) of various brain regions and kidney have been tested at a ^{3}H-5HT concentration of 4.10^{-9} M.
Each column represents the mean of 3 to 4 experiments each in triplicates, bars show standard errors.

For homogenates originating from the whole brain, the percentage of specific to total binding was lower (20 to 30 %) than in the case of synaptic membranes (60 to 80 %). It also varied amongst the various brain areas, i.e. 48 % in the striatum, 40 % in cortex, 33 % in raphe : in kidney it was only 12 %.

Discussion

Our results demonstrate the existence of a saturable, high affinity, specific binding of 5-hydroxytryptamine in rat brain synaptic plasma membranes. The half saturation concentration is close to 1.10^{-9} M, equivalent to the K_D ($1.5\ 10^{-9}$ M) obtained from a Scatchard plot for the high affinity component of the 5HT total binding.

This high affinity binding has been clearly distinguished from the non-displaceable, non-saturable 5HT binding which, in the same concentration range, progresses linearly. Bound radioactivity had the same Rf as 5HT in the different solvent systems. The specificity was established by the fact that tryptamines were found to be very effective displacers of bound ^{3}H-5HT, the ID_{50} being 1.10^{-8} M for non-radioactive 5HT, 4.10^{-8} M for methoxytryptamine, 2.10^{-7} M for tryptamine, and 5.10^{-7} M for dimethyltryptamine, whereas 5-hydroxyindole acetic acid is ineffective at 10^{-6} M and noradrenaline, dopamine, octopamine and tetrahydroisoquinoline at 10^{-5} M.

Such saturable, specific, high affinity binding is also observed using rat brain homogenates (crude mitochondrial fraction). The temperature dependence is different from that found with synaptic membranes; for the latter, binding increases from 0 to 30°C, whereas for homogenates it decreases between 10 and 50°C. The fact that the content of degradative enzymes which can be actived by increasing temperature is much higher in the homogenate than in the synaptic membrane preparation might partly explain this difference. On the other hand, the binding capacity expressed in picomoles 5HT per mg protein is lower for the whole brain homogenate than for synaptic membranes (0.05 to 0.1 and 0.4 to 0.5 respectively); similarly, the percentage of specific to total binding varied from 20 to 30 % in homogenates to 60 to 80 % in synaptic membranes. Thus, an increase in the specific binding capacity of the preparation was observed in parallel with the purification of synaptic membranes; this fact tends to support the view that specific binding sites are located on synaptic membranes. Moreover, the distribution of these specific sites suggests that they might be involved in serotonergic function, since it is similar to that described for serotonergic nerve endings or 5HT content (10-11-12): the values obtained for 5HT concentrations and for specific binding capacity are highest in striatum (0.4 to 0.7 µg 5HT per g tissue (10-12) and 0.165 ± 0.011 picomole 5HT per mg protein respectively) and lowest in cerebellum (0.07 to 0.25 µg 5HT per g (11-12) and 0.059 ± 0.017 picomole per mg protein respectively); they are intermediate in hippocampus, diencephalon, cortex and raphe (0.3 to 0.6 µg/g and 0.08 to 0.14 picomole/mg protein respectively). This indicates that specific binding is probably related to the serotonergic input in the different brain areas studied.

Some similarities exist between the 5HT-specific binding described here and the high affinity saturable binding reported for ^{3}H-LSD by Farrow and van Vunakis (13) using synaptosomes and by Benett and Aghajanian (14) using rat brain homogenates and, in some experiments, synaptosomes. The affinities are of the same order (9.10^{-9} M and 4.10^{-9} M respectively), bound LSD can be displaced by 5HT, other tryptamines and d-LSD but noradrenaline and

dopamine have little or no effect. The distribution of the corresponding sites is similar - i.e. maximal binding is observed in striatum and decreases in the order : cortex, hippocampus, diencephalon, raphe, cerebellum and kidney.

According to a current view (15-16-17-18-19-20), Benett and Aghajanian suggested that LSD binding sites might correspond to 5HT-specific receptors. However, there are some differences between the results of these authors and our own, which might indicate that the two populations of binding sites are not quite identical. We found a definite 5HT-binding capacity in the raphe, while Benett and Aghajanian found a very weak d-LSD specific binding. This would indicate that this region contains 5HT receptors which do not bind LSD, possibly because they are presynaptic, as suggested by Benett and Aghajanian. This might also be true for hippocampus. Moreover, in our experiments 5HT was more effective than LSD in displacing ^{3}H-5HT, whereas in Benett and Aghajanian's studies, LSD was more effective than 5HT in displacing LSD. This might also argue in the same direction. However, differences in experimental conditions, have to be taken in account - those used by Benett and Aghajanian (homogenates and an incubation temperature of 37°C) are more favorable for metabolic modifications (i.e. inactivation of 5HT or eventual activation of LSD or other drugs) than ours (synaptic membranes and an incubation temperature of 0°C).

Differences in experimental conditions might also be taken in account for the fact that d-LSD was approximately 40 times less potent in displacing bound 5HT in our experiments than in those of Snyder and Bennett (3) the dissociation constant for 5HT binding being approximately 10 times higher.

In conclusion, our results demonstrate the existence of a high affinity binding, specific for 5HT, saturable, 5HT displaceable ; it is not randomly distributed within the brain, but seems related to the serotonergic input in the area considered. Thus the characteristics of these binding sites fulfil certain criteria for 5HT receptor sites and, whilst not definitely identifying them as such, at least provide some arguments in favour of such an hypothesis.

References

1. R.M. MARCHBANKS, Biochem. Pharmacol. 16, 1971-1979 (1967)
2. S. FISZER and E. de ROBERTIS, J. Neurochem. 16, 1201-1209 (1969)
3. S. SNYDER and J.P. BENNETT, in Pre and Postsynaptic Receptors eds E. URDIN and W.E. BUNNEY, Jr, Debber, N.Y. (1975)
4. C.W. COTMAN and D.A. MATTHEWS, Biochim. Biophys. Acta. 249, 380-394 (1971)
5. V.P. WHITTAKER, Progr. Biophys. Mol. Biol. 15, 39-96 (1965)
6. J. GLOWINSKI and L.L. IVERSEN, J. Neurochem. 13, 655-669 (1966)
7. C.B. PERT and S.H. SNYDER, Science 179, 1011-1014 (1973)
8. O.H. LOWRY, H.J. ROSEBROUGH, A.L. FARR and R.J. RANDALL, J. Biol. Chem. 193, 265-275 (1971)
9. G. SCATCHARD, Ann. N.Y. Acad. Sci. 51, 660 (1949)
10. K. FUXE, Acta phys. Scand. 64, suppl. 247, 39-85 (1965)
11. N.E. ANDEN, Europ. J. Pharmacol. 7, 7 (1967)

12. P.J. KNOTT, C.A. MARSDEN and G. CURZON, Advances in Biochem. Psychopharmacol. 11, 109-114 (1974)
13. J.T. FARROW and H. van VUNAKIS, Nature 237, 164-165 (1972)
14. J.L. BENETT and G.K. AGHAJANIAN, Life Sci. 15, 1935-1944 (1974)
15. J.H. GADDUM, Ann. N.Y. Acad. Sci. 66, 643-647 (1957)
16. N.J. GIARMAN and D.W. FREEDMAN, Pharmac. Rev. 17, 1-26 (1965)
17. C. CHOTHIA and P. PAULING, Proc. Nat. Acad. Sci. USA 63, 1063-1070 (1969)
18. S. KANG and J.P. GREEN, Proc. Nat. Acad. Sci. USA 67, 62-67 (1970)
19. R.W. BAKER, C. CHOTHIA, P. PAULING and H.P. WEBER, Mol. Pharmacol. 9, 23-32 (1973)
20. M.J. BERRIDJE and W.T. PRINCE, Brit. J. Pharmacol. 51, 269-278 (1974)