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IN SITU MOLECULAR SIZES OF THE VARIOUS TYPES OF 5-HT BINDING SITES IN THE RAT BRAIN

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Abstract—The radiation inactivation technique has been used to estimate the molecular weights of 5-HT binding sites in various regions of the rat brain. Using ^{3}H-5-HT or ^{3}H-8-OH-DPAT as the ligand, the same molecular weight of 55,000–60,000 daltons was calculated for the postsynaptic 5-HT_{1A} and 5-HT_{1B} sites in the hippocampus and cerebral cortex. Studies with ^{3}H-ketanserin as the selective ligand indicated a molecular weight in the same range for the post-synaptic 5-HT_2 binding site in the cerebral cortex. In contrast, a higher value (67,000 daltons) was found for the presynaptic 5-HT_3 site selectively labelled by ^{3}H-8-OH-DPAT in the striatum and cerebral cortex.

The curvilinear pattern of the radiation-induced inactivation of 5-HT_{1A} and 5-HT_{1B} binding sites suggested that both sites belong to complex polymeric structures. In contrast, the 5-HT_2 and 5-HT_3 sites may correspond to less cooperative structures since simple monoexponential inactivation curves were observed upon irradiation.

Several subtypes of postsynaptic serotonin (5-HT) binding sites seem to exist in mammalian brain (see [1] for review). Originally, it was thought that only two subtypes 5-HT_1 and 5-HT_2 characterized respectively by a high (nanomolar) and a low (micromolar) affinity for 5-HT and related agonists are present in the CNS [2]. However, recent investigations made this scheme no longer valid since evidence has been reported for the existence of possibly three different subtypes of 5-HT_1 sites [3–8]. According to Pedigo *et al.* [5], two of these subtypes called 5-HT_{1A} and 5-HT_{1B} can be easily distinguished by their respective affinity for the butyrophenone antagonist, spiperone: the 5-HT_{1A} subtype having an nM affinity for spiperone whereas the 5-HT_{1B} subtype is only poorly recognized by this drug (IC_{50} in the μM range). Further support to this classification appeared recently with reports demonstrating that specific 5-HT agonists acting selectively on 5-HT_{1A} or 5-HT_{1B} subtype can be identified on the basis of appropriate *in vitro* and *in vivo* tests [3, 9–12].

In addition to these postsynaptic sites, evidence has been provided recently for the presence of 5-HT binding sites onto serotoninergic terminals [13]. This third category of binding sites has been called 5-HT_3 [14] and is characterized also by an nM affinity for 5-HT and related agonists [14, 15]. The possible identity of these presynaptic 5-HT_3 sites with the presynaptic autoreceptors controlling 5-HT release is still the matter of debate [3, 14, 15, 16], as the pharmacological properties of 5-HT autoreceptors seem to correspond better with those of the 5-HT_{1B} subtype [16, 17]. However, recent autoradiographic investigations [18] have demonstrated that 5-HT_{1B} binding sites cannot be detected on presynaptic serotoninergic terminals.

So far, the present classification of 5-HT binding sites in the central nervous system is based on pharmacological distinctions, and no real proof of the existence of several receptors corresponding to these different binding sites has been provided yet. Studies on other neurotransmitter binding sites have shown that measurement of *in situ* molecular weights can give evidence opposing or supporting that distinct pharmacological subtypes correspond to separate receptor protein complexes in brain membranes [19–25]. This led us to select the same approach in the case of 5-HT binding sites. For this purpose, frozen tissues were submitted to high energy irradiation and the resulting inactivation of 5-HT_{1A}, 5-HT_{1B}, 5-HT_2 and 5-HT_3 binding sites was examined in three regions of the rat brain, the cerebral cortex, hippocampus and striatum.

MATERIALS AND METHODS

^{3}H-5-hydroxytryptamine (^{3}H-5-HT generally labelled, 10.5–13.5 Ci/mmol, Amersham International plc) was purified just before use for binding assays as described elsewhere [3].

^{3}H-8-hydroxy-2-(di-*n*-propylamino)tetralin (^{3}H-8-OH-DPAT, 105–125 Ci/mmol) was prepared by the Service des Molécules Marquées (CEA Saclay, France) as described previously [13].

^{3}H-ketanserin (65.8 Ci/mmol, New England Nuclear) was used directly on receipt for the selective labelling of 5-HT_2 sites [26].

Other drugs were: Spiperone (Janssen), methysergide (Sandoz), "cold" 8-OH-DPAT (CEA, Sandoz), "cold" 5-HT (Merck). Other compounds were of the purest grade available (Sigma, Merck, Prolabo).

Irradiation

Male Wistar rats weighing 200–250 g were killed

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by decapitation and their brains were dissected rapidly in the cold (4°) [27]. Isolated structures (cerebral cortex, hippocampi, striata) from each rat were then frozen (−20°) and exposed to highly accelerated (10 MeV) electrons from a linear accelerator (Risø, Denmark) [20]. The samples were kept frozen (−10–15°) during irradiation which was delivered in doses of 0.5–2 Mrad. The dose of radiation was determined using a calibrated thermodosimeter (water).

Calibration

Molecular weights were calculated by comparison with the decay rate of the activity of enzymes of known molecular weights (the enzymes were either present in or added to the frozen tissue sample). The radiation inactivation of enzymes or receptors follows the decay curve: $A = A_0 \times e^{-k.D}$, where A is the activity after the dose D Mrad, A_0 is the activity in the non-irradiated sample and k is the radiation inactivation constant [28]. The logarithmic transcription of the decay curve ($\ln A = -k.D + \ln A_0$) estimates k $Mrad^{-1}$ as the slope of the line in a plot of ln A versus D.

The radiation inactivation constant was estimated for (MW in daltons): yeast alcohol dehydrogenase (148,000, $k = 0.202$), *E. coli* beta-galactosidase (116,248, $k = 0.157$), pyruvate kinase (114,000, $k = 0.152$), horse liver alcohol dehydrogenase (72,000, $k = 0.095$), lactate dehydrogenase (70,000, $k = 0.108$), choline esterase (70,000, $k = 0.087$) and glutamic acid decarboxylase (67,000, $k = 0.098$). A mean calibration factor of $K = 730{,}000$ daltons × Mrad was determined from the expression $K = MW/k$ applied to all the enzymes studied. The K value of 730,000 daltons × Mrad is closely similar to a value calculated from the data of Kempner and Haigler [29]: 640,000 × 1.2 = 768,000 daltons × Mrad (1.2 is the temperature factor at −10° [29]). Apparent molecular weights in the present study were calculated according to MW = 730,000 × k daltons (see also ref. 23).

Preparation of membranes

Frozen irradiated tissues were homogenized in 40 vol. (w/v) of ice-cold 50 mM Tris–HCl (pH 7.4 at 23°) using a Polytron disrupter (PT10 OD) and centrifuged at 40,000 *g* for 20 min. The supernatant was discarded and the pellet was washed twice by resuspension in 40 vol. of Tris–HCl followed by centrifugation. The resulting pellet was homogenized in 40 vol. of Tris–HCl and incubated at 37° for 10 min to remove endogenous 5-HT [30]. Membranes were then collected by centrifugation and washed twice more before final resuspension in 10 vol. of 50 mM Tris–HCl, pH 7.4. Binding assays were performed using 50 μl aliquots of this suspension (equivalent to 0.25–0.30 mg protein).

Binding assays

^{3}H-5-*HT*. For the measurement of ^{3}H-5-HT binding to 5-$HT_{1(A+B)}$ sites, membranes were incubated under optimal conditions for the specific labelling of postsynaptic receptors [15, 31], i.e. in 0.5 ml of 50 mM Tris–HCl, pH 8.2, containing 1 mM $MnCl_2$ and various concentrations of the labelled ligand (2.0–12.3 nM). Incubations proceeded for 30 min at 23° before the addition of 3.5 ml of ice-cold Tris–HCl buffer and filtration through Whatman GF/B filters under vacuum. Filters were then rinsed twice with 3.5 ml of the same buffer, dried and finally vigorously shaken in scintillating vials containing 10 ml of Aquasol® (New England Nuclear) for radioactivity counting. Non-specific binding was defined as that persisting in similar samples supplemented with an excess (10 μM) of "cold" 5-HT.

The specific binding to 5-HT_{1A} subsites was determined as the difference between total ^{3}H-5-HT binding minus that persisting in the presence of 1 μM spiperone or 0.3 μM 8-OH-DPAT. Previous data have shown that either drug at these selected concentrations completely displaces ^{3}H-5-HT bound to 5-HT_{1A} subsites without affecting that bound to 5-HT_{1B} subsites [3, 32]. Therefore, the specific binding of ^{3}H-5-HT to 5-HT_{1B} subsites was defined as the difference between the radioactivity bound in the presence of 1 μM spiperone or 0.3 μM 8-OH-DPAT minus that persisting in the presence of 10 μM 5-HT.

^{3}H-8-*OH-DPAT*. The specific binding of ^{3}H-8-OH-DPAT to postsynaptic 5-HT_{1A} sites was carried out as described for ^{3}H-5-HT (see above, and [15]). For the measurement of ^{3}H-8-OH-DPAT binding to presynaptic 5-HT_3 sites [14], membranes were suspended in 0.5 ml of 50 mM Tris–HCl, pH 7.4, containing 1 mM *N*-ethyl-maleimide (NEM, Sigma) to inactivate postsynaptic sites [31], and various concentrations of the labelled ligand (1.0–21.7 nM). Samples were incubated for 10 min at 37°, and then diluted with 3.5 ml of ice-cold Tris–HCl buffer before being filtered through GF/B filters. After washing (see above), filters were dried and their radioactivity counted. Non-specific binding was considered to be that persisting in similar samples incubated in the presence of 10 μM "cold" 5-HT.

^{3}H-*ketanserin*. Aliquots (50 μl) of the membrane suspensions were incubated with ^{3}H-ketanserin (0.1–4.4 nM) at 25° for 30 min in 50 mM Tris–HCl, pH 7.4 (final volume = 1 ml). Assays were stopped by filtering samples through Whatman GF/C filters. The washing and counting procedures were as described for ^{3}H-5-HT and ^{3}H-8-OH-DPAT (see above). Non-specific binding was obtained by adding methysergide (10 μM) to separate samples.

Triplicate determinations were made for each binding assay condition.

Proteins were determined using the Folin phenol procedure [33] and bovine serum albumin (Sigma) as the standard.

Calculations

Slopes of the curves obtained by plotting the specific binding (in ln (cpm/mg membrane protein)) to a given 5-HT site as a function of the radiation dose (in Mrad) were calculated by linear regression analysis. Molecular weights were then derived according to the formula: MW = 730,000 × k daltons, as explained above. Means ± S.E.M. of a given value were calculated from estimations made in n individual experiments.

Data from saturation studies (Scatchard) were analysed by non-linear computer-assisted curve fitting ("Hypmic" [34]). Results were subjected to multigroup analysis of variance (ANOVA) to deter-

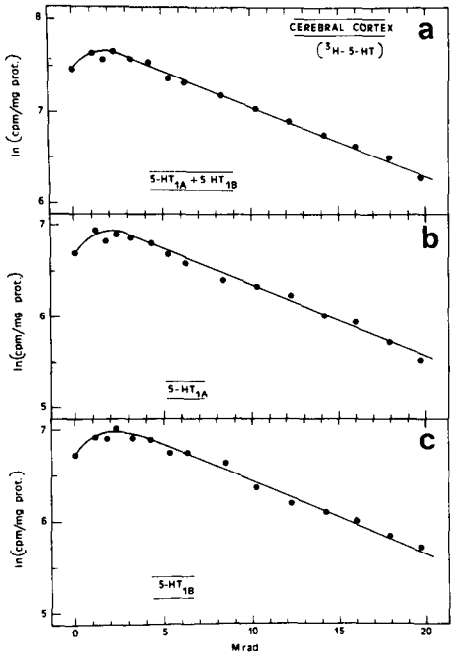

Fig. 1. Radiation inactivation of specific ^{3}H-5-HT binding to 5-HT$_{1(A+B)}$, 5-HT$_{1A}$ and 5-HT$_{1B}$ sites in cortical membranes. Frozen cerebral cortices were irradiated by high energy electrons (10 MeV) as described in Materials and Methods. Binding assays were carried out in 50 mM Tris–HCl, pH 8.2, containing 1 mM $MnCl_2$ and 2.0 nM ^{3}H-5-HT. Non specific binding was determined with 10 μM 5-HT (a) or 0.3 μM 8-OH-DPAT (b) for estimating ^{3}H-5-HT binding to 5-HT$_{1(A+B)}$ or 5-HT$_{1A}$ sites respectively. The specific binding to 5-HT$_{1B}$ sites (c) corresponded to ^{3}H-5-HT still bound in the presence of 0.3 μM 8-OH-DPAT but displaced by 10 μM 5-HT. Each point is the mean of triplicate determinations in a typical experiment. Similar results were obtained in 5 independent experiments. Ordinate: ^{3}H-5-HT specifically bound in ln(cpm/mg membrane prot.); abscissa: dose of radiation in Mrad.

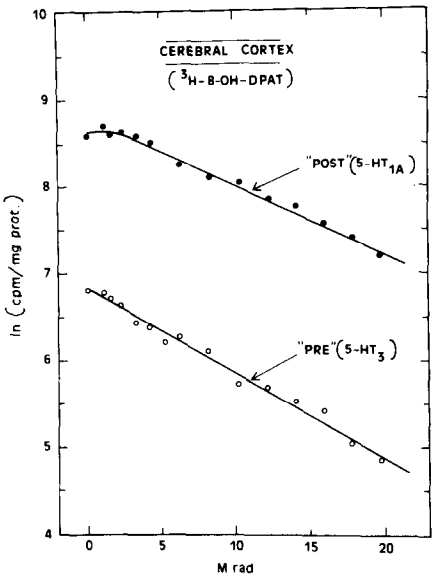

Fig. 2. Radiation inactivation of specific ^{3}H-8-OH-DPAT binding to postsynaptic 5-HT$_{1A}$ and presynaptic 5-HT$_3$ sites in cortical membranes. Binding assays were carried out with 1.0 nM ^{3}H-8-OH-DPAT under the following conditions: ●, 5-HT$_{1A}$: 50 mM Tris–HCl, pH 8.2, containing 1 mM $MnCl_2$; 30 min at 23°; ○, 5-HT$_3$: 50 mM Tris–HCl, pH 7.4, containing 1 mM NEM; 10 min at 37°. Each point is the mean of triplicate determinations of specifically bound ^{3}H-8-OH-DPAT in ln(cpm/mg membrane prot.). This experiment has been reproduced 5 times (see Table 2).

mine if group data were significantly different. Where mean values were different ($P < 0.05$), group data were compared to respective control values using Dunnett's test [35].

RESULTS

Radiation inactivation of 5-HT *binding sites in brain membranes*

As illustrated in Fig. 1, irradiation of the cerebral cortex exerted a biphasic effect on ^{3}H-5-HT binding to 5-HT$_{1A}$ and/or 5-HT$_{1B}$ sites. Low doses ($\leq$ 3 Mrad) produced a slight enhancement of ^{3}H-5-HT binding to each subsite (+30–35%) whereas a dose-dependent reduction occurred for higher doses. For this second portion of the curves, a close parallelism was noted between the inactivation of 5-HT$_{1(A+B)}$, 5-HT$_{1A}$ and 5-HT$_{1B}$ sites (Fig. 1).

As for ^{3}H-5-HT as the ligand, tissue irradiation affected ^{3}H-8-OH-DPAT binding to postsynaptic 5-HT$_{1A}$ subsites in a complex manner. Low doses of radiations ($\leq$3 Mrad) did not alter or even slightly enhanced (+15–20%) ^{3}H-8-OH-DPAT binding, whereas a progressive decrease was observed for higher doses (Fig. 2). In contrast, a clear monophasic effect was noted when assay conditions were chosen for the selective labelling of presynaptic 5-HT$_3$ sites (Fig. 2). Furthermore, the slope of the curve obtained by plotting the specific binding versus the dose of radiations (in Mrad) was regularly steeper in the case of presynaptic 5-HT$_3$ sites compared to postsynaptic 5-HT$_{1A}$ sites (Fig. 2).

As noted for 5-HT$_3$ sites, a monophasic decay of ^{3}H-ketanserin binding to 5-HT$_2$ sites was observed in cortical membranes submitted to increasing doses of radiations (Fig. 3). However, the slope of the

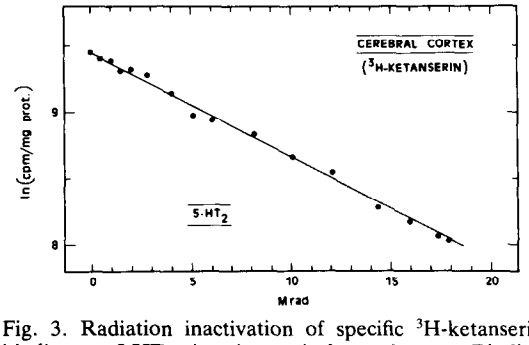

Fig. 3. Radiation inactivation of specific ^{3}H-ketanserin binding to 5-HT$_2$ sites in cortical membranes. Binding assays were carried out with 1.0 nM ^{3}H-ketanserin, and 10 μM methysergide was used for the determination of non-specific binding. Each point is the mean of triplicate determinations in a typical experiment. Similar results were obtained in 3 independent experiments. Ordinate: ^{3}H-ketanserin specifically bound in ln(cpm/mg membrane prot.); abscissa: dose of radiation in Mrad.

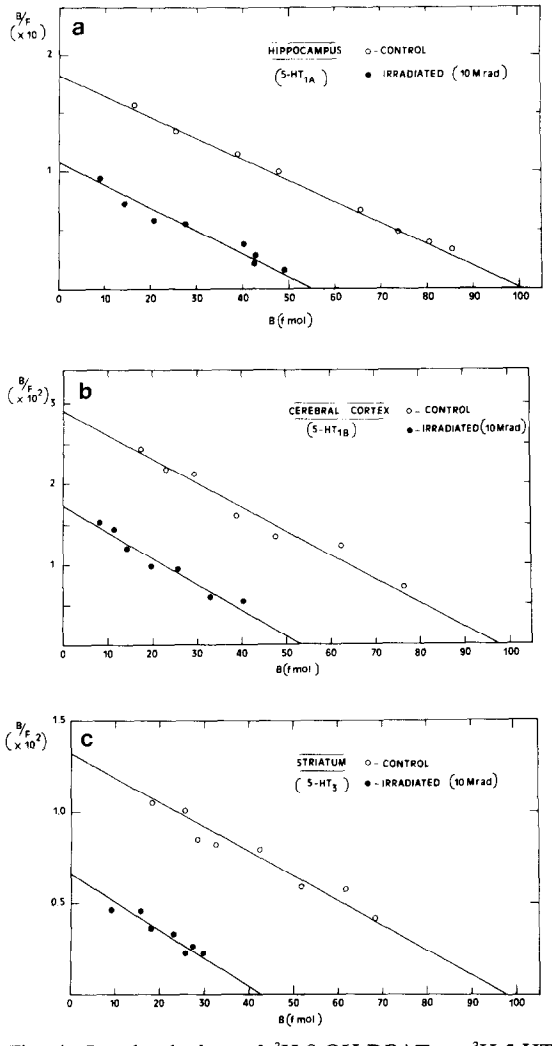

Fig. 4. Scatchard plots of ^{3}H-8-OH-DPAT or ^{3}H-5-HT binding to various 5-HT sites in membranes from control or irradiated tissues. Binding assays were performed on membranes prepared from non-irradiated tissues (○) or from those exposed to 10 Mrad (●). (a) ^{3}H-8-OH-DPAT binding to 5-HT$_{1A}$ sites in hippocampal membranes: assays were performed with 8 different concentrations of the labelled ligand (0.20–5.37 nM). B: ^{3}H-8-OH-DPAT (in fmol.) specifically bound per sample (corresponding to 0.27 mg membrane prot.); F: unbound ^{3}H-ligand (in fmol.). (b) ^{3}H-5-HT binding to 5-HT$_{1B}$ sites in cortical membranes: assays were performed in triplicate with 7 different concentrations of ^{3}H-5-HT (2.4–12.3 nM). B: ^{3}H-5-HT (in fmol.) specifically bound per sample (corresponding to 0.30 mg membrane prot.). (c) ^{3}H-8-OH-DPAT binding to 5-HT$_3$ sites in striatal membranes: assays were performed in triplicate with ^{3}H-8-OH-DPAT concentrations ranging between 3.4 nM and 21.7 nM. B: ^{3}H-8-OH-DPAT (in fmol.) specifically bound per sample (corresponding to 0.31 mg membrane prot.).

curve obtained by plotting the specific binding versus the dose of radiations was much closer ($k = 0.078$) to that found for 5-HT$_1$ ($k = 0.079$) than for 5-HT$_3$ sites ($k = 0.092$).

Scatchard plots of ^{3}H-5-HT, ^{3}H-8-OH-DPAT or ^{3}H-ketanserin binding to the various 5-HT binding classes presently studied indicated that radiation-induced inactivation was associated with a marked decrease in the B_{max} with no significant alteration in the K_d. A typical example of the changes produced by exposure to 10 Mrad is given in Fig. 3 for 5-HT$_{1A}$ sites in the hippocampus (Fig. 4A), 5-HT$_{1B}$ sites in the cerebral cortex (Fig. 4B) and 5-HT$_3$ sites in the striatum (Fig. 4C). In all cases, the loss of specific binding sites due to irradiation approximated 50%, as observed for 5-HT$_2$ sites in the cerebral cortex (Table 1).

Table 1 recapitulates the characteristics of ^{3}H-5-HT, ^{3}H-8-OH-DPAT and ^{3}H-ketanserin binding to membranes from control or irradiated tissues. In particular, B_{max} values for 5-HT$_{1A}$ sites in control as well as in irradiated samples were not significantly different whether the specific binding was assayed with ^{3}H-5-HT or ^{3}H-8-OH-DPAT, therefore confirming that the latter ligand is specific of this subclass of sites [3]. Indeed, the affinity of 5-HT$_{1A}$ subsites for ^{3}H-8-OH-DPAT was even regularly (3–5 times) higher than for ^{3}H-5-HT (Table 1). However, a much lower affinity for this ligand was noted in the case of presynaptic 5-HT$_3$ sites (Table 1).

Estimation of in situ *molecular weights of* 5-HT *binding sites in the rat brain*

From series of experiments similar to those illustrated in Figs. 1–3, we could calculate the molecular weights of the various classes of 5-HT binding sites in the hippocampus, cerebral cortex and striatum (Table 2). As expected for the specific labelling of the same class of binding sites, i.e. 5-HT$_{1A}$, similar molecular weights of 55,000–60,000 daltons were obtained when ^{3}H-8-OH-DPAT or ^{3}H-5-HT was used as the ligand, and 10 μM 5-HT or 0.3 μM 8-OH-DPAT was used to determine non-specific binding respectively. This MW was in the same range as that found for the 5-HT$_{1B}$ and 5-HT$_2$ sites, but was regularly lower (−10–23%) than that found for the presynaptic 5-HT$_3$ site labelled with ^{3}H-8-OH-DPAT in the striatum and cerebral cortex: 67,000 daltons (Table 2). However, this difference was small and barely reached the critical level of significance ($P = 0.05$).

DISCUSSION

The radiation inactivation technique offers the great advantage of allowing the determination of the molecular weights of proteins in *intact* frozen tissues, i.e. without any of the purification steps required for using the conventional biochemical techniques [28]. In the case of membrane bound receptors, biochemical methods are often extremely difficult since their application first involves the solubilisation of membrane proteins, a procedure which generally results in low yield due to the destruction of the functional properties of proteins out of their natural environment. These difficulties largely explain why the radiation inactivation technique has been frequently selected for the determination of the molecular weights of neurotransmitter receptors in the CNS (references in the Introduction).

The present application of the radiation inactivation technique to the study of 5-HT binding sites

Table 1. Effects of high energy radiation inactivation on the characteristics of 5-HT binding sites in various regions of the rat brain

5-HT binding sites (ligand)	Structure		Control	Irradiated
	Postsynaptic sites			
5-HT_{1A}				
(^{3}H-8-OH-DPAT)	Hippocampus	K_d	0.91 ± 0.13	1.06 ± 0.07
		B_{max}	379.8 ± 31.4	192.6* ± 9.7
(^{3}H-8-OH-DPAT)	Cerebral cortex	K_d	1.72 ± 0.26	1.32 ± 0.20
		B_{max}	134.4 ± 13.0	67.6* ± 5.5
(^{3}H-5-HT)	Cerebral cortex	K_d	5.43 ± 1.48	6.17 ± 0.87
		B_{max}	162.1 ± 18.2	79.9* ± 6.4
5-HT_{1B}				
(^{3}H-5-HT)	Cerebral cortex	K_d	6.32 ± 1.05	5.89 ± 1.27
		B_{max}	353.4 ± 38.2	191.9* ± 24.1
5-HT_2				
(^{3}H-ketanserin)	Cerebral cortex	K_d	1.06 ± 0.19	0.97 ± 0.06
		B_{max}	371.6 ± 19.9	182.1* ± 10.8
	Presynaptic sites			
5-HT_3				
(^{3}H-8-OH-DPAT)	Striatum	K_d	14.2 ± 3.1	11.3 ± 1.4
		B_{max}	365.8 ± 42.3	147.8* ± 9.3
(^{3}H-8-OH-DPAT)	Cerebral cortex	K_d	17.3 ± 4.1	15.6 ± 2.8
		B_{max}	370.3 ± 18.9	166.9* ± 12.8

Radiation of 10 Mrad was applied to frozen tissues as described in "Materials and Methods". Binding assays were performed using at least 7 different concentrations of the labelled ligand for each condition. In the case of ^{3}H-8-OH-DPAT binding to 5-HT_{1A} or 5-HT_3 sites, non-specific binding was defined as that persisting in the presence of 10 μM 5-HT. Using ^{3}H-5-HT as the ligand, the specific binding to 5-HT_{1A} sites corresponded to the difference of total binding minus that found in the presence of 0.3 μM 8-OH-DPAT or 1 μM spiperone. ^{3}H-5-HT binding to 5-HT_{1B} sites consisted of that found in the presence of 0.3 μM 8-OH-DPAT (or 1 μM spiperone) minus that persisting when 10 μM 5-HT was added to the assay mixture. Using ^{3}H-ketanserin for labelling 5-HT_2 sites, non-specific binding was defined as that persisting in the presence of 10 μM methysergide.

Each value of K_d (in nM) or B_{max} (in fmol./mg protein) is the mean ± S.E.M. of at least 3 separate determinations. *$P < 0.05$ when compared to respective control values.

indicated that the molecular weights of the functional subunits with 5-HT binding capacity approximate 60,000 daltons for the so-called 5-HT_{1A}, 5-HT_{1B}, 5-HT_2 and 5-HT_3 sites in the CNS. Comparison with values already reported using classical biochemical approaches reveals some coincidence. Thus Van den Berg *et al.* [36] calculated a MW of 58,000 daltons for the 5-HT_1 binding site solubilised from bovine cortical membranes and partially purified by Sephacryl S300 chromatography and glycerol gradient sedimentation. SDS-gel electrophoresis of proteins irreversibly labelled by ^{3}H-5-HT [37] or a photosensitive derivative of 8-OH-DPAT (^{3}H-8-methoxy-nitroazidophenyl-PAT [38]) in brain membranes exposed to u.v. light also gave a molecular weight around 60,000 daltons for the band with 5-HT binding capacity similar to that of postsynaptic 5-HT_1 sites. However, using another photosensitive molecule, ^{3}H-nitroazidophenyl-5-HT, Cheng and Shih [39] described the irreversible labelling of three distinct membrane proteins with different molecular weights: 80,000, 49,000 and 38,000 daltons. Indirect pharmacological observations led these authors to postulate that the 80,000 and 38,000 MW proteins correspond to postsynaptic receptors whereas the 49,000 MW protein is presynaptic [40]. Thus far, the possible relationships between these proteins and the high affinity 5-HT_{1A}, 5-HT_{1B} and 5-HT_3 sites have never been examined by Shih and coworkers.

In the case of cortical 5-HT_2 receptors, density gradient centrifugation of ^{3}H-ketanserin binding sites solubilized by cholamidopropyl-dimethylammonio-1-propane sulfonate (CHAPS) indicated a Svedberg coefficient of 5 S [41]. However, no obvious correspondence exists between this coefficient and the MW of a given protein, and the present estimate of 57,000 daltons cannot be compared yet with any value inferred from direct biochemical investigations. It can be emphasized however that Wouters *et al.* [42] have recently synthesized a photosensitive probe of 5-HT_2 receptors which should be of great help for MW determination using "classical" biochemical methods.

On the basis of the present determination of the

Table 2. *In situ* molecular weights of 5-HT binding sites in various regions of the rat brain

5-HT receptor binding type	N	Ligand	Structure	Molecular weight (daltons)
Postsynaptic sites				
5-$HT_{1(A+B)}$	5	^{3}H-5-HT	Cerebral cortex	59,400 ± 3500
	1	^{3}H-5-HT	Hippocampus	57,100
5-HT_{1A}	6	^{3}H-8-OH-DPAT	Cerebral cortex	59,900 ± 2700
	7	^{3}H-8-OH-DPAT	Hippocampus	58,400 ± 1600
	3	^{3}H-5-HT	Cerebral cortex	61,000 ± 4200
	1	^{3}H-5-HT	Hippocampus	53,000
5-HT_{1B}	5	^{3}H-5-HT	Cerebral cortex	52,100 ± 1500
	1	^{3}H-5-HT	Hippocampus	57,700
5-HT_2	3	^{3}H-ketanserin	Cerebral cortex	56,900 ± 2500
Presynaptic sites				
5-HT_3	5	^{3}H-8-OH-DPAT	Striatum	67,900 ± 3300
	5	^{3}H-8-OH-DPAT	Cerebral cortex	66,500 ± 2200

Radiation inactivation was performed on frozen tissues as described in "Materials and Methods". Using ^{3}H-5-HT as the labelled ligand, the distinction of 5-HT_{1A} and 5-HT_{1B} binding sites was made with 0.3 μM 8-OH-DPAT or 1 μM spiperone which completely occupies the 5-HT_{1A} sites but does not interact with the 5-HT_{1B} sites [29]. Each value is the mean ± S.E.M. of N independent determinations.

MW of the various 5-HT binding sites in the rat brain, it can be proposed that the 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 pharmacological types either correspond to only one protein or to distinct proteins with identical molecular weights. In the case of presynaptic 5-HT_3 sites, the protein involved was probably different since its *in situ* MW was 10–25% higher than those of other 5-HT sites. Previous investigations on the respective physicochemical properties of the different classes of high affinity 5-HT binding sites support this conclusion. Thus Hall *et al.* [15, 31] noted that the temperature and pH dependences are similar for the ligand binding to 5-HT_{1A} and 5-HT_{1B} subsites but strikingly different in the case of ^{3}H-8-OH-DAPT binding to the presynaptic 5-HT_3 sites. Furthermore, experiments with NEM revealed that SH groups play a critical role for the ligand binding to postsynaptic 5-HT_{1A} and 5-HT_{1B} subsites but not to presynaptic 5-HT_3 sites [31].

The difference concerning the respective molecular weights of 5-$HT_{1(A\text{ or }B)}$ and 5-HT_3 sites was, however, rather small and barely reach the critical level of significance. Further confirmation using appropriate chromatographic and electrophoretic techniques to identify membrane proteins selectively labelled by photosensitive probes will be necessary to validate this difference.

In addition to the slope differences suggesting distinct molecular weights as discussed above, the curves of irradiation-induced inactivation of 5-$HT_{1(A\text{ or }B)}$, 5-HT_2 and 5-HT_3 binding sites exhibited marked differences in the range of low doses of radiations. Indeed, with doses of 3 Mrad or less, ^{3}H-5-HT or ^{3}H-8-OH-DPAT binding to 5-$HT_{1(A\text{ or }B)}$ sites did not decrease but in fact slightly increased compared to that found with non-irradiated tissues. In contrast, ^{3}H-ketanserin binding to 5-HT_2 sites and ^{3}H-8-OH-DPAT binding to 5-HT_3 sites decreased already with such low doses, and the corresponding slope was similar to that obtained with a higher dose range.

Curvilinear radiation inactivations have already been observed for other receptor types, notably the insulin receptor [43] and the benzodiazepine site labelled by convulsant beta-carbolines [21]. In the present case, we have not investigated whether the enhanced 5-HT_{1A} and 5-HT_{1B} binding found after low doses of radiation ($\leq$ 3 Mrad) (Figs. 1 and 2) was caused by increase in binding affinity or increase in number of specific sites. However, since no significant change in K_D was found at 10 Mrad compared to non-irradiated samples (Table 1), the initial increase in binding observed at low doses of radiation might indicate that new ("hidden") binding sites appeared in membranes. As discussed previously for the insulin receptor [43] and the benzodiazepine binding site [21], the increased binding due to low radiation doses might result from the primary destruction of a high molecular weight component regulating (and in fact masking) a fraction of specific binding sites. Other evidence for such a high molecular weight component involved in the modulation of 5-HT_1 binding sites in brain membranes has not been reported yet. However, Heron *et al.* [44] and Shih and Ohsawa [45] have mentioned that alterations in membrane fluidity (by incorporation of cholesterol or stearic acid) can increase the density of ^{3}H-5-HT specific binding sites, and it can be speculated that this effect might result from the dissociation of a putative regulatory protein from 5-HT_1 binding sites. Further experiments will be necessary for testing this interesting possibility.

In contrast to that of 5-HT_1 binding sites, the organisation of 5-HT_2 and 5-HT_3 sites does not prob-

ably involve a high molecular weight complex since a simple monoexponential decay of respective binding capacities was only observed upon radiation inactivation. Alternatively, all 5-HT_2 and 5-HT_3 binding sites were already accessible to their respective ligands even in polymeric complex, and the selective radiation inactivation of (putative) high molecular weight component(s) of such complex would affect neither the affinity nor the number of these sites.

In conclusion, the radiation inactivation technique allowed not only the determination of *in situ* molecular weights of the various classes of 5-HT binding sites in brain but contributed to demonstrate further that these sites correspond to different molecular structures.

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Note added in proof: During submission of the present paper, Nishino and Tanaka (*Life Sci.* **37**, 1167, 1985) reported MW of 57,000 and 150,000 for 5-HT_1 and 5-HT_2 binding sites respectively in bovine brain membranes. Differences in their radiation inactivation procedure (high energy gamma ray focused on freeze-dried membranes) probably accounted for their higher MW estimate of 5-HT_2 sites than that presently reported.

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