0026-895X/96/030556-04$3.00/0

MOLECULAR PHARMACOLOGY, 49:556–559 (1996).

Are 5-Hydroxytryptamine$_7$ Receptors Involved in [^{3}H]5-Hydroxytryptamine Binding to 5-Hydroxytryptamine$_{1nonA\text{-}nonB}$ Receptors in Rat Hypothalamus?

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Received July 31, 1995; Accepted December 7, 1995

SUMMARY

We assayed [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) binding in rat hypothalamic membranes to confirm the possibility of measuring 5-HT$_7$ receptors. Binding was tested in the presence of 3 μM (±)-pindolol, a concentration higher than previously suggested for the same purpose (0.1 μM). This higher concentration was, however, needed to fully saturate 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors without interaction with 5-HT$_7$ receptors. Under these conditions, [^{3}H]5-HT binding could be further inhibited with methiothepin (used to determine nonspecific binding) and with 5-HT, with an IC$_{50}$ of 1.4 nM and a slope of 1. The inhibition curves of (±)-8-hydroxy-dipropylaminotetralin, ritanserin, and mianserin were shallow (slopes, 0.35–0.58) and could be better analyzed with the two-site model, indicating that the pindolol-insensitive [^{3}H]5-HT binding sites in rat hypothalamic membranes are heterogeneous. Although the IC$_{50}$ of the compounds tested suggests that one population of sites is actually associated with 5-HT$_7$ receptors, our data clearly indicate that this binding assay does not selectively label 5-HT$_7$ receptors in native tissues. These results challenge a previous report and suggest that the proposed down-regulation of 5-HT$_7$ receptors after fluoxetine treatment should be considered with caution. The development of more selective and sensitive binding assays will probably offer significant advantage.

A novel 5-HT receptor subtype, termed 5-HT$_7$, has recently been cloned from humans (1), guinea pigs (2), and rats (3, 4). It contains seven predicted transmembrane domains, is positively coupled to adenylate cyclase (1–3, 5), but is distinct from any previously described class of 5-HT receptors in its primary structure and pharmacology (1, 3, 4). For example, when expressed in mammalian cells, the rat 5-HT$_7$ receptor has high affinity for both 8-OH-DPAT (a 5-HT$_{1A}$ agonist; K_i = 35–52 nM) and ritanserin (a 5-HT$_{2A/C}$ antagonist; K_i = 15 nM). Northern blot analysis in the rat brain (3, 4) indicated the highest abundance of 5-HT$_7$ mRNA in the hypothalamic region, with high expression also in various limbic and cortical brain areas.

A binding assay has been proposed to measure 5-HT$_7$ receptors in rat hypothalamus (6). [^{3}H]5-HT was used as labeled ligand in the presence of 100 nM pindolol to mask 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors because this compound is inactive at 5-HT$_7$ receptors (1, 3). Pharmacological analysis of these binding sites showed a good correlation with the binding site described in cells transfected with the rat 5-HT$_7$ receptor (4), suggesting that 5-HT$_7$ could be measured in animal tissue. Interestingly, these receptors were down-regulated after chronic administration of fluoxetine (6), so they may have a functional role in the pathophysiology of some forms of depression.

However, two important pitfalls have not been considered that could invalidate the conclusions of Sleight *et al.* (6): (i) 100 nM pindolol seemed to be too low a concentration to completely mask 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors because the K_i of the active isomer is ~20 nM for both subtypes (7), and (ii) [^{3}H]5-HT could also label other 5-HT receptor subtypes, e.g., similar incubation conditions were used in autoradiographic studies to label 5-HT$_{2C}$ receptors (8).

The current study was designed to verify these points to validate the proposed use of [^{3}H]5-HT to label 5-HT$_7$ receptors in rat hypothalamus.

Materials and Methods

We used male CRL:CD(SD)BR rats (Charles River, Calco, CO, Italy) weighing ~150 g. Procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with Italian [1] and international[2] laws and policies.

This work was supported by Consiglio Nazionale delle Ricerche, Rome, Italy, Convenzione Psicofarmacologia.

[1] D.L. n. 116, G.U., suppl. 40, February 18, 1992.
[2] EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; United States National Institutes of Health "Guide for the Care and Use of Laboratory Animals," National Institutes of Health publication No. 85–23 (1985).

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-dipropylaminotetralin.

The preparation of rat hypothalamic membranes and the binding assay were conducted as described previously (6), except for the concentration of pindolol (see below).

The rats were killed by decapitation, and their hypothalami were rapidly dissected out and frozen at −20° until use. Hypothalami were homogenized in ~20 volumes of ice-cold 50 mM Tris·HCl buffer, pH 7.4, with the use of an Ultra Turrax TP-1810 homogenizer (2 × 20 sec) and centrifuged at 40,000 × *g* for 10 min at 4°. The pellet was suspended in fresh buffer and centrifuged as before. The pellet was resuspended in the same volume of fresh Tris·HCl buffer, incubated for 10 min at 37°, and centrifuged as before. The last pellet was resuspended in ~20 volumes of ice-cold buffer consisting of 50 mM Tris·HCl with 10 μM pargyline, 0.1% ascorbic acid, 4 mM $CaCl_2$, and 3 μM (±)-pindolol [this concentration, higher than that used previously (6), was chosen based on the concentration-effect curve shown in Results]. Aliquots of the suspension (4 mg of original wet tissue weight) were incubated with 1 nM [^{3}H]5-HT (specific activity, 25.4 Ci/mmol; NEN) in the absence or presence of different drug concentrations in final volumes of 1 ml. Nonspecific binding was determined with 10 μM methiothepin. After 120 min at 23°, the incubation was stopped by rapid filtration under vacuum through GF/B fiber filters, which were washed with ice-cold Tris·HCl buffer (three times in 4 ml), dried, and counted in 4 ml of Filter Count (Packard) in a liquid scintillation spectrometer with a counting efficiency of ~60%.

Inhibition curves were first analyzed with the use of nonlinear curve fitting according to the logistic equation (sigmoid curve) and with the use of the ALLFIT program (9). The data were fitted either without or with the slope constrained to 1: comparison of the two fittings with the use of an *F* test, according to the "extra-sum of squares" principle (9, 10), indicated whether the curves had a slope significantly <1. These curves were then analyzed according to the two-site model equation, with the use of the GraphPAD program (developed by H. Motulsky; ISI software).

5-HT was obtained from Fluka (Buchs, Switzerland); (±)-pindolol was obtained from Sigma Chemical Co. (St. Louis, MO); and 8-OH-DPAT, ritanserin, mianserin, and methiothepin were obtained from R.B.I. (Natick, MA).

Results

Fig. 1 shows the inhibition curves obtained with increasing concentrations of methiothepin and (±)-pindolol on [^{3}H]5-HT (1 nM) binding in rat hypothalamic membranes. Analysis of these curves indicated IC_{50} values of 82 ± 8 and 38 ± 4 nM for (±)-pindolol and methiothepin, respectively. Methiothepin maximally inhibited 76% of total binding (corresponding to a specific binding of 655 cpm/sample, 4.8 fmol/mg tissue), whereas (±)-pindolol (up to 10 μM) maximally inhibited 50% of total binding (428 cpm/sample). Thus, 26% of total [^{3}H]5-HT binding (227 cpm/sample) was methiothepin sensitive but pindolol insensitive.

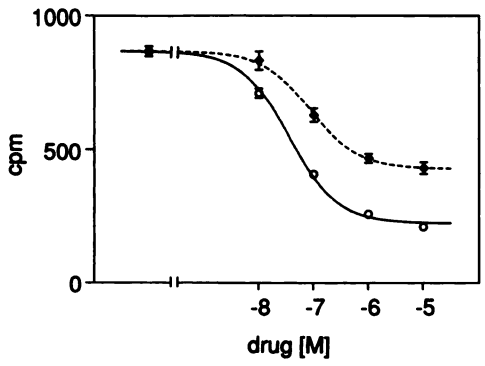

Fig. 1. Inhibition of [^{3}H]5-HT binding in rat hypothalamic membranes by methiothepin (○) or (±)-pindolol (♦). *Values*, mean ± standard deviation of the experiment performed in triplicate.

The inhibition curve of (±)-pindolol had a slope of 1.1 ± 0.1, and the plateau of maximal inhibition was obtained with concentrations of 1–10 μM. Because pindolol does not bind to 5-HT$_7$ receptors at concentrations of <10 μM (1), we decided to perform the following [^{3}H]5-HT binding assays in the presence of 3 μM (±)-pindolol. Under this condition, the total pindolol-insensitive binding was 419 ± 142 cpm/sample (mean ± standard deviation; four experiments), and nonspecific binding (obtained with 10 μM methiothepin) was 171 ± 39 cpm/sample. Specific binding to the pindolol-insensitive sites was 248 ± 112 cpm/sample, corresponding to 1.8 ± 0.8 fmol/mg tissue.

Fig. 2 and Table 1 report the results of experiments designed to achieve pharmacological characterization of these sites. The inhibition curve of 5-HT (Fig. 2A) had a slope of 1.1 ± 0.3 and an IC_{50} of 1.4 ± 0.2 nM. Fig. 2B shows the inhibition curve of 8-OH-DPAT, which was obtained by pooling the results of three experiments. Analysis of the single experiments with the use of the logistic function indicated a slope significantly <1 (0.41; Table 1), apparently due to a biphasic curve with an intermediate plateau between 0.3 and 10 μM 8-OH-DPAT. Fitting with the two-site model (Table 1) indicated 59% high affinity binding sites (IC_{50} = 104 nM) and 41% low affinity binding sites (IC_{50} = 35 μM). The inhibition curve of ritanserin (Fig. 2C) also appeared shallow (slope = 0.35) or even biphasic. In this case, fitting with the two-site model indicated 32% high affinity binding sites (IC_{50} = 1.6 nM) and 68% low affinity binding sites (IC_{50} = 302 nM).

Fig. 2D shows the inhibition curve of mianserin, which was obtained by pooling the results of three experiments. Analysis of the single experiments with the logistic equation showed a slope of <1 (0.58; Table 1), whereas the two-site model indicated 67% high affinity binding sites (IC_{50} = 88 nM) and 33% low affinity binding sites (IC_{50} = 10 μM). It should be noted that 10 nM mianserin did not inhibit [^{3}H]5-HT binding (see Discussion).

Discussion

[^{3}H]5-HT binding to rat hypothalamic membranes was inhibited by (±)-pindolol, with an IC_{50} of 82 nM and a slope not significantly different than 1. In accordance with previous data indicating that the K_i of the active isomer of pindolol for both 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors is ~20 nM (7), the inhibition is likely to be due to displacement of [^{3}H]5-HT from these receptors [note that assuming a K_d of 1 nM for [^{3}H]5-HT, K_i values correspond to approximately half of the value of IC_{50} (11)]. The (±)-pindolol inhibition curve also showed that to reach maximal inhibition, and therefore to completely inhibit binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, the concentration had to be between 1 and 10 μM. With 0.1 μM pindolol, the concentration used previously (6), inhibition was not maximal, indicating that 5-HT$_{1A}$ and 5-HT$_{1B}$ were not completely masked.

We therefore used 3 μM (±)-pindolol, a concentration that is inactive on transfected 5-HT$_7$ receptors (1, 3). In the presence of this concentration, [^{3}H]5-HT binds to methiothepin-sensitive binding sites. To pharmacologically characterize these 5-HT$_{1nonA/nonB}$ binding sites and to validate the possibility of selectively measuring [^{3}H]5-HT binding to 5-HT$_7$

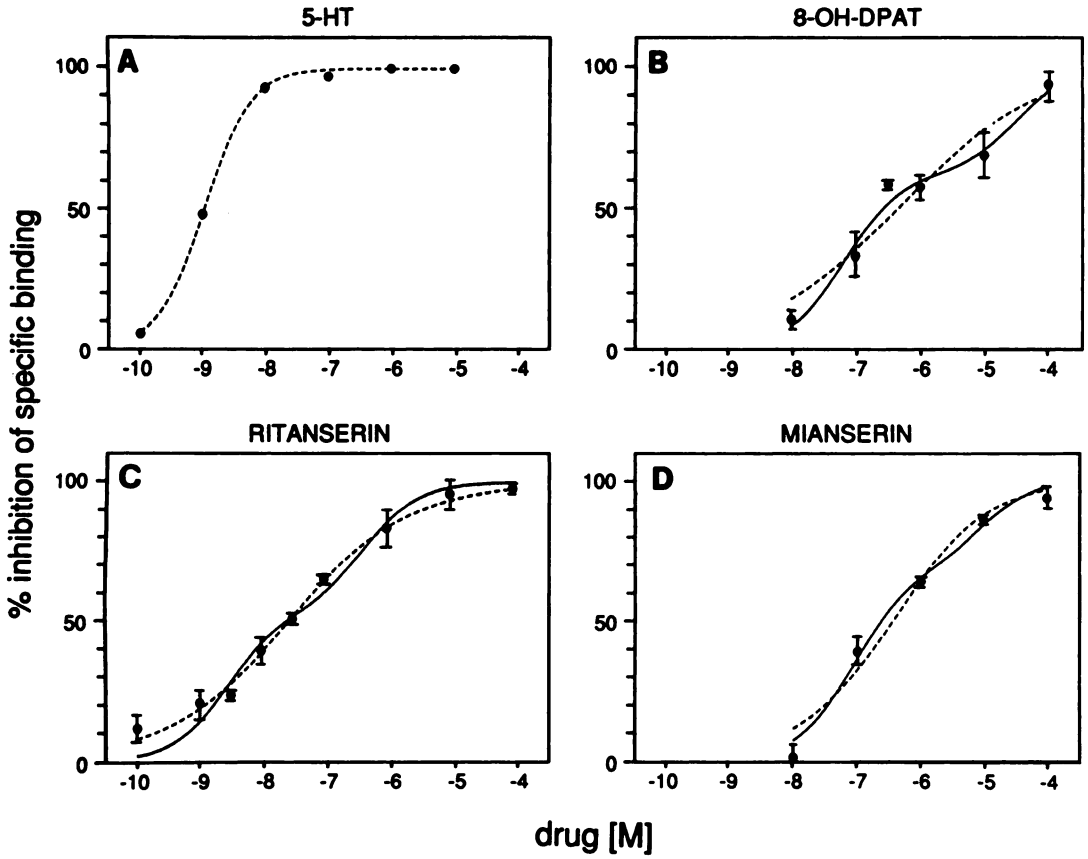

Fig. 2. Inhibition of [^{3}H]5-HT binding in rat hypothalamic membranes by different drugs. (±)-Pindolol (3 μM) was present in all assays, and nonspecific binding was defined in the presence of 10 μM methiothepin. *Values*, mean ± standard error percentage of inhibition of specific binding in one (5-HT) or three (8-OH-DPAT, ritanserin, and mianserin) experiments, each performed in triplicate. *Dotted lines*, nonlinear curve fitting according to the logistic equation; *solid lines*, nonlinear fitting with the two-site model equation. Fitting was always constrained to have the plateau values at 0% and 100% inhibition of specific binding, relative to the lower and the higher drug concentrations, respectively. The results of analysis of the single experiments are given in Table 1.

TABLE 1

Inhibition of [^{3}H]5-HT binding in rat hypothalamic membranes by different drugs

(±)-Pindolol (3 μM) was present in all assays, and nonspecific binding was defined in the presence of 10 μM methiothepin. Three different experiments were performed for each drug, and the inhibition curves were analyzed either with the logistic function, giving their slopes, or with the two-site model. Values are the mean ± SD of these three experiments.

Compound	Slope	High affinity		Low affinity	
		IC_{50}		IC_{50}	
		nM	%	μM	%
8-OH-DPAT	0.41 ± 0.04	104 ± 56	59 ± 6	35 ± 18	41 ± 6
Ritanserin	0.35 ± 0.15	1.6 ± 0.8	32 ± 9	0.30 ± 0.17	68 ± 9
Mianserin	0.58 ± 0.11	88 ± 4	67 ± 5	10 ± 2	33 ± 5

receptors (6), mainly considering the possible interference of 5-HT_{2C} receptor binding, we evaluated the inhibitory effect of 8-OH-DPAT, a compound with high affinity for 5-HT_7 [pK_i = 7.4 (3, 4)] but not for 5-HT_{2C} [pK_i = 5.2 (7)]; ritanserin and mianserin, compounds with higher affinity for 5-HT_{2C} [pK_i = 8.6 and 8.0, respectively (7)] than for 5-HT_7 [pK_i = 7.8 and 7.3, respectively (3, 4)]; and 5-HT, a nonselective compound.

As expected, the inhibition curve of 5-HT had a slope not significantly different than 1, whereas those of 8-OH-DPAT, ritanserin, and mianserin were significantly shallower. These findings appear to differ from previous data of Sleight *et al.* (6), who do not describe biphasic or shallow inhibition curves and interpret their results as suggesting a homogeneous population of 5-HT_7 receptors. We do not know the reason for this qualitative difference, because we used exactly the same experimental procedure; the only difference was that we increased the concentration of pindolol, as explained above.

The two-site model was therefore used to fit the inhibition curves, although the parameters found (i.e., the proportion of the two subpopulations and the relative affinities of the inhibiting compounds) should only considered estimates. This is because of the small number of concentrations tested and mainly because we do not know the affinities of [^{3}H]5-HT on each putative subtype, even though the inhibition curve of unlabeled 5-HT has a slope of 1 (12). These values are needed to make correct estimates of the unlabeled-ligand affinities or the receptor proportions (12).

However, fitting our inhibition curves with the two-site model suggests that one population corresponds to the 5-HT_7 receptors because the high affinity component of 8-OH-DPAT and mianserin had IC_{50} values of 104 and 88 nM, respectively, which are similar to those reported for rat 5-HT_7 (3, 4). Regarding ritanserin, we found two components whose IC_{50} values (1.6 and 320 nM) differ by 1 order of magnitude from that described for ritanserin at 5-HT_7 [K_i = 15 nM (4)]. This confounding result could be due to the presence of more than two sites labeled by [^{3}H]5-HT; 5-$HT_{1D\alpha}$ (13), 5-HT_{1E} (14),

5-HT$_{1F}$ (15), 5-HT$_{2B}$ (16), 5-HT$_{2C}$ (7), and 5-ht$_{5A}$ (17) are all candidates. The higher affinity might correspond to 5-HT$_{2C}$ receptors, although this is unlikely due to the lack of an inhibitory effect of 10 nM mianserin, being the K_i for the 5-HT$_{2C}$ subtype (7). 5-HT$_{2B}$ receptors, whose affinity for the compounds used here is similar to that of 5-HT$_{2C}$ (16), could be involved, but 5-HT$_{2B}$ mRNA was not found in the rat brain (18). Similarly, low expression of 5-HT$_{1D\alpha}$ (19), 5-HT$_{1F}$ (20), and 5-ht$_{5A}$ (17) receptor mRNAs was found in the rat brain.

In summary, our data show that pindolol-sensitive [^{3}H]5-HT binding (likely 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors) accounts for two thirds of the methiothepin-sensitive [^{3}H]5-HT binding. The remaining pindolol-insensitive [^{3}H]5-HT binding is to heterogeneous sites, of which one population might be 5-HT$_7$ receptors. Under these experimental conditions, given the very low specific binding and low-specific-to-total ratio, it is very difficult to identify the populations of pindolol-insensitive sites, and our data are not sufficient to draw clear conclusions on this point. However, they do indicate that this binding assay does not selectively label 5-HT$_7$ receptors in native tissues, such as the rat hypothalamus; moreover, even if the other [^{3}H]5-HT subpopulations could be identified and opportunely masked, the resulting 5-HT$_7$ receptor measurement would be very critical. The development of more selective and sensitive binding assays (21) will probably offer significant advantage. For example, it was recently reported that under appropriate conditions [i.e., in the presence of (−)-cyanopindolol and sumatriptan], [^{3}H]5-carboxamidotryptamine labels a single population of binding sites whose pharmacological profile is similar to that obtained in transfected cells expressing guinea pig 5-HT$_7$ receptors (21). All of the drugs tested inhibited the binding, with n_H values not different than 1, and the following K_i values were found (in transfected cells and guinea pig cortex, respectively): 8-OH-DPAT, 41–135 nM; ritanserin, 45–191 nM; mianserin, 107–661 nM; and (−)-pindolol, >10,000 (21).

Finally, our findings that 0.1 μM pindolol does not completely mask 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors and that the population of pindolol-insensitive [^{3}H]5-HT receptors in rat hypothalamus appear to be heterogeneous suggest that the proposed down-regulation of 5-HT$_7$ receptors after fluoxetine treatment (6) should be considered with caution and confirmed with the use of more appropriate binding assays.

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