British Journal of Pharmacology (1999) 127, 236–242
http://www.stockton-press.co.uk/bjp

Pindolol-insensitive [^{3}H]-5-hydroxytryptamine binding in the rat hypothalamus; identity with 5-hydroxytryptamine$_7$ receptors

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1 Pindolol-insensitive [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) binding to rat hypothalamic membranes was pharmacologically and functionally characterized to resolve whether this procedure selectively labels 5-HT$_7$ receptors.

2 Consistent with a previous report, 3 μM and not 100 nM pindolol was required to occupy fully 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. Remaining [^{3}H]-5-HT binding was saturable (K_D, 1.59$\pm$0.21 nM; B_{max}, 53.8$\pm$3.1 fmol.mg protein^{-1}).

3 Displacement of [^{3}H]-5-HT with metergoline and 5-CT revealed shallow Hill slopes ($<$0.5) but seven other compounds had slopes $>$0.8 and pK_i values and the rank order of affinity were significantly correlated (r=0.81 and 0.93, respectively) with published [^{3}H]-5-HT binding to rat recombinant 5-HT$_7$ receptors.

4 In the presence of pindolol, 5-HT-enhanced accumulation of [^{32}P]-cyclic AMP was unaffected by the 5-HT$_4$ antagonist RS39604 (0.1 μM) or the 5-ht$_6$ antagonist Ro 04-6790 (1 μM) but significantly attenuated by mesulergine (250 nM), ritanserin (450 nM) or methiothepin (200 nM) which have high affinity for the 5-HT$_7$ receptor.

5 Intracerebroventricular pretreatment with the serotonergic neurotoxin 5,7-dihydroxytryptamine, 5,7-DHT, elevated the [^{3}H]-5-HT B_{max} 2 fold, indicating that the hypothalamic 5-HT$_7$ receptor is post-synaptic to 5-HT nerve terminals and regulated by synaptic 5-HT levels.

6 These results suggest that, in the presence of 3 μM pindolol, [^{3}H]-5-HT selectively labels hypothalamic binding sites consistent with functional 5-HT$_7$ receptors.

Keywords: 5-HT$_7$ receptors; 5,7-dihydroxytryptamine; serotonin; hypothalamus; 5-HT; adenylyl cyclase; [^{3}H]-5-HT binding

Abbreviations: 5-CT, 5-carboxamidotryptamine; 5,7-DHT, 5,7-dihydroxytryptamine; GMP-PNP, guanylyl-imidophosphate; HPLC-ED, high performance liquid chromatography with electrochemical detection; 5-HT, 5-hydroxytryptamine; i.c.v., intracerebroventricular; 5-MeOT, 5-methoxytryptamine; 8-OH-DPAT, (+)8-hydroxy-dipropylaminotetralin; STEED; (300 mM sucrose, 40 mM Tris-HCl (pH 7.7) containing 2.5 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol), WAY 100635, *N*-[2[[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride

Introduction

The neurotransmitter 5-HT mediates its effects *via* interactions with seven distinct families of receptors, termed 5-HT$_1$-5-HT$_7$ (Boess & Martin, 1994; Hoyer *et al*., 1994). The mammalian 5-HT$_7$ receptor has been cloned from human (Bard *et al*., 1993; Heidmann *et al*., 1997; Jasper *et al*., 1997), rat (Lovenberg *et al*., 1993; Meyerhof *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993), mouse (Plassat *et al*., 1993) and guinea-pig (Tsou *et al*., 1994) cDNA libraries. Recently, at least four different 5-HT$_7$ receptor isoforms, produced by alternative splicing and differing in their C-terminal tails, have been identified in both human and rat tissues (Heidmann *et al*., 1997; Stam *et al*., 1997). So far, no pharmacological differences have been identified between these isoforms, although they may show different rates of desensitization (Clemett *et al*., 1997) and/or internalization. The 5-HT$_7$ receptor mediates its effects *via* activation of adenylyl cyclase and has a pharmacological profile which is distinct from that of other 5-HT receptor subtypes (Bard *et al*., 1993; Lovenberg *et al*., 1993; Plassat *et al*., 1993; Ruat *et al*., 1993; Shen *et al*., 1993; Tsou *et al*., 1994).

In the periphery, 5-HT$_7$ receptor mRNA is expressed in vascular smooth muscle (Ullmer *et al*., 1995) and activation appears to mediate relaxation in several vascular beds across a variety of species (see reviews, Eglen *et al*., 1997; Saxena *et al*., 1998) and may account for the prolonged hypotensive effect of intravenous 5-HT in anaesthetized rats (De Vries *et al*., 1997). The 5-HT$_7$ receptor also appears to mediate 5-HT-induced relaxation in non-vascular smooth muscle such as the guinea-pig ileum (Carter *et al*., 1995) and porcine myometrium (Kitazawa *et al*., 1998). Within the central nervous system 5-HT$_7$ receptor mRNA is also widely distributed, being especially abundant in the thalamus and hippocampus with high levels also present in the hypothalamus and amygdala (Gustafson *et al*., 1996; Vizuete *et al*., 1997). Autoradiographic studies using [^{3}H]-5-carboxamidotryptamine ([^{3}H]-5-CT) have localized 5-HT$_7$ receptor binding sites in several areas of the hypothalamus of both rat (Gustafson *et al*., 1996) and guinea-pig (To *et al*., 1995). [^{3}H]-5-CT has also been used to determine 5-HT$_7$ receptor density in the guinea-pig cortex by radioligand binding (Boyland *et al*., 1995; To *et al*., 1995), although this radioligand appeared to label a heterogeneous population of binding sites in the rat cortex (Boyland *et al*., 1995). In contrast, Sleight *et al*. (1995) used [^{3}H]-5-HT, in the presence of 100 nM pindolol, to label 5-HT$_7$ receptors in the rat

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hypothalamus. However, Gobbi *et al*. (1996) suggested that this binding assay does not selectively label the 5-HT$_7$ receptor in native tissues as 100 nM pindolol was found to be insufficient to completely mask 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor binding sites and, at a concentration of pindolol (3 μM) which fully saturates these subtypes, the remaining specific pindolol-insensitive sites were still suggested to be heterogeneous (Gobbi *et al*., 1996).

In view of these conflicting reports we have re-evaluated the characteristics of pindolol-insensitive [^{3}H]-5-HT binding to resolve the validity of using this radioligand to label hypothalamic 5-HT$_7$ receptors in the rat. To further confirm the pharmacological profile of the binding site we have also established whether the pindolol-insensitive [^{3}H]-5-HT binding site in rat hypothalamic membranes is positively coupled to adenylyl cyclase. In addition, the effect of intracerebroventricular (i.c.v.) pretreatment of rats with the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) was studied to determine whether chronic reduction in synaptic levels of 5-HT altered hypothalamic 5-HT$_7$ receptor density, and so assess whether these receptors are located pre- or post-synaptic in relation to serotonergic nerve terminals. A preliminary report of these data has been presented (Fone *et al*., 1997).

Methods

Tissue preparation

Previously frozen brains from adult male hooded Lister rats (250–300 g, Charles River) were allowed to defrost and the hypothalami were rapidly dissected out. The hypothalami were immediately homogenized in 20 v of 50 mM Tris (pH 7.4 at 23°C) and the homogenate centrifuged (Sigma 3K20) at 36,000 × *g* for 15 min at 4°C. The supernatant was removed and the resulting pellet resuspended in Tris before being resuspended and centrifuged twice more, with an intervening 15 min incubation at 37°C to remove endogenous 5-HT. The resulting pellets were frozen at −80°C until required.

5-HT$_7$ binding assay

The 5-HT$_7$ binding assay was performed using the method of Sleight *et al*. (1995), modified by increasing the concentration of pindolol to prevent fully binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ sites.

Prepared hypothalamic membranes were resuspended in assay buffer (50 mM Tris containing 10 μM pargyline, 4 mM $CaCl_2$ and 0.58 M ascorbic acid, pH 7.4 at 23°C) to a concentration of approximately 500 μg protein in 50 μl. Saturation analysis was performed in a total assay volume of 1 ml using [^{3}H]-5-HT (0.08–10 nM, 11.2 Ci mmol^{-1}, Amersham) with 3 μM pindolol to mask binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ sites. Non-specific binding was determined using 10 μM methiothepin. For competition experiments aliquots of membranes were incubated in the presence of 3 μM pindolol with 2 nM [^{3}H]-5-HT and at least seven concentrations of displacing drug. After incubation at 23°C for 2 h (to achieve equilibrium for [^{3}H]-5-HT binding, (Sleight *et al*., 1995)) the reaction was terminated by rapid filtration through Whatman GF/B filters followed by three 4 ml washes with ice-cold Tris (50 mM, pH 7.4 at 4°C). Radioactivity bound to the filters was measured by liquid scintillation counting (1214 RackBeta, LKB Wallac) in 4 ml scintillation fluid (Emulsifier Scintillator Plus, Packard). The protein content of samples was determined using the method of Lowry *et al*. (1951) with bovine serum albumin used as standard. All assays were performed in duplicate in at least three separate experiments.

Adenylyl cyclase assay

The adenylyl cyclase activity in hypothalamic membranes was determined using the extraction procedure described by Tsou *et al*. (1994), the assay protocol of Williams & Kelly (1995) and cyclic AMP isolated and quantified according to Alvarez & Daniels (1990). In brief, hypothalami from 36 adult hooded Lister rats were weighed and homogenized (12 strokes by hand at 300 mg wet weight per 10 ml) in ice cold STEED (in mM: sucrose 300, Tris-HCl (pH 7.7) 40 containing EDTA 2.5, EGTA 1 and dithiothreitol 1) and centrifuged for 10 min at 39,000 × *g* and 4°C. The pellet was resuspended in 5 ml STEED containing spiperone 100 μM (to enhance 5-HT-induced adenylyl cyclase stimulation (Tsou *et al*., 1994)) and incubated at 37°C for 15 min. Membranes were washed four times prior to resuspension in STEED at 1.5 mg original wet weight in 40 μl, for direct use in the assay. For assay, each tube (in triplicate) contained Tris (10 μl) 50 mM, 5-HT agonist (10 μl) and 5-HT antagonist (10 μl in 50 mM Tris) or an equivalent volume of Tris, 30 μl of reagents (in mM: Tris-HCl at pH 7.5 50 containing $MgCl_2$ 5, cyclic AMP 1, ATP 1, creatine phosphate 20, rolipram 200 μM, GTP 1 μM, 130 U ml^{-1} creatine phosphokinase and 2 μCi [α^{32}P]-ATP (30 Ci mmol^{-1}, Amersham)) and the reaction was initiated by addition of hypothalamic membranes (40 μl containing approximately 50 μg protein). Incubations proceeded for 12 min at 37°C and the reaction was terminated by addition of 200 μl 1 M HCl. [8-^{3}H]-cyclic AMP was added to evaluate cyclic AMP recovery and [^{32}P]-ATP (8 ml 5 mM HCl) and [^{32}P]-cyclic AMP (3.5 ml 100 μM ammonium acetate) were sequentially eluted from preactivated alumina columns. Scintillation fluid was added to the ammonium acetate eluate prior to quantification of [^{32}P]-cyclic AMP using a Packard β counter as described for the binding assay above.

5,7-DHT treatment

Male hooded Lister rats (185–285 g, $n=8$, each group) were implanted with guide cannulae into the left lateral ventricle (A −0.9, L +1.4, D −1.8 mm from bregma, according to the atlas of Paxinos & Watson (1986) under halothane anaesthesia (2% v v^{-1} in nitrous oxide). Four and seven days after surgery all animals were treated with maprotiline (8 mg kg^{-1}, i.p.) 1 h prior to i.c.v. injection of vehicle (0.1 M ascorbic acid in 0.154 M saline) or 5,7-DHT (150 μg in 2 μl). Twenty-one days after the second treatment the animals were killed by stunning and decapitation and the hypothalami removed and membranes prepared as described above. Hypothalami from the same treatment group were pooled and saturation analysis performed to determine 5-HT$_7$ receptor density. The depletion of 5-HT was confirmed by HPLC with electrochemical detection. Individual brain regions (brainstem, amygdala and hippocampus) were sonicated in 1 ml 0.1 M perchloric acid (30 s; Soniprep 150, MSE) and centrifuged (2500 × *g*, for 10 min at 0°C; Mistral 6000, MSE). The supernatant was filtered through 0.45 μm filters (Millipore) and injected onto a reverse phase column (Hypersil C18, 3 μm particle size, 10 cm × 4.6 mm; Technicol) with 0.15 M NaH_2PO_4 containing 1 mM EDTA, 1 mM octane sulphonic acid and 14% v v^{-1} methanol, pH 3.6 as mobile phase. The glassy carbon working electrode was held at a potential of +0.65 V against an Ag/AgCl working electrode.

Data analysis

Binding data was analysed with non-linear curve fitting programmes (GraphPad Prism, version 2.01; GraphPad Software, Inc.), generating K_D and B_{max} values for saturation experiments. Individual competition experiments were analysed using a variable slope analysis to obtain Hill coefficients and IC_{50} values which were then converted into inhibition constants (K_i) using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). Values are reported as mean ± s.e.mean of three separate experiments unless otherwise stated. Correlation analysis was used to compare the pK_i values and rank order of affinities with those obtained in COS-7 cells expressing the rat 5-HT$_7$ receptor (Shen *et al*., 1993). For adenylyl cyclase determination values are expressed as the mean percentage increase in [^{32}P]-cyclic AMP above that in the absence of 5-HT or pindolol, unless otherwise stated. For the 5,7-DHT treatment, B_{max} and K_D are reported as the mean of two or three saturation experiments (5,7-DHT and control groups, respectively). HPLC and adenylyl cyclase data were analysed using one way ANOVA followed by a *post hoc* Duncan's New Multiple Range test.

Drugs

(±)Pindolol, methiothepin mesylate, metergoline, 5-carboxamidotryptamine (5-CT) maleate, pergolide methanesulphonate, 5-methoxytryptamine (5-MeOT) hydrochloride, (±)8-hydroxy-dipropylaminotetralin (8-OH-DPAT) hydrobromide and mesulergine hydrochloride were purchased from Research Biochemicals International. 5-HT creatinine sulphate, maprotiline hydrochloride and 5,7-DHT hydrochloride were purchased from Sigma Chemical Company. [8-^{3}H]-adenosine 3′,5′ cyclic phosphate (26 Ci mmol^{-1}) and [α^{32}P]-ATP (30 Ci mmol^{-1}) was supplied by Amersham Pharmacia Biotech. GMP-PNP was purchased from Boehringer Mannheim. WAY 100635 (Wyeth (U.K.) Ltd.), ritanserin (Janssen) and clozapine (Sandoz) were obtained as gifts.

Results

Pindolol-insensitive [^{3}H]-5-HT binding

Initial competition curves involving displacement of [^{3}H]-5-HT by increasing concentrations of pindolol showed that maximal inhibition was achieved between 1 and 10 μM (Figure 1) and subsequent experiments were performed in the presence of 3 μM pindolol. In the presence of 3 μM pindolol the remaining pindolol-insensitive [^{3}H]-5-HT binding was found to be saturable and homogeneous (Figure 2), with K_D and B_{max} values of 1.59 ± 0.21 mM and 53.8 ± 3.1 fmol mg protein^{-1}, respectively. In competition experiments specific binding represented approximately 50% of total; pharmacological analysis of these binding sites is shown in Table 1 and Figure 3. All compounds displaced [^{3}H]-5-HT to the same plateau level (Figure 3). However, displacement curves obtained using 5-CT and metergoline produced low Hill coefficients (<0.5) which could represent the presence of an additional low affinity binding site, although this is impossible to define given the very high concentrations of drugs required. A comparison of the affinity values from rat hypothalamic membranes and from published data for [^{3}H]-5-HT binding to rat recombinant 5-HT$_7$ receptors (Shen *et al*., 1993) generated a highly significant correlation (Pearson $r = 0.81$, $P < 0.01$; Figure 4). Comparison of the rank order of affinities also yielded a very highly

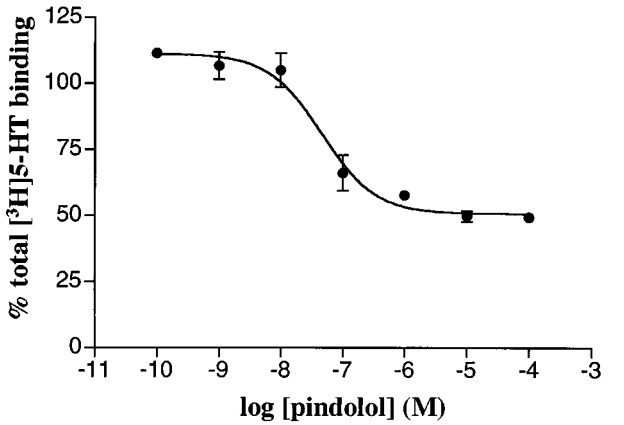

Figure 1 Representative displacement of 2 nM [^{3}H]-5-HT binding to rat hypothalamic membranes by (±) pindolol. Values represent mean ± standard deviation of the experiment performed in duplicate. Essentially identical results were obtained in two additional experiments. Note that the Hill slope = −1.00.

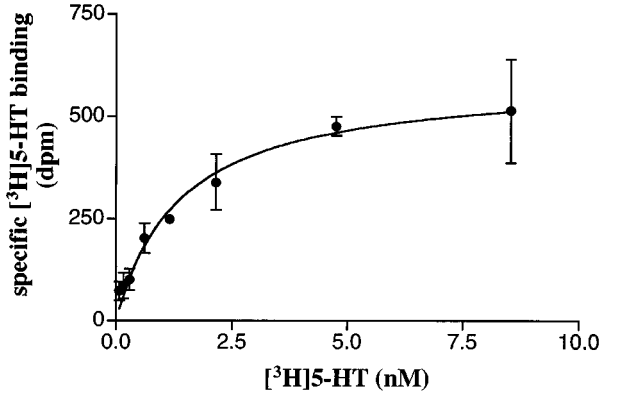

Figure 2 Saturation analysis of [^{3}H]-5-HT binding to rat hypothalamic membranes in the presence of 3 μM (±) pindolol. Non-specific binding was determined in the presence of 10 μM methiothepin. Values represent mean ± standard deviation of a single experiment performed in duplicate. Similar results were obtained in two additional experiments.

Table 1 Estimated affinity values (pK_i) and Hill coefficients of displacing ligands for rat hypothalamic pindolol-insensitive [^{3}H]-5-HT binding sites

Compound	*Hill coefficient*	*pK_i*
5-CT	0.44 ± 0.03	8.48 ± 0.24
5-MeOT	0.80 ± 0.14	8.40 ± 0.26
Methiothepin	1.09 ± 0.11	7.34 ± 0.13
Pergolide	0.92 ± 0.27	7.32 ± 0.15
Metergoline	0.39 ± 0.07	7.32 ± 0.07
Mesulergine	0.80 ± 0.01	7.17 ± 0.05
8-OH-DPAT	0.76 ± 0.09	7.13 ± 0.51
Ritanserin	0.87 ± 0.09	6.93 ± 0.28
Clozapine†	0.82 ± 0.16	6.39 ± 0.31
WAY 100635	–	<6

Hypothalamic binding assays were performed in the presence of 3 μM pindolol and non-specific binding was determined using 10 μM methiothepin. Displacement curves were analysed using a variable slope analysis. pK_i values were used to construct the correlation graph shown in Figure 3. Values are mean ± s.e.mean of three separate experiments (except †, $n = 4$).

significant correlation (Spearman $r=0.93$, $P<0.001$). Competition studies with the selective 5-HT$_{1A}$ antagonist WAY 100635 showed that this compound has a very low affinity for this binding site.

5-HT-induced adenylyl cyclase in hypothalamic membranes

Forskolin (10 μM) induced a 253±23% ($n=4$) increase in cyclic AMP accumulation above basal levels in hypothalamic membranes. As shown in Figure 5, in the absence of pindolol, 5-HT (10 μM) failed to elevate [^{32}P]-cyclic AMP (109± 2%) above basal. In the presence of pindolol (3 μM), which as in the binding assay should prevent the action of 5-HT on G$_i$-linked 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, 5-HT (10 μM) produced a small, consistent and significantly greater production (119±4% above basal) of cyclic AMP. This response was too small to permit a full dose-response analysis (being only 110±2% above basal with 1 μM 5-HT) but was evident in every experiment permitting pharmacological characterization of the stimulation with a single concentration of 5-HT (Figure 5). The 5-HT-induced stimulation of adenylyl cyclase was not prevented by incubation with either the selective 5-HT$_4$ antagonist, 1-(4-amino-5-chloro-2-(3,5-dimethoxy)benzyloxy-phenyl)-3-[1-((2-methylsulphonylamino)ethyl)-piperidin-4-yl]1-propanone (RS36904, 100 nM, (Hegde *et al*., 1995)) or the 5-ht$_6$ antagonist, 4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790, 1 μM, (Sleight *et al*., 1998)) at concentrations approximately ten times their respective pA$_2$ values determined in rat tissues. In contrast,

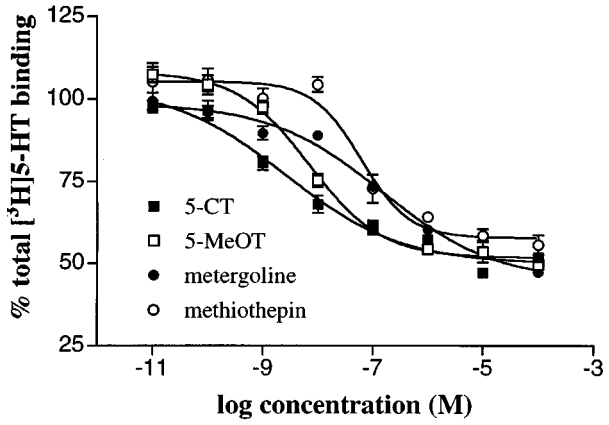

Figure 3 Representative competition curves for selected serotonergic drugs versus [^{3}H]-5-HT specific binding to hypothalamic membranes in the presence of 3 μM pindolol.

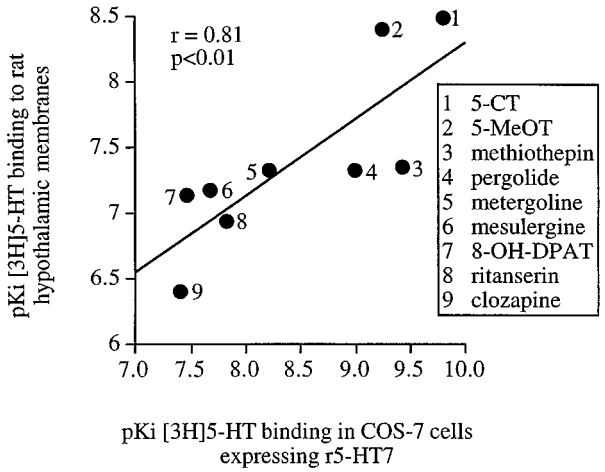

Figure 4 Correlation of affinity values at the rat hypothalamic binding site with those reported for [^{3}H]-5-HT binding to rat 5-HT$_7$ receptors expressed in COS-7 cells (data from Shen *et al*., 1993). Pearson $r=0.81$, $P<0.01$.

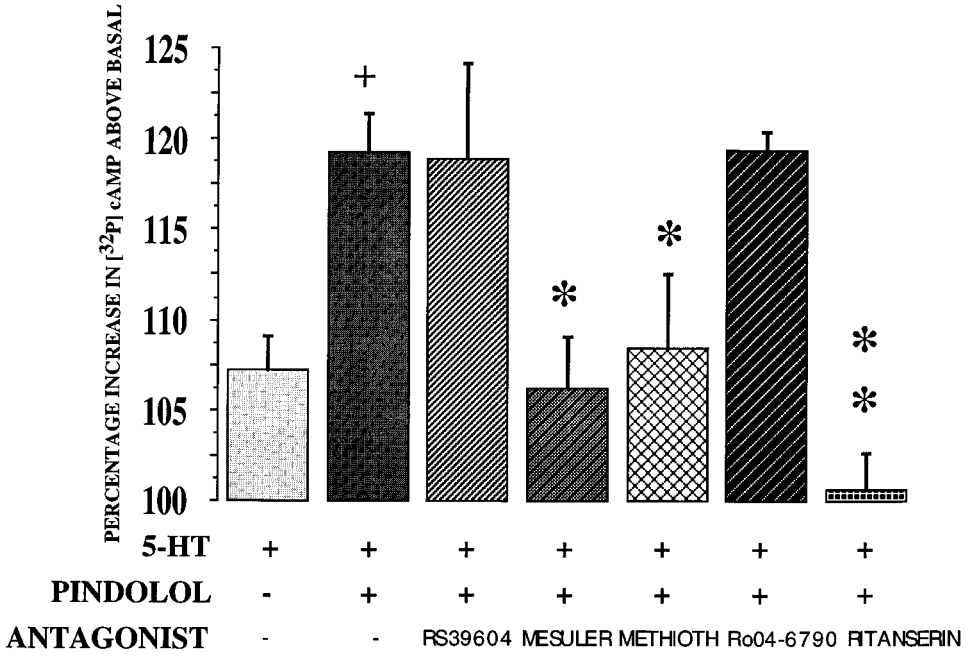

Figure 5 Percentage increase (mean±s.e.mean, $n=5-8$ each in triplicate) in [^{32}P]-cyclic AMP accumulation above basal (in the absence of 5-HT or pindolol) induced by 5-HT (10 μM) alone or combination with pindolol (3 μM) or with pindolol and either RS39604 (0.1 μM), mesulergine (MESULER, 250 nM), methiothepin (METHIOTH, 200 nM), Ro 04-6790 (1 μM) or ritanserin (450 nM). $^+P<0.05$ from 5-HT alone and $^{**}P<0.01$ and $^*P<0.05$ from 5-HT and pindolol, Duncan's New Multiple Range following ANOVA $F_{(6,38)}=6.31$, $P=0.0001$.

Table 2 Effect of 5,7-DHT on [^{3}H]-5-HT binding in hypothalamic membrance and on 5-HT levels determined by HPLC-ED in individual brain regions

	B_{max}		*5-HT* (pmol mg tissue^{-1})		
Treatment	(fmol mg protein^{-1})	K_D (nM)	*Amygdala*	*Hippocampus*	*Brainstem*
Vehicle	54.4	1.66	15.03 ± 1.11	3.01 ± 0.26	7.44 ± 0.54
5,7-DHT	112.7	4.12	3.89 ± 1.09**	0.34 ± 0.05**	7.59 ± 0.40

Four and seven days after implantation of i.c.v. cannulae rats all rats were treated with maprotiline (8 mg kg^{-1}, i.p.) 1 h prior to i.c.v. injection of vehicle or 5,7-DHT (150 μg in 2 μl). Twenty-one days after the second treatment animals were killed and the hypothalami, brainstem, hippocampi and amygdala removed. For [^{3}H]-5-HT binding, hypothalami from each treatment group were pooled to obtain saturation curves ($n=3$, vehicle; $n=2$, 5,7-DHT; mean values). For HPLC-ED analysis of 5-HT levels, measurements were made in individual brain regions (values are mean ± s.e.mean, $n=7$, vehicle; $n=6$, 5.7-DHT). **$P<0.01$ from vehicle, Duncan's New Multiple range following ANOVA.

the 5-HT-induced stimulation was significantly attenuated by the presence of either mesulergine (250 nM), ritanserin (450 nM) or methiothepin (200 nM) all of which have high affinity, and some selectivity, for the 5-HT$_7$ compared with either the 5-HT$_4$ or 5-ht$_6$ receptor (Bard *et al*., 1993).

5,7-DHT-induced changes in hypothalamic pindolol-insensitive [^{3}H]-5-HT binding

Following 5,7-DHT treatment, saturation analysis of pooled hypothalamic membranes demonstrated a 2 fold increase in 5-HT$_7$ receptor level from a mean value of 54.4 fmol mg protein^{-1} in control animals to 112.7 fmol mg protein^{-1} following 5,7-DHT (ranges being 45.7 – 62.4 and 96.9 – 128.6 fmol mg protein^{-1}, respectively), without any marked alteration in the K_D value (ranges being 1.57 – 1.74 and 3.13 – 5.10 nM, respectively). HPLC analysis of 5-HT levels in brainstem, hippocampus and striatum showed significant depletion of 5-HT in serotonergic neuronal projection areas (hippocampus and amygdala, 89.7 and 73.1% depletion from vehicle, respectively) though not in the brainstem raphe nuclei (Table 2), confirming nerve terminal depletion without loss of cell bodies.

Discussion

5-HT receptors are recognized as important therapeutic targets for an array of CNS disorders. Recently, several antidepressant and antipsychotic drugs, most notably clozapine (Ruat *et al*., 1993; Shen *et al*., 1993; Roth *et al*., 1994), have been reported to have high affinity for the 5-HT$_7$ receptor, which has led to the suggestion of a role for this subtype in the therapeutic efficacy of these compounds. To establish any potential role in the therapy of such disorders it is necessary to be able to localize and quantify individual receptor subtypes within the central nervous system; this has proved difficult due to the lack of high affinity, selective ligands for the 5-HT$_7$ receptor. By using [^{3}H]-5-CT autoradiography, in the presence of suitable masking compounds, 5-HT$_7$ binding sites have been identified in both guinea-pig (To *et al*., 1995; Waeber & Moskowitz, 1995) and rat brain (Waeber & Moskowitz, 1995), including several areas within the hypothalamus. This radioligand has also been used to quantify 5-HT$_7$ receptor levels in guinea-pig cortex (Boyland *et al*., 1995; To *et al*., 1995), although a heterogeneous population of binding sites was labelled in rat cortex (Boyland *et al*., 1995). As an alternative, Sleight *et al*. (1995) described a binding assay using [^{3}H]-5-HT to measure 5-HT$_7$ receptor levels in rat hypothalamus. However, it was subsequently suggested by Gobbi *et al*. (1996) that the concentration of pindolol (100 nM) used in this assay was insufficient to completely mask 5-HT$_{1A}$ and 5-HT$_{1B}$ sites, resulting in the measurement of a heterogeneous population of receptors, and casting doubt on the observation that chronic treatment with fluoxetine down-regulates the 5-HT$_7$ receptor. In view of these conflicting reports, this study re-evaluated the homogeneity of pindolol-sensitive [^{3}H]-5-HT binding to establish whether this ligand can be used to selectively label 5-HT$_7$ receptors in the rat hypothalamus.

In accordance with the findings of Gobbi *et al*. (1996), our studies confirmed that the displacement of [^{3}H]-5-HT binding by pindolol reaches a plateau between 1 and 10 μM (Figure 1), hence the 100 nM concentration used by Sleight *et al*. (1995) would have been insufficient to mask completely binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ sites. Consequently, in the current study, the concentration of pindolol was increased to 3 μM to determine whether the remaining pindolol-insensitive [^{3}H]-5-HT binding represented a single population of receptors. Under these conditions [^{3}H]-5-HT binding to rat hypothalamic membranes was saturable, with K_D and B_{max} values of 1.59 ± 0.21 nM and 53.8 ± 3.1 fmol mg protein^{-1}, respectively. Additionally, saturation analysis does not indicate the presence of more than one binding site in the current study. However, the saturation curve would still model a single site if [^{3}H]-5-HT has a similar affinity for two sites in this preparation, so this alone is not conclusive proof of a single site. Of the nine compounds which displaced [^{3}H]-5-HT binding, seven showed monophasic curves with Hill coefficients $>0.8<1.1$. Competition data obtained with metergoline and 5-CT revealed lower Hill slopes; both compounds appearing to have a high affinity component corresponding to a 5-HT$_7$ binding site and a possible additional very low affinity site. The possibility that these sites might represent two affinity states of the 5-HT$_7$ receptor is unlikely since the agonist 5-MeOT did not produce a competition curve with a low slope. The putative low affinity site is unlikely to represent a previously identified 5-HT receptor as metergoline has a p$K_i>6$ at all of the subtypes for which mRNA has been detected in the hypothalamus (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1F}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_{5A}$ and 5-HT$_6$ see reviews (Boess & Martin, 1994; Hoyer *et al*., 1994)) except for the 5-HT$_{5A}$ receptor. Furthermore, as both 8-OH-DPAT and mesulergine have pK_i values of <6 at the 5-HT$_{5A}$ receptor it would be expected that these compounds would also produce low Hill slopes if this site was a very low affinity component contributing to binding in the current assay. Similarly such a site is unlikely to be anything other than a 5-HT receptor, as the radioligand (5-HT) is present at nanomolar concentrations, but it could conceivably represent some other previously uncharacterized 5-HT binding site. Interestingly, 5-CT has recently been shown to recognize an additional, as yet unidentified binding site in guinea-pig and human brain which

would not be expected to be masked by pindolol (Castro *et al*., 1997), but the affinity of 5-CT for this site would have to be substantially lower in the rat than in the other species (pK_D 7.8) for this to account for the current data.

Our findings that 8-OH-DPAT and ritanserin did not produce shallow slopes are in contrast to those of Gobbi *et al*. (1996) who, under the same assay conditions, reported the presence of a low Hill slope and more than one binding site. However, for 8-OH-DPAT, only five ligand concentrations were used by Gobbi *et al*. (1996) and maximum and minimum displacement values were not adequately defined. These workers also based their conclusion that [^{3}H]-5-HT does not selectively label 5-HT$_7$ receptors on the evaluation of only four compounds.

A comparison of pK_i values and rank order of affinities determined in the present hypothalamic binding assay with those reported by Shen *et al*. (1993) for [^{3}H]-5-HT binding to rat 5-HT$_7$ receptors expressed in COS-7 cells revealed highly significant correlations ($P<0.01$ and $P<0.001$, respectively). However, all affinity values determined in hypothalamic membranes were lower than those reported for the recombinant receptor. This finding is similar to that of To *et al*. (1995) who noted lower affinities for the guinea-pig 5-HT$_7$ receptor in cortical membranes than when expressed in CHO-K1 cells. These workers suggested that this apparent discrepancy may be due to the use of an agonist radioligand or to differences in receptor-G protein coupling in brain membranes compared with an over-expressed cell system. Furthermore, if sites other than 5-HT$_7$ receptors were being measured in the current assay, pK_i values for specific compounds would be expected to differ from those obtained in recombinant cells rather than the approximate 10 fold lower affinity obtained for all nine compounds examined.

In the present study only approximately 50% of total pindolol-insensitive [^{3}H]-5-HT binding was specific (displaced by 10 μM methiothepin). This is lower than the value of 70% reported by Sleight *et al*. (1995) using a lower concentration of the masking compound pindolol, with the difference presumably representing binding to 5-HT$_{1A}$ and/or 5-HT$_{1B}$ receptors. In view of this, it cannot be concluded that the 27% decrease in [^{3}H]-5-HT binding observed following chronic fluoxetine treatment (Sleight *et al*., 1995) represents solely a down-regulation of 5-HT$_7$ receptors. Further studies are required to address the potential for regulation of this receptor by chronic administration of fluoxetine.

To further validate the assay we have attempted to demonstrate that the pindolol-insensitive sites present in the hypothalamus represent functional receptors by examining 5-HT-induced stimulation of adenylyl cyclase in these preparations. In agreement with previous accounts, it is extremely difficult to elicit 5-HT induced activation of adenylyl cyclase in rat hypothalamic membranes (Barbaccia *et al*., 1983); the maximal effect of 10 μM 5-HT in the presence of pindolol (3 μM) being $<20\%$ above basal. Only three mammalian 5-HT receptors are known to be positively coupled to adenylyl cyclase (5-HT$_4$, 5-ht$_6$ and 5-HT$_7$). Under the current assay conditions neither the 5-HT$_4$ nor 5-ht$_6$ selective antagonists (RS36904 and Ro 04-6790, respectively (Hegde *et al*., 1995; Sleight *et al*., 1998)) prevented 5-HT-induced increase in adenylyl cyclase stimulation, making it unlikely that either of these receptors contribute to the resultant elevation in cyclic AMP. Because no selective 5-HT$_7$ receptor antagonist was available to us, we examined the effects of non-selective 5-HT antagonists which have high affinity, and some selectivity, for the 5-HT$_7$ over either 5-HT$_4$ or 5-ht$_6$ receptors. Mesulergine, methiothepin and ritanserin, all of which have low nanomolar affinity for the 5-HT$_7$ receptor (Bard *et al*., 1993), all significantly attenuated the cyclic AMP response, compatible with it being a 5-HT$_7$-mediated response.

We have recently shown that twice daily i.c.v. injection of a 5-HT$_7$ receptor-directed antisense oligonucleotide significantly (approximately 45%) reduced hypothalamic pindolol-insensitive [^{3}H]-5-HT binding from that in either vehicle or mismatch treated control rats (Clemett *et al*., 1998), further verifying the use of this assay to measure 5-HT$_7$ receptor density. To determine the cellular localization of hypothalamic 5-HT$_7$ receptors, animals were treated with the neurotoxin 5,7-DHT, which decreased synaptic 5-HT levels in 5-HT neuronal projection areas, and caused an apparent up-regulation of 5-HT$_7$ receptors, resulting in a 2 fold increase in receptor density. Although this does not rule out the existence of some pre-synaptic receptors (the up-regulation could be an underestimate), these results indicate that the 5-HT$_7$ receptor is mainly located post-synaptic to 5-HT nerve terminals in the rat hypothalamus, where it appears to be regulated by altered synaptic levels of the endogenous neurotransmitter. In agreement with this suggestion, there is close similarity between the distribution of [^{3}H]-5-CT binding and 5-HT$_7$ mRNA in all rat hypothalamic nuclei examined (Gustafson *et al*., 1996). In contrast, in other brain areas the 5-HT$_7$ receptor may have a pre-synaptic location, for instance, 5-HT$_7$ mRNA expression is very high in the CA3 region of the hippocampus where [^{3}H]-5-CT binding is relatively low (Gustafson *et al*., 1996).

In summary, our findings indicate, that in the presence of 3 μM pindolol, [^{3}H]-5-HT can label a single population of binding sites in the rat hypothalamus with a pharmacological and functional profile that correlates well with that of the 5-HT$_7$ receptor. However, in view of the low level of specific binding produced under these assay conditions it would be beneficial, when available, to use a radiolabelled selective antagonist. Nevertheless, the ability to detect and quantify these post-synaptic receptors using radioligand binding will help to elucidate the role of the 5-HT$_7$ receptor in the therapeutic efficacy of antidepressant and antipsychotic drugs.

We acknowledge the support of the MRC and Wyeth-Ayerst Research. We would also like to thank Dr A.J. Sleight and F. Hoffmann-La-Roche for providing Ro 04-6790 and Dr R. Eglen for advice on the adenylyl cyclase protocol.

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(Received November 3, 1998
Revised January 25, 1999
Accepted January 29, 1999)