0026-895X/98/030577-07$3.00/0

MOLECULAR PHARMACOLOGY, **54**:577–583 (1998).

The 5-Hydroxytryptamine$_6$ Receptor-Selective radioligand [^{3}H]Ro 63–0563 Labels 5-Hydroxytryptamine Receptor Binding Sites in Rat and Porcine Striatum

FRANK G. BOESS, CLAUS RIEMER, MICHAEL BÖS, JANE BENTLEY, ANNE BOURSON, and ANDREW J. SLEIGHT

Pharma Division, Preclinical Research, F. Hoffmann-La Roche, Ltd., 4070 Basel, Switzerland

Received February 17, 1998; Accepted June 5, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

Ro 63–0563 [4-amino-*N*-(2,6 bis-methylamino-pyridin-4-yl)-benzene sulfonamide] is a high affinity 5-hydroxytryptamine$_6$ (5-HT$_6$) receptor antagonist with more than 100-fold selectivity for the 5-HT$_6$ receptor compared with 69 other receptors and binding sites. The present study describes the properties of [^{3}H]Ro 63–0563, the first selective 5-HT$_6$ receptor radioligand. Specific binding of [^{3}H]Ro 63–0563 (nonspecific binding defined in the presence of 10 μM methiothepin) to recombinant rat and human 5-HT$_6$ receptors was saturable, rapid, and reversible with equilibrium dissociation constants (K_d) of 6.8 nM and 4.96 nM, respectively. The pharmacological profile of the rat 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563 (methiothepin > D-lysergic acid diethylamide > clozapine ~ Ro 63–0563 > lisuride > ergotamine ~ Ro 04–6790 > 5-HT > amitriptyline ~ metergoline ~ mianserin ~ ritanserin > methysergide > mesulergine) was similar to that obtained by using either [^{3}H]D-lysergic acide diethylamide or [^{3}H]5-HT as radioligand. In equilibrium binding studies with rat striatal membranes, [^{3}H]Ro 63–0563 labeled a single binding site with K_d and B_{max} values of 11.7 nM and 175 fmol/mg protein, respectively. In porcine striatal membranes, [^{3}H]Ro 63–0563 also labeled a single binding site with K_d and B_{max} values of 8.0 nM and 130 fmol/mg protein, respectively. The affinities of 14 5-HT$_6$ receptor ligands at this binding site were similar to those found for the recombinant rat and human 5-HT$_6$ receptor, which suggested the presence of 5-HT$_6$ receptors in porcine striatum.

The effects of the neurotransmitter serotonin (5-HT) are mediated by at least 14 different receptors: one ligand-gated ion channel (the 5-HT$_3$ receptor) and 13 G protein-coupled receptors (Boess and Martin, 1994; Hoyer and Martin, 1997). Of these, at least five are coupled to inhibition of adenylyl cyclase (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{1F}$), three are linked to phosphoinositide hydrolysis (5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$), and three have been shown to stimulate adenylyl cyclase activity (5-HT$_4$, 5-HT$_6$, 5-HT$_7$). Unlike the classical 5-HT receptors, the 5-HT$_6$ receptor was not recognized as a pharmacological entity in physiological or radioligand binding experiments before its cloning from rat striatal cDNA (Monsma *et al.*, 1993; Ruat *et al.*, 1993). The highest levels of 5-HT$_6$ receptor mRNA were detected in the olfactory tubercle, nucleus accumbens, striatum, and hippocampus (Monsma *et al.*, 1993; Ruat *et al.*, 1993; Ward *et al.*, 1995; Gérard *et al.*, 1996). To determine the distribution of 5-HT$_6$ receptor protein, Gérard *et al.* (1997) raised polyclonal antibodies against a synthetic peptide corresponding to part of the carboxyl-terminal domain of the 5-HT$_6$ receptor. They observed high levels of 5-HT$_6$ receptor-like immunoreactivity in olfactory tubercle, piriform cortex, nucleus accumbens, islands of Calleja, striatum, hippocampus (CA1 and dentate gyrus), and the molecular layer of the cerebellum.

Many nonselective compounds, including several tricyclic antidepressant drugs, antipsychotic agents, and tryptamine and ergoline derivatives interact with the 5-HT$_6$ receptor, as was shown in binding studies on recombinant rat and human receptors using [^{3}H]LSD, ^{125}I-LSD, and [^{3}H]5-HT as radioligands (Monsma *et al.*, 1993; Roth *et al.*, 1994; Boess *et al.*, 1997; reviewed in Sleight *et al.*, 1997).

Putative 5-HT$_6$-like receptors positively coupled to adenylyl cyclase have been described in the mouse neuroblastoma-derived cell lines N18TG2 and NCB20 (Berry-Kravis and Dawson, 1983; Conner and Mansour, 1990; Unsworth and Molinoff, 1994). In addition, responses with a 5-HT$_6$ receptor-like profile have been observed in pig caudate membranes (Schoeffter and Waeber, 1994) and in mouse embryonic striatal neurons (Sebben *et al.*, 1994).

Until recently, the physiological role of the 5-HT$_6$ receptor was not known, because of the lack of selective ligands. However, the functional significance of this receptor has been investigated by using intracerebroventricular injections of 5-HT$_6$ receptor-specific antisense oligonucleotides. This

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; LSD, D-lysergic acid diethylamide; Ro 63–0563, 4-amino-*N*-(2,6-bis-methylamino-pyridin-4-yl)-benzene sulfonamide; Ro 04–6790, 4-amino-*N*-(2,6-bis-methylamino-pyrimidin-4-yl)-benzene sulfonamide); HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

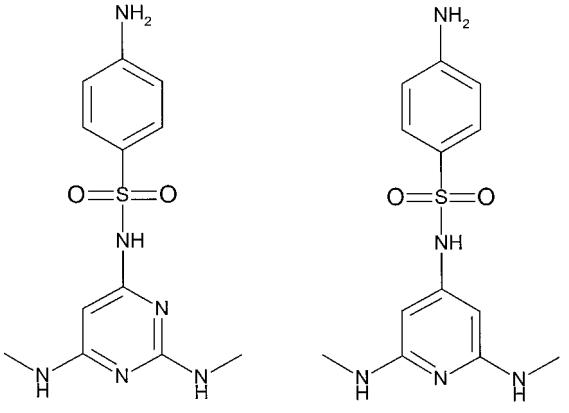
treatment, which should abolish or reduce the expression of 5-HT_6 receptor protein, produced a behavioral syndrome consisting of yawning, stretching, and chewing (Bourson *et al.*, 1995; Sleight *et al.*, 1996).

Recently, we have described two selective 5-HT_6 receptor antagonists, Ro 04–6790 and Ro 63–0563, and demonstrated the ability of Ro 04–6790 to produce behavioral effects similar to those observed after antisense treatment (Sleight *et al.*, 1998). In the current study, we present the first selective 5-HT_6 receptor radioligand [^{3}H]Ro 63–0563 and demonstrate that it labels recombinant rat and human 5-HT_6 receptors as well as 5-HT_6 receptor binding sites in rat and porcine striatal membranes.

Experimental Procedures

Materials. HeLa cells expressing human 5-HT_6 receptors were obtained from Dr. David Sibley (National Institutes of Health, Bethesda, MD) under licensing agreement. 5-HT was purchased from Fluka (Buchs, Switzerland); ergotamine from Sigma (Buchs, Switzerland); mesulergine, metergoline, methysergide, lisuride, methiothepin, clozapine, amitriptyline, ritanserin, mianserin, and pargyline from Research Biochemicals (Natick, MA). Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, streptomycin, and geneticin were obtained from Gibco Life Technologies (Basel, Switzerland). Ro 20–1724, Ro 63–0563 (Fig. 1), Ro 04–6790 (Fig. 1), and LSD were synthesized at F. Hoffmann-La Roche AG (Basel, Switzerland). [^{3}H]Ro 63–0563 (specific activity, 29 Ci/mmol) was kindly prepared by Dr. Philipp Huguenin at F. Hoffmann-La Roche (Basel).

Preparation of membranes from cells expressing recombinant 5-HT_6 receptor. Membranes were prepared from HEK 293 cells stably transfected with the rat 5-HT_6 receptor (Boess *et al.*, 1997) or HeLa cells stably expressing a human 5-HT_6 receptor clone (Kohen *et al.*, 1996). HEK 293 cells were grown in Dulbecco's modified Eagle medium + 10% fetal bovine serum containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and 0.5 mg/ml geneticin in a humidified atmosphere (5% CO_2). HeLa cells were grown in exactly the same conditions except that geneticin was omitted from the media. The cells were detached with phosphate-buffered saline containing 1 mM EDTA, washed with phosphate-buffered saline (Gibco Life Technologies) by two centrifugations (10 min, 500 $\times$ *g*) and the resulting pellet was resuspended in ice-cold 50 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$ and 0.5 mM EDTA by using a Polytron homogenizer (15 sec at maximal speed) at a concentration corresponding to 2 $\times$ 10^6 cells/ml. This homogenate was centrifuged at 100,000 $\times$ *g* for 60 min, the resulting pellet was resuspended in the same buffer to obtain a concentration corresponding to 4 $\times$ 10^7 cells/ml, and aliquots were stored at −80°.



Ro 04-6790 Ro 63-0563

Fig. 1. Chemical structures of selective 5-HT_6 receptor antagonists Ro 04–6790 and Ro 63–0563.

Preparation of rat and porcine striatal membranes. Before dissection, rat brains (male Ibm:RoRo; Biological Research Laboratories, Füllinsdorf, Switzerland) and porcine brains (from a local abattoir) were kept on ice. Striatal tissue (in 3-g aliquots) was homogenized in 26 ml of 0.32 M sucrose with a glass/teflon homogenizer and centrifuged for 10 min at 1000 $\times$ *g* (4°). The pellet was discarded and the supernatant centrifuged for 30 min at 25,000 $\times$ *g*. The pellet was resuspended in 26 ml of 50 mM Tris·HCl, pH 7.4, centrifuged for 15 min at 25,000 $\times$ *g*, resuspended in 50 mM Tris·HCl, incubated for 15 min at 37° and centrifuged for 15 min at 25,000 $\times$ *g*. The pellet was washed two times by resuspension in 50 mM Tris·HCl and centrifugation for 15 min at 25,000 $\times$ *g* and the final pellet was stored frozen at −80°.

[^{3}H]Ro 63–0563 binding assays. Membranes prepared from cells expressing recombinant rat and human 5-HT_6 receptor were resuspended in assay buffer (50 mM Tris·HCl, 10 μM pargyline, 5 mM $MgCl_2$, 0.5 mM EDTA, and 0.1% ascorbic acid, pH 7.4). Binding assays consisted of 100 μl of membrane suspension (corresponding to 4 $\times$ 10^5 cells per assay tube), 50 μl of [^{3}H]Ro 63–0563 (specific activity, 29 Ci/mmol), and 50 μl of displacing drug or assay buffer (final assay volume, 200 μl). Nonspecific binding was measured in the presence of 10 μM methiothepin. Saturation experiments were performed using eight concentrations of [^{3}H]Ro 63–0563 (final concentrations of 0.31 nM to 40 nM). In competition assays, seven to 14 concentrations of the displacing ligands were tested (3 $\times$ 10^{-11} to 10^{-4} M) at a final [^{3}H]Ro 63–0563 concentration of 5 nM. At this concentration, specific binding corresponded to 70% of total binding. Incubations were performed at room temperature for 80 min. Association and dissociation experiments were conducted in the presence of 5 nM [^{3}H]Ro 63–0563. Association experiments were carried out by incubating samples with [^{3}H]Ro 63–0563 in the absence or presence of 10 μM methiothepin for 1 min to 120 min. Dissociation was initiated by addition of 10 μM methiothepin after equilibration with [^{3}H]Ro 63–0563 for 120 min and allowed to proceed for 1 to 120 min.

For binding experiments with rat and porcine striatal membranes, the pellets were resuspended in 26 ml of assay buffer. Binding assays were performed using 0.5 ml of the membrane suspension (corresponding to 1.5 mg of protein per assay tube) in a final assay volume of 1 ml using a shaking incubator. Otherwise, the assay conditions and the ligand concentrations examined were similar to the experiments with recombinant 5-HT_6 receptors with the exception of the final concentration of [^{3}H]Ro 63–0563 used in the competition and kinetic experiments (2 nM). At this concentration, specific binding corresponded to 20% of total binding.

The incubations were terminated by rapid filtration through Whatmann GF/B filters pretreated with polyethyleneimine (0.3%). The filters were washed with 3 $\times$ 2 ml of cold Tris·HCl (50 mM, pH 7.4) and the radioactivity retained on the filters was measured by liquid scintillation counting in 2 ml of scintillation fluid. All experiments were performed in triplicate and repeated at least three times (unless indicated otherwise). Values are given as mean $\pm$ standard error. Data were analyzed using the programs EBDA and LIGAND (Munson and Rodbard, 1980; McPherson, 1985). Protein concentrations were determined using either the bicinchoninic acid method (Pierce, Munich, Germany) or a Coomassie Brilliant Blue G-250 based assay (Biorad).

Results

[^{3}H]Ro 63–0563 binding to recombinant rat 5-HT_6 receptor. At a concentration of 5 nM, 70% of total [^{3}H]Ro 63–0563 binding to membranes prepared from HEK 293 cells stably expressing the rat 5-HT_6 receptor was prevented by the presence of 10 μM methiothepin. No specific binding was

detected to nontransfected HEK 293 cells. Specific binding reached a maximum within 30 min with an association rate constant of k_1 = 6.01 ± 0.36 × 10^6 M^{-1} min^{-1} (mean ± standard error, three experiments). The binding of [^{3}H]Ro 63–0563 was reversible (dissociation rate constant k_{-1} = 6.75 ± 0.36 × 10^{-2} min^{-1}, mean ± standard error, four experiments) and apparently to a single site (Fig. 2). The dissociation constant (K_d = k_{-1}/k_1) determined from these values was 11.2 nM. In equilibrium binding studies with recombinant rat 5-HT$_6$ receptor, specific binding of [^{3}H]Ro 63–0563 was saturable in the range of 0.31 to 40 nM, whereas nonspecific binding increased linearly with increasing ligand concentration (Fig. 3). [^{3}H]Ro 63–0563 labeled a single binding site with an equilibrium dissociation constant (K_d) of 6.8 ± 0.9 nM (mean ± standard error, eleven experiments) and a B_{max} of 2.17 ± 0.09 pmol/mg protein (five experiments).

[^{3}H]Ro 63–0563 binding to recombinant human 5-HT$_6$ receptor. In membranes prepared from HeLa cells stably expressing the human 5-HT$_6$ receptor, [^{3}H]Ro 63–0563 also labeled a single binding site. The binding of [^{3}H]Ro 63–0563 was rapid (association rate constant k_1 = 1.59 ± 0.20 × 10^7 M^{-1} min^{-1}, mean ± standard error, three experiments) and reversible (dissociation rate constant k_{-1} = 4.79 ± 0.38 × 10^{-2} min^{-1}, mean ± standard error, three experiments) (results not shown). The affinity constant (K_d = k_{-1}/k_1) determined from these values was 3.0 nM. In equilibrium binding studies with recombinant human 5-HT$_6$ receptor, [^{3}H]Ro 63–0563 labeled a single binding site with a K_d of 4.96 ± 0.97 nM (mean ± standard error, six experiments) and a B_{max} of 1.59 ± 0.04 pmol/mg protein (Fig. 4).

Pharmacological profile of [^{3}H]Ro 63–0563 binding to rat and human 5-HT$_6$ receptors. Table 1 shows the pK_i values and Hill slopes measured in competition assays with various 5-HT$_6$ receptor ligands in the presence of 5 nM [^{3}H]Ro 63–0563. The pharmacological profile of [^{3}H]Ro 63–0563 binding to the recombinant rat 5-HT$_6$ receptor (methiothepin > LSD > clozapine > lisuride > ergotamine > 5-HT > amitriptyline ~ metergoline ~ mianserin ~ ritanserin > methysergide > mesulergine) was similar to that determined with [^{3}H]LSD using the same cells (Table 1). The Hill slopes of both antagonists (e.g., methiothepin, clozapine, mianserin, ritanserin) and agonists (e.g., 5-HT, LSD, lisuride), as well as those of unlabeled Ro 63–0563 and the structurally related 5-HT$_6$ receptor-selective antagonist Ro 04–6790, were close to one and competition curves with 14 ligand concentrations did not suggest the presence of multiple binding sites (Fig. 5). The affinities of the ligands tested at the human 5-HT$_6$ receptor were very similar to those determined for the recombinant rat 5-HT$_6$ receptor (correlation coefficient r = 0.97, 14 experiments) (Table 1; Fig. 6).

[^{3}H]Ro 63–0563 binding to 5-HT$_6$ receptor-like binding sites in rat striatum. In equilibrium binding studies with rat striatal membranes, [^{3}H]Ro 63–0563 labeled a single binding site with a K_d of 11.7 ± 1.1 nM (mean ± standard error, four experiments) and a B_{max} of 175 ± 26 fmol/mg protein (Fig. 7). Competition assays were carried out in the presence of 2 nM [^{3}H]Ro 63–0563. At this concentration, specific binding corresponded to 20% of total binding. In an initial characterization with a limited set of ligands (tested at seven different concentrations in the range of 10^{-10} to 10^{-4} M), we measured the following pK_i values (three or four experiments, mean ± standard error) for Ro 04–6790 (7.10 ± 0.36), 5-HT (7.31 ± 0.43), amitriptyline (6.90 ± 0.53), clozapine (7.50 ± 0.14), and methiothepin (7.98 ± 0.05). Comparison with the pK_i values measured at the recombinant rat receptor (Table 1) showed a statistically significant correlation (r = 0.9, six experiments, $p < 0.01$), indicating that this [^{3}H]Ro 63–0563 binding site had a pharmacological profile similar to the 5-HT$_6$ receptor. Because of the relatively large quantity of tissue needed per assay, a more detailed pharmacological characterization was carried out using porcine striatal tissue.

[^{3}H]Ro 63–0563 binding to 5-HT$_6$ receptor-like binding sites in porcine striatum. Specific binding of [^{3}H]Ro 63–0563 to porcine striatal membranes reached a maximum within 30 min with an association rate constant of k_1 = 6.75 ± 1.74 × 10^7 M^{-1} min^{-1} (mean ± standard error, three experiments). The binding of [^{3}H]Ro 63–0563 was reversible (dissociation rate constant k_{-1} = 1.28 ± 0.33 × 10^{-1} min^{-1}, mean ± standard error, three experiments). The affinity constant (K_d = k_{-1}/k_1) determined from these values was 2 nM. In equilibrium binding studies with porcine striatal membranes, [^{3}H]Ro 63–0563 labeled a single binding site with a K_d of 8.01 ± 0.63 nM (mean ± standard error, 16 experiments) and a B_{max} of 130 ± 25 fmol/mg protein (eight experiments) (Fig. 8). The affinities of a range of 5-HT$_6$ receptor ligands at this [^{3}H]Ro 63–0563 binding site were similar to those found for the recombinant rat and human 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563. There was a statistically significant ($p < 0.01$) correlation of the pK_i values of the 14 ligands tested at the binding site in porcine striatal membranes with the values measured for both rat (correlation coefficient r = 0.76) and human recombinant 5-HT$_6$ receptor (r = 0.78). High concentrations of all ligands

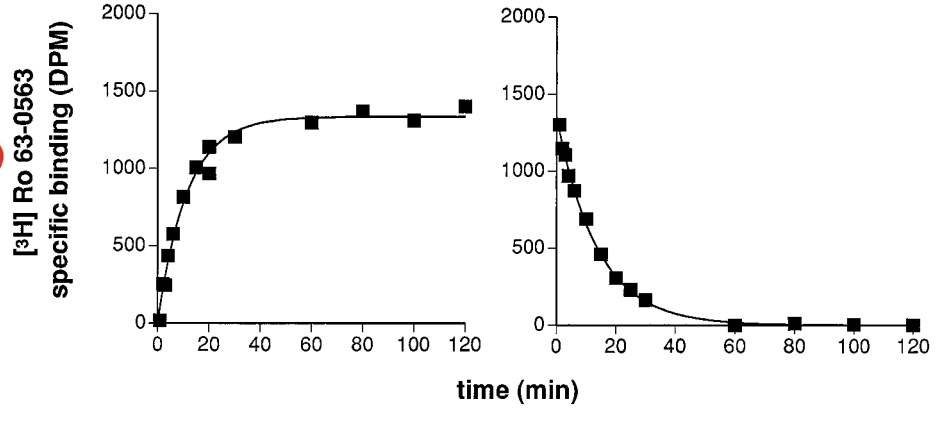


Fig. 2. Association and dissociation kinetics of [^{3}H]Ro 63–0563 binding to rat 5-HT$_6$ receptors stably expressed in HEK 293 cells, performed as described in Experimental Procedures. The data shown (k_1 = 5.37 × 10^6 M^{-1} min^{-1}, k_{-1} = 7.23 × 10^{-2} min^{-1}) are representative of three similar experiments, each performed in triplicate.

tested displaced [^{3}H]Ro 63–0563 binding to the same level as the nonspecific binding defined in the presence of 10 μM methiothepin. In addition, the Hill slopes of the ligands tested were not significantly different from one (i.e., the mean Hill slope value and unity were within 2 standard deviation units from each other), which suggests that [^{3}H]Ro 63–0563 labels a homogenous population of binding sites (Table 1, Fig. 9).

Discussion

Ro 63–0563 is a high affinity 5-HT$_6$ receptor antagonist with more than 100-fold selectivity for the 5-HT$_6$ receptor compared with 69 other receptors and binding sites (Sleight *et al.*, 1998). [^{3}H]Ro 63–0563 binds with nanomolar affinity to a single binding site in membranes prepared from HEK 293 cells stably expressing recombinant rat 5-HT$_6$ receptors (K_d = 7 nM) and HeLa cells stably expressing recombinant human 5-HT$_6$ receptors (K_d = 5 nM). The number of binding sites labeled by [^{3}H]Ro 63–0563 in HEK 293 cells stably expressing rat 5-HT$_6$ receptor (2.17 pmol/mg protein) is similar to that measured previously with the (nonselective) agonist radioligands [^{3}H]5-HT (2.54 pmol/mg protein) and [^{3}H]LSD (2.22 pmol/mg protein) in the same cell line (Boess *et al.*, 1997). This suggests that these radioligands do not distinguish between different agonist high and low affinity states of the recombinant rat 5-HT$_6$ receptor expressed in HEK 293 cells.

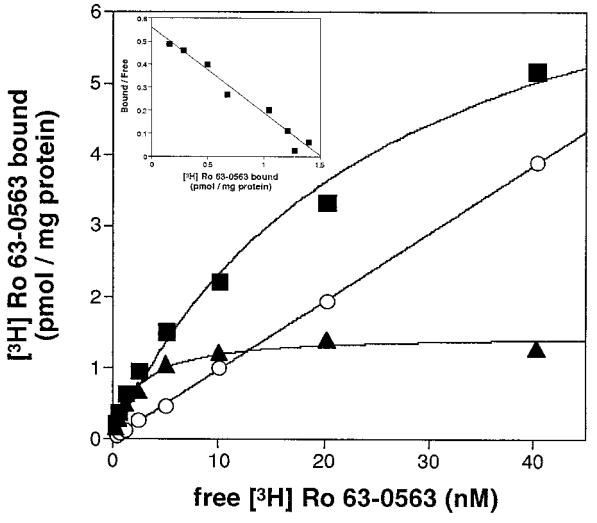


Fig. 3. Equilibrium binding of [^{3}H]Ro 63–0563 to rat 5-HT$_6$ receptor stably expressed in HEK 293 cells, performed as described in Experimental Procedures. ■, Total binding; ○, nonspecific binding; ▲, specific binding. *Inset*, Scatchard transformation of the saturation binding data. The data shown are from one of 11 similar experiments, each performed in triplicate.

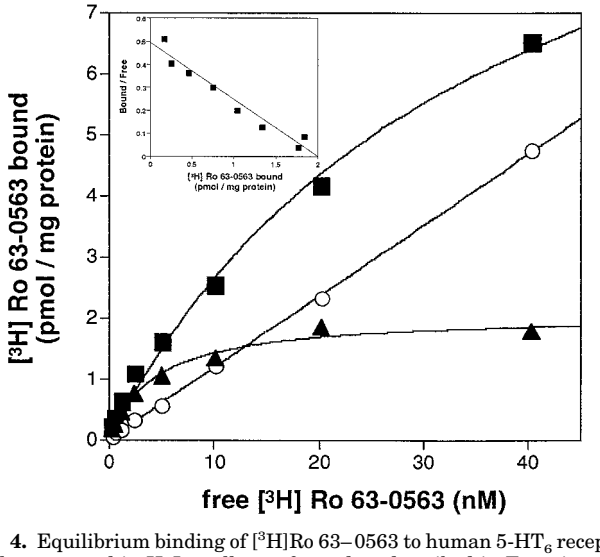


Fig. 4. Equilibrium binding of [^{3}H]Ro 63–0563 to human 5-HT$_6$ receptor stably expressed in HeLa cells, performed as described in Experimental Procedures. ■, Total binding; ○, nonspecific binding; ▲, specific binding. *Inset*, Scatchard transformation of the saturation binding data. The data shown are from one of six similar experiments, each performed in triplicate.

The pharmacological profile of the rat 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563 (methiothepin > LSD > clozapine ~ Ro 63–0563 > lisuride > ergotamine ~ Ro 04–6790 > 5-HT > amitriptyline ~ metergoline ~ mianserin ~ ritanserin > methysergide > mesulergine) was very similar to that of the human 5-HT$_6$ receptor (methiothepin > Ro 63–0563 ~ LSD > lisuride > ergotamine > Ro 04–6790 ~ clozapine > 5-HT ~ metergoline > ritanserin > mianserin ~ amitriptyline > methysergide > mesulergine) (correlation coefficient r = 0.97, 14 experiments). As expected, there was also a significant correlation between the pK_i values for the rat recombinant 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563 measured in the present study and the values obtained with the same cell line using either [^{3}H]LSD (r = 0.87, 14 experiments) or [^{3}H]5-HT (r = 0.92, 12 experiments) as the radioligand (Boess *et al.*, 1997).

Because Northern blots (Monsma *et al.*, 1993), *in situ* hybridization (Ward *et al.*, 1995), and reverse-transcription polymerase chain reaction (Gérard *et al.*, 1996) had demonstrated especially high levels of 5-HT$_6$ receptor mRNA in the rat striatum and high levels of 5-HT$_6$ receptor-like immunoreactivity had also been observed in this brain region (Gérard *et al.*, 1997), we looked for [^{3}H]Ro 63–0563 binding sites in rat striatal membranes. [^{3}H]Ro 63–0563 labeled a single binding site with a K_d of 11.7 nM and a B_{max} of 175 fmol/mg protein. A comparison of the pK_i and pK_d values of the ligands tested in rat striatal membranes (including [^{3}H]Ro 63–0563) with the values determined for the recombinant rat 5-HT$_6$ receptor showed a significant correlation (r = 0.9, six experiments, $p < 0.01$) suggesting that this [^{3}H]Ro 63–0563 binding site actually corresponds to the native rat 5-HT$_6$ receptor. Because of the relatively large quantity of tissue needed per assay, a more detailed pharmacological characterization was carried out using porcine striatal membranes, a tissue in which 5-HT-mediated stimulation of adenylyl cyclase activity with a 5-HT$_6$ receptor-like profile has been reported (Schoeffter and Waeber, 1994). The amount of specific binding observed in porcine striatal membranes (20–30%) was generally higher than that measured with rat striatal membranes (10–20%), which facilitated a more detailed characterization of the [^{3}H]Ro 63–0563 binding in the porcine tissue. We did attempt to improve the level of specific binding by changing pH, the buffer used for the binding assay (HEPES instead of Tris), and the concentrations of the various salts in the incubation buffer. None of these changes, however, significantly improved specific binding (results not shown). In equilibrium binding studies [^{3}H]Ro 63–0563 labeled a single binding site in porcine striatal membranes with a K_d of 8 nM and a B_{max} of 130 fmol/mg protein. The pK_i

values of the 5-HT$_6$ receptor ligands tested at the [^{3}H]Ro 63–0563 binding site in porcine striatal membranes showed a statistically significant correlation with the values measured for both rat (correlation coefficient $r = 0.76$, 14 experiments, $p < 0.01$) and human recombinant 5-HT$_6$ receptor ($r = 0.78$, 14 experiments, $p < 0.01$). Although the overall pharmacological profile was quite similar to that of the rat and human receptor, methiothepin, clozapine, and amitriptyline had about 10-fold lower affinity for the binding site in porcine brain. This is in agreement with the low pK_b values of methiothepin and clozapine for the inhibition of 5-HT mediated stimulation of adenylyl cyclase in porcine striatal membranes reported by Schoeffter and Waeber (1994). High concentrations of all ligands tested displaced [^{3}H]Ro 63–0563 binding to the same level as the nonspecific binding defined in the presence of 10 μM methiothepin. The Hill slopes of the ligands tested, including the selective 5-HT$_6$ receptor ligand Ro 04–6790, were not significantly different from unity, suggesting that [^{3}H]Ro 63–0563 labels a homogenous population of binding sites. Furthermore, we attempted to displace [^{3}H]Ro 63–0563 with 5-HT in both the presence and absence of guanylyl-imidodiphosphate (200 μM). Guanylyl imidodiphosphate had no effect on either the amount of specific binding, the affinity of 5-HT, or the Hill slope of the displacement (results not shown).

We have previously demonstrated that the apparent affinity of certain compounds, particularly tryptamine derivatives, was dependent upon whether [^{3}H]5-HT or [^{3}H]LSD was used as the radioligand (Boess *et al.*, 1997). Because the purpose of the present study was to determine whether 5-HT$_6$ receptors are expressed in the rat and porcine striatum, we specifically chose ligands that had consistent affinities with respect to both [^{3}H]5-HT and [^{3}H]LSD binding. It would be interesting, however, to determine the affinity of a number of tryptamine derivatives for the 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563 and compare them with the affinities obtained with [^{3}H]5-HT and [^{3}H]LSD binding.

These results demonstrate for the first time the presence of 5-HT$_6$ receptor binding sites in membranes prepared from rat and porcine brain. Previously, a [^{3}H]clozapine binding site with a pharmacological profile resembling that of the 5-HT$_6$ receptor had been identified in rat brain membranes (Glatt *et al.*, 1995). However, 5-HT displayed a very low affinity (K_i = 200,000 nM) for this [^{3}H]clozapine binding site in rat brain compared with an affinity of 150 nM at recombinant rat 5-HT$_6$ receptors (Glatt *et al.*, 1995), which suggests that these binding sites may not correspond to the 5-HT$_6$ receptor, but to a distinct receptor with similar pharmacological properties. In contrast, 5-HT has a high affinity for both the recombinant rat and human 5-HT$_6$ receptor labeled with [^{3}H]Ro 63–0563 (K_i = 34 and 44 nM, respectively), as well as for the [^{3}H]Ro 63–0563 binding sites in rat and porcine striatal membranes (19 and 40 nM, respectively).

Our data show that 5-HT$_6$ receptors are expressed in the rat and porcine striatum. In agreement with our results, 5-HT-mediated stimulation of adenylyl cyclase activity with a 5-HT$_6$ receptor-like pharmacological profile has been observed in porcine striatal membrane preparations (Schoeffter and Waeber, 1994) and mouse striatal neurons in primary culture (Sebben *et al.*, 1994). Sebben *et al.* (1994) studied the affinity of a limited number of compounds for a 5-HT-sensitive [^{125}I]LSD binding site in membranes prepared from mouse striatal neurons after 11 days of primary culture and concluded that they interacted with more than one population of binding sites. Our results suggest that at least some of the [^{125}I]LSD binding sites did in fact correspond to 5-HT$_6$ receptors. The number of receptors measured in the membrane preparations of mouse striatal neurons with [^{125}I]LSD (B_{max} = 16 fmol/mg) (Sebben *et al.*, 1994) was approximately 10-fold lower than the value we observed in membranes prepared from freshly dissected rat and porcine striatum (175 and 130 fmol/mg protein, respectively). This may indicate that the expression of 5-HT$_6$ receptors actually diminishes during primary culture or that species differences exist in the levels of 5-HT$_6$ receptor expression.

In summary, [^{3}H]Ro 63–0563 is a 5-HT$_6$ receptor selective radioligand that binds with high affinity to recombinant rat and human 5-HT$_6$ receptors. The pharmacological profile of the [^{3}H]Ro 63–0563 binding site is similar to that of the 5-HT$_6$ receptor labeled with either [^{3}H]LSD or [^{3}H]5-HT. We have identified [^{3}H]Ro 63–0563 binding sites with a 5-HT$_6$ receptor-like pharmacological profile in the rat and porcine

TABLE 1

Pharmacological profile of [^{3}H]Ro 63-0563 binding

pK_i (* or pK_d) values (mean ± standard error, > three experiments) and Hill slopes measured with rat 5-HT$_6$ receptor expressed in HEK 293 cells and human 5-HT$_6$ receptor expressed in HeLa cells and porcine striatal membranes. Data from [^{3}H]LSD and [^{3}H]5-HT binding studies with recombinant rat 5-HT$_6$ receptor (Boess *et al.*, 1997 and [a]Sleight *et al.*, 1998) are presented for comparison.

Ligand	[^{3}H]Ro 63-0563						Rat 5-HT$_6$ receptor	
	Rat 5-HT$_6$ receptor		Human 5-HT$_6$ receptor		Porcine striatal membranes		[^{3}H]LSD	[^{3}H] 5-HT
	pK_i	Hill slope	pK_i	Hill slope	pK_i	Hill slope	pK_i	pK_i
5-HT	7.49 ± 0.09	1.02 ± 0.06	7.46 ± 0.20	0.74 ± 0.05	7.45 ± 0.15	0.77 ± 0.12	6.63	7.90*
Ro63-0563	8.00 ± 0.09	0.81 ± 0.09	8.40 ± 0.13	0.95 ± 0.22	8.12* ± 0.04	0.97 ± 0.01	7.83[a]	
Ro04-6790	7.64 ± 0.08	1.02 ± 0.07	7.87 ± 0.11	1.03 ± 0.22	7.63 ± 0.13	1.21 ± 0.08	7.35[a]	
Methiothepin	8.26 ± 0.16	0.82 ± 0.21	8.71 ± 0.12	1.15 ± 0.15	7.01 ± 0.08	0.99 ± 0.06	8.82	7.53
LSD	8.20 ± 0.09	1.22 ± 0.10	8.36 ± 0.01	1.14 ± 0.21	8.28 ± 0.11	1.00 ± 0.05	8.73*	7.78
Clozapine	8.03 ± 0.06	0.94 ± 0.13	7.86 ± 0.14	0.86 ± 0.11	7.05 ± 0.09	0.88 ± 0.10	7.95	7.51
Lisuride	7.93 ± 0.06	1.04 ± 0.10	8.07 ± 0.10	1.42 ± 0.16	7.81 ± 0.16	1.11 ± 0.10	8.14	7.52
Ergotamine	7.65 ± 0.06	1.16 ± 0.22	7.98 ± 0.03	1.08 ± 0.08	7.84 ± 0.15	1.09 ± 0.19	8.63	6.97
Amitriptyline	7.08 ± 0.09	0.91 ± 0.10	6.88 ± 0.05	0.89 ± 0.13	6.00 ± 0.15	0.88 ± 0.18	7.14	6.47
Metergoline	7.05 ± 0.09	0.90 ± 0.15	7.44 ± 0.10	1.01 ± 0.12	6.54 ± 0.09	1.26 ± 0.37	7.30	6.58
Mianserin	7.01 ± 0.02	0.85 ± 0.03	6.91 ± 0.12	0.94 ± 0.14	6.96 ± 0.11	0.73 ± 0.11	7.22	6.91
Ritanserin	6.96 ± 0.18	1.06 ± 0.12	6.99 ± 0.08	0.95 ± 0.06	6.81 ± 0.01	0.76 ± 0.03	7.47	6.50
Methysergide	6.46 ± 0.14	0.81 ± 0.02	6.59 ± 0.11	0.88 ± 0.11	6.35 ± 0.18	0.92 ± 0.13	6.59	6.47
Mesulergine	5.45 ± 0.03	1.06 ± 0.13	5.66 ± 0.04	0.83 ± 0.04	6.03 ± 0.15	0.74 ± 0.11	5.76	5.10

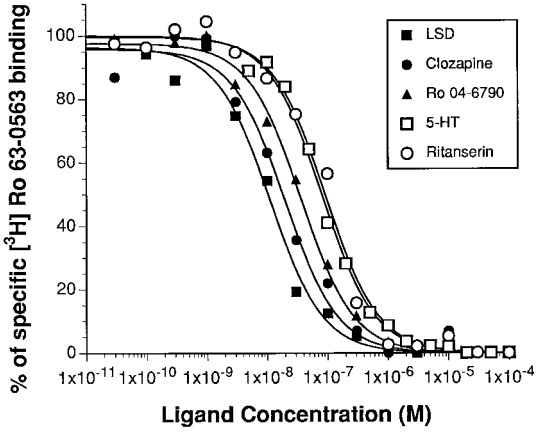


Fig. 5. Pharmacological profile of [^{3}H]Ro 63–0563 binding to rat 5-HT$_6$ receptors stably expressed in HEK 293 cells. Competition experiments with LSD (■), clozapine (●), Ro 04–6790 (▲), 5-HT (□), and ritanserin (○) were performed as described in Experimental Procedures. The data shown are from one of three or four similar experiments, each performed in triplicate.

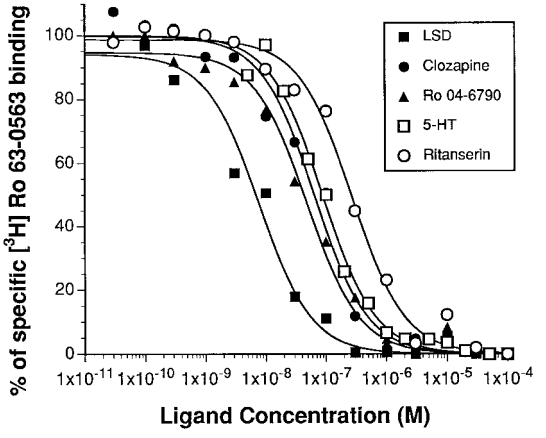


Fig. 6. Pharmacological profile of [^{3}H]Ro 63–0563 binding to human 5-HT$_6$ receptors stably expressed in HeLa cells. Competition experiments with LSD (■), clozapine (●), Ro 04–6790 (▲), 5-HT (□), and ritanserin (○) were performed as described in Experimental Procedures. The data shown are from one of three or four similar experiments, each performed in triplicate.

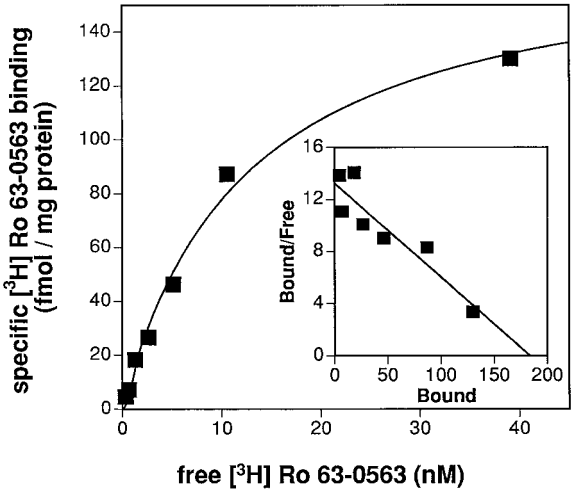


Fig. 7. Equilibrium binding of [^{3}H]Ro 63–0563 to 5-HT$_6$ receptor-like binding sites in rat striatal membranes, performed as described in Experimental Procedures. *Inset*, Scatchard transformation of the saturation binding data. The data shown are from one of three similar experiments, each performed in triplicate.

striatum, confirming the expression of 5-HT$_6$ receptors in this brain region in agreement with the distribution of 5-HT$_6$ receptor mRNA and immunoreactivity. [^{3}H]Ro 63–0563 will therefore be a useful tool to determine the expression level of 5-HT$_6$ receptors in different brain regions. In addition, our data support the results of studies using antisense oligonucleotides (Bourson *et al.*, 1995; Sleight *et al.*, 1996) and the 5-HT$_6$ receptor selective antagonist Ro 04–6790 (Sleight *et al.*, 1998) that first suggested the presence of functional 5-HT$_6$ receptors in the rat brain. Indeed, given the previously reported data from our group (Bourson *et al.*, 1995; Sleight *et al.*, 1996, 1998) and the data reported here, we feel justified in abandoning lower case letters for 5-HT$_6$ receptor nomenclature.

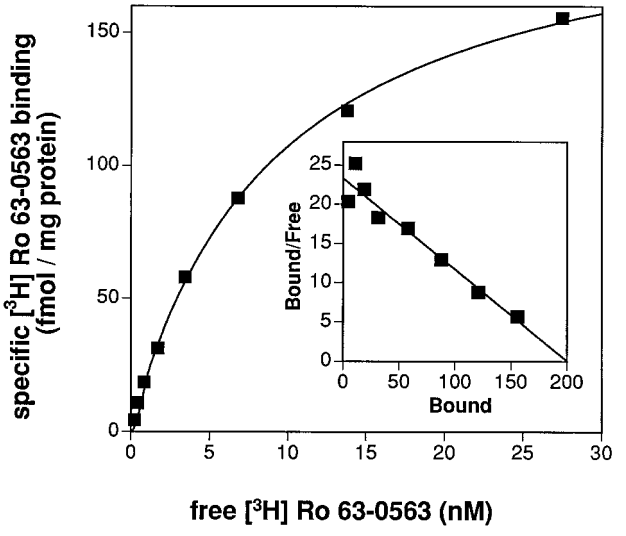


Fig. 8. Equilibrium binding of [^{3}H]Ro 63–0563 to 5-HT$_6$ receptor-like binding sites in porcine striatal membranes, performed as described in Experimental Procedures. *Inset*, Scatchard transformation of the saturation binding data. The data shown are from one of eight similar experiments, each performed in triplicate.

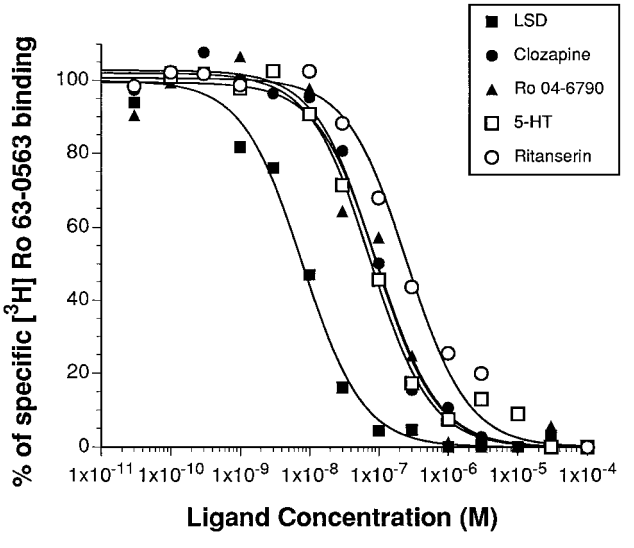


Fig. 9. Pharmacological profile of [^{3}H]Ro 63–0563 binding to 5-HT$_6$ receptor-like binding sites in porcine striatal membranes. Competition experiments with LSD (■), clozapine (●), Ro 04–6790 (▲), 5-HT (□), and ritanserin (○) were performed as described in Experimental Procedures. The data shown are from one of three to six similar experiments, each performed in triplicate.

Acknowledgments

We would like to thank Catherine Ardati, Catherine Carolo, and Alain Rudler for expert technical assistance and Dr. Philipp Huguenin for the radiolabeling of [^{3}H]Ro 63–0563.

References

Berry-Kravis E and Dawson G (1983) Characterization of an adenylate cyclase-linked serotonin (5-HT$_1$) receptor in a neuroblastoma X brain explant hybrid cell line (NCB-20). *J Neurochem* **40:**977–985.

Boess FG and Martin IL (1994) Molecular biology of 5-HT receptors. *Neuropharmacology* **133:**275–317.

Boess FG, Monsma FJ Jr., Carolo C, Meyer V, Rudler A, Zwingelstein C, and Sleight AJ (1997) Functional and radioligand binding characterization of rat 5-HT$_6$ receptors stably expressed in HEK 293 cells. *Neuropharmacology* **36:**713–720.

Bourson A, Borroni E, Austin RH, Monsma FJ, and Sleight AJ (1995) Determination of the role of the 5-HT$_6$ receptor in the rat brain: a study using antisense oligonucleotides. *J Pharmacol Exp Ther* **274:**173–180.

Conner DA and Mansour TE (1990) Serotonin receptor-mediated activation of adenylate cyclase in the neuroblastoma NCB.20: a novel 5-hydroxytryptamine receptor. *Mol Pharmacol* **37:**742–751.

Gérard C, El Mestikawy S, Lebrand C, Adrien J, Ruat M, Traiffort E, Hamon M, and Martres M-P (1996) Quantitative RT-PCR distribution of serotonin 5-HT$_6$ receptor mRNA in the central nervous system of control or 5,7-dihydroxytryptamine-treated rats. *Synapse* **23:**164–173.

Gérard C, Martres M-P, Lefèvre K, Miquel M-C, Vergé D, Lanfumey L, Doucet E, Hamon M, and El Mestikawy S (1997) Immuno-localization of serotonin 5-HT$_6$ receptor-like material in the rat central nervous system. *Brain Res* **746:**207–219.

Glatt CE, Snowman AM, Sibley DR, and Snyder SH (1995) Clozapine: selective labeling of sites resembling 5-HT$_6$ serotonin receptors may reflect psychoactive profile. *Mol Med* **1:**398–406.

Hoyer D and Martin G (1997) 5-HT receptor classification and nomenclature: towards a harmonization with the human genome. *Neuropharmacology* **46:**419–428.

Kohen R, Metcalf MA, Khan N, Druck T, Huebner K, Lachowicz JE, Sibley DR, Roth BL, and Hamblin MW (1996) Cloning, characterization and chromosomal localization of a human 5-HT$_6$ serotonin receptor. *J Neurochem* **66:**47–56.

McPherson GA (1985) *KINETIC, EBDA, LIGAND, LOWRY: a collection of radioligand binding analysis programs.* Biosoft, Cambridge, UK.

Monsma FJ, Shen Y, Ward RP, Hamblin MW, and Sibley DR (1993) Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol Pharmacol* **43:**320–327.

Munson PJ and Rodbard D (1980) LIGAND: a versatile computerized approach for characterisation of ligand-binding systems. *Anal Biochem* **107:**220–239.

Roth BL, Craigo SC, Choudhary MS, Uluer A, Monsma FJ, Shen Y, Meltzer HY, and Sibley DR (1994) Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptor. *J Pharmacol Exp Ther* **268:**1403–1410.

Ruat M, Traiffort E, Arrang J-M, Tardivel-Lacombe J, Diaz J, Leurs R, and Schwartz J-C (1993) A novel serotonin (5-HT$_6$) receptor: molecular cloning, localisation and stimulation of cAMP accumulation. *Biochem Biophys Res Comm* **193:**269–276.

Schoeffter P and Waeber C (1994) 5-Hydroxytryptamine receptors with a 5-HT$_6$ receptor-like profile stimulating adenylyl cyclase activity in pig caudate membranes. *Naunyn-Schmiedebergs Arch Pharmacol* **350:**356–360.

Sebben M, Ansanay H, Bockaert J, and Dumuis A (1994) 5-HT$_6$ receptors positively coupled to adenylyl cyclase in striatal neurones in culture. *Neuroreport* **5:**2553–2557.

Sleight AJ, Boess F, Bös M, Levet-Trafit B, Riemer C, and Bourson A (1998) Ro 04–6790 and Ro 63–0563: potent and selective antagonists at human and rat 5-HT$_6$ receptors. *Br J Pharmacol* **124:**556–562.

Sleight AJ, Boess FG, Bourson A, Sibley DR, and Monsma FJ (1997) 5-HT$_6$ and 5-HT$_7$ receptors: molecular biology, functional correlates and possible therapeutic indications. *Drug News Perspect* **10:**214–224.

Sleight AJ, Monsma FJ, Borroni E, Austin RH, and Bourson A (1996) Effects of altered 5-HT$_6$ expression in the rat: functional studies using antisense oligonucleotides. *Behav Brain Res* **73:**245–248.

Unsworth CD and Molinoff PB (1994) Characterization of a 5-hydroxytryptamine receptor in mouse neuroblastoma N18TG2 cells. *J Pharmacol Exp Ther* **269:**246–255.

Ward RP, Hamblin MW, Lachowicz JE, Hoffman BJ, Sibley DR, and Dorsa DM (1995) Localization of serotonin subtype 6 receptor messenger RNA in the rat brain by in situ hybridization histochemistry. *Neuroscience* **64:**1105–1111.

Send reprint requests to: Dr. Andrew J. Sleight, Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., 4070 Basel, Switzerland. E-mail: anelrew.john.sleight@roche.com