0026-895X/95/010099-05$03.00/0

MOLECULAR PHARMACOLOGY, 47:99–103, 1995.

Identification of 5-Hydroxytryptamine$_7$ Receptor Binding Sites in Rat Hypothalamus: Sensitivity to Chronic Antidepressant Treatment

ANDREW J. SLEIGHT, CATHERINE CAROLO, NADINE PETIT, CATHERINE ZWINGELSTEIN, and ANNE BOURSON

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., 4002 Basel, Switzerland

Received August 24, 1994; Accepted October 13, 1994

SUMMARY

Due to the high level of expression of mRNA for the 5-hydroxytryptamine (5-ht$_7$) receptor in the hypothalamus and the high affinity of 5-HT for this receptor, [^{3}H]5-HT binding was performed in rat hypothalamus to determine whether 5-ht$_7$ receptor binding sites are present in animal tissue. [^{3}H]5-HT binding was performed in the presence of 100 nM pindolol, which is inactive at 5-ht$_7$ receptors but prevents the binding of [^{3}H]5-HT to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor binding sites. Under these conditions, [^{3}H]5-HT bound to a binding site with an affinity of 1.94 nM. Displacement studies showed the pharmacology of the hypothalamic binding site to correlate well with the published pharmacology of the 5-ht$_7$ receptor (r = 0.921). The treatment of rats with fluoxetine (5 mg/kg/day, orally) for 21 days caused a significant reduction in the number of hypothalamic 5-ht$_7$ receptor binding sites. These data suggest that the 5-ht$_7$ receptor binding site is expressed in rat hypothalamus and that this receptor binding site is down-regulated after a chronic increase in the synaptic level of 5-HT.

The effects of the neurotransmitter 5-HT are mediated through a number of receptor subtypes and are thought to play important roles in the pathology of a number of psychiatric disorders, including depression and anxiety (1). These receptor subtypes have been grouped into a number of families, termed 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$ (2). In addition, a number of novel 5-HT receptors, i.e., 5-ht$_5$ (3), 5-ht$_6$ (4), and 5-ht$_7$ (5–7) receptors, have been identified with molecular biological techniques. Although antisense technology has been used to demonstrate the expression and function of the 5-ht$_6$ receptor (8), it is not known whether the 5-ht$_5$ and 5-ht$_7$ receptors are present in the brain.

When expressed in cell lines, the 5-ht$_7$ receptor is positively coupled to adenylate cyclase (5–7). Its pharmacology is unique, in that it has high affinity for both the 5-HT$_{1A}$ agonist 8-OH-DPAT and the 5-HT$_2$ antagonist ritanserin. Northern blot analysis and *in situ* hybridization of the mRNA for the receptor have shown the presence of 5-ht$_7$ mRNA in the cortex, striatum, hippocampus, brainstem, and thalamus of rat brain, with a high concentration in the hypothalamic region (5–7). Therefore, it was the purpose of the present study to determine whether the 5-ht$_7$ receptor binding site could be identified in rat hypothalamic membranes. Hypothalamic binding sites were labeled with the radioligand [^{3}H]5-HT. Pindolol (100 nM), a compound that is inactive at the 5-ht$_7$ receptor (6, 7) but that has nanomolar affinity for the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (9), was used to mask the binding of [^{3}H]5-HT to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. It was not possible to use a higher concentration of pindolol, because literature values for its activity at 5-ht$_7$ receptors note it as inactive at 1 μM concentrations (6, 7). Therefore, to be certain that the compound does not occupy 5-ht$_7$ receptors, the concentration of 100 nM was chosen. At this concentration (a concentration approximately 1 log unit greater than the affinity of pindolol for the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors), pindolol should occupy approximately 100% of the specific [^{3}H]5-HT binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. The effect of chronic treatment with fluoxetine was also studied to determine whether chronically elevated synaptic levels of 5-HT caused a down-regulation of these binding sites.

Materials and Methods

Animals. Male rats (Ibm:RORO; Biological Research Laboratories, Füllinsdorf, Switzerland) weighing 260–280 g at the beginning of the experiment were used in all experiments. The animals were maintained on a 12-hr light/dark cycle, with all tests being performed during the light phase. Ambient temperature was approximately 21° and the relative humidity was 55–65%. Rats had free access to both food (standardized 25–343 pellets; KLIBA, Kaiseraugst, Switzerland) and water.

Tissue preparation for the pharmacological validation of the 5-ht$_7$ binding assay. Rats were killed, the brains were removed, and the hypothalami were dissected. The hypothalami were homogenized in 20 volumes of 50 mM Tris·HCl, and the homogenate was centrifuged at 40,000 × g for 10 min at 4°. The pellet produced was resuspended in the same volume of buffer and centrifuged as before. The pellet pro-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy(di-*n*-propylamino)tetralin; MCPP, metachlorophenylpiperazine.

duced from this second centrifugation was resuspended in the same volume of buffer and incubated for 15 min at 37°. After this incubation, the homogenate was centrifuged as before and the pellet was stored at −80° until used in experiments.

5-ht$_7$ receptor binding assay. The final tissue concentration for 5-ht$_7$ receptor binding was 4 mg/assay. All assays were carried out in an incubation buffer consisting of 50 mM Tris·HCl, 10 μM pargyline, 0.1% ascorbic acid, and 4 mM $CaCl_2$. The radioligand used was [^{3}H]5-HT in the presence of 100 nM pindolol. Saturation assays were performed using eight concentrations of [^{3}H]5-HT (10, 5, 2.5, 1.25, 0.63, 0.32, 0.13, and 0.06 nM) and consisted of 0.1 ml of [^{3}H]5-HT, 0.1 ml of buffer or methiothepin (10 μM, to define nonspecific binding), and 0.8 ml of tissue. The kinetics of the binding assay at room temperature (23°) were determined by association and dissociation experiments. For association analysis the amount of specific binding was determined at various time points after the addition of 1 nM [^{3}H]5-HT; for dissociation analysis the same concentration of radioligand was allowed to bind to hypothalamic membranes and the amount of specific binding remaining at various time points after the addition of a high concentration of a displacing compound (10 μM methiothepin) was determined. Displacement experiments were performed using 1 nM [^{3}H]5-HT and consisted of 0.1 ml of [^{3}H]5-HT, 0.1 ml of buffer or displacing drug, and 0.8 ml of tissue. Displacement curves were determined with at least six concentrations of each drug. The assay tubes were incubated at 23° for 2 hr, after which time the tube contents were rapidly filtered, under vacuum, through GF/B filters, with two 5-ml washes with Tris·HCl buffer (50 mM, pH 7.4 at 23°). The radioactivity retained on the filters was measured by scintillation counting in 4 ml of scintillation fluid (Emulsifier Safe; Packard). All assays were performed in triplicate, in at least three separate experiments.

Preparation of tissue from animals treated chronically with fluoxetine. Fluoxetine was dissolved in saline solution, and a volume of 4 ml/kg was administered orally to rats. Groups of 30 rats were treated for 21 days with either saline or fluoxetine (5 mg/kg, orally). Forty-eight hours after the final treatment, the rats were killed by decapitation and the hypothalami were removed. The hypothalami from five animals within the same treatment group were pooled and the hypothalamic membranes were prepared in the same way as described above. Saturation analysis, also as described above, to determine the dissociation constant and the total number of binding sites (K_d and B_{max}, respectively) was carried out with each tissue preparation, allowing six separate determinations of the binding parameters for each treatment group.

Data analysis. The K_d and B_{max} values were estimated from saturation experiments using the nonlinear regression program EBDA/LIGAND (10, 11). The affinity constants (K_i) of the displacing compounds were estimated from the results of displacement experiments, which were analyzed using the nonlinear regression program LIGAND. This analysis assumes that the Hill coefficient does not differ from unity. Data analysis was performed for each separate displacement curve, and K_i values are given as means of at least three experiments. Differences in the K_d and B_{max} values obtained from membranes prepared from animals treated chronically with antidepressants were analyzed using the unpaired Student's t test.

Chemicals. All chemicals were either synthesized at F. Hoffmann-La Roche or purchased from either Sigma or Research Biochemicals. [^{3}H]5-HT (25.7 Ci/mmol) was purchased from New England Nuclear. For displacement studies, all compounds were dissolved in 10% dimethylsulfoxide to a concentration of 10^{-3} M and diluted in assay buffer.

Results

The binding of [^{3}H]5-HT (in the presence of 100 nM pindolol) to rat hypothalamic membranes was found to have reached equilibrium after 2 hr at room temperature (23°) (Fig. 1), and therefore this was the incubation time used in all subsequent

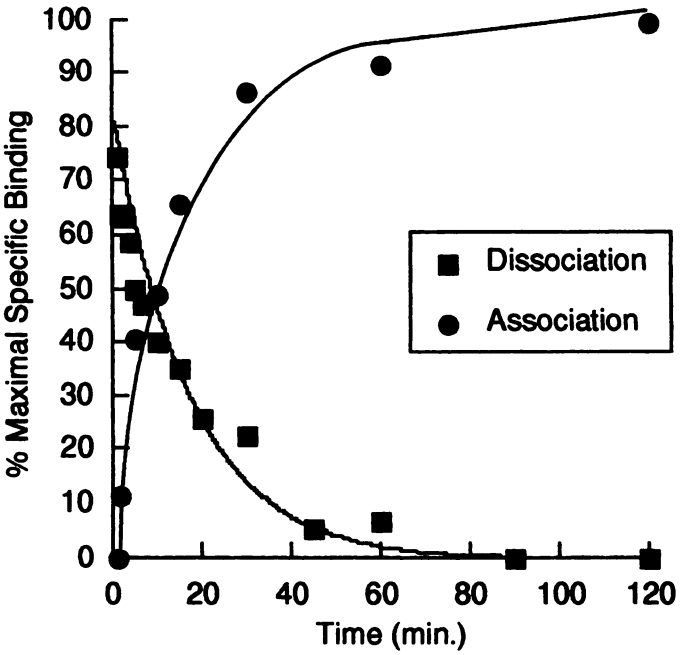

Fig. 1. Association and dissociation of [^{3}H]5-HT with rat hypothalamic membranes.

experiments. The dissociation constant (K_d) calculated from these experiments was 3.7 nM. Saturation analysis of the binding of [^{3}H]5-HT to rat hypothalamic membranes in the presence of 100 nM pindolol showed a saturable population of binding sites. Scatchard analysis of these data showed that there appeared to be a single population of homogeneous binding sites for [^{3}H]5-HT, with a K_d value of 1.94 nM and a maximum number of binding sites (B_{max}) of 8.15 fmol/mg of tissue. These data were in agreement with the K_d calculated from the association and dissociation experiments. An example of a single saturation binding experiment is shown in Fig. 2.

Pharmacological analysis of the binding site in rats shows that it has a close correlation with the binding site described in cells transfected with the rat 5-ht$_7$ receptor ($r = 0.921$, $p < 0.01$). This includes a high affinity for both ritanserin and 8-OH-DPAT. Specific binding was approximately 70% of the total binding, and it was because of this small signal (approximately 600 dpm of specific binding) that the LIGAND program was used to calculate the affinities of the compounds. The affinities of the compounds tested are given in Table 1, and examples of some displacement curves are given in Fig. 3. A graphical representation of the correlation between the affinities calculated for rat hypothalamic membranes and those given in the literature (7), using [^{125}I]-lysergic acid diethylamide as radioligand, is shown in Fig. 4. There was no statistically significant correlation between the data obtained with hypothalamic membranes and results for any other known 5-HT receptor (data not shown).

In membranes from animals treated chronically with fluoxetine (5 mg/kg, orally), a significant reduction in the number of 5-ht$_7$ receptor binding sites was observed ($p < 0.05$, compared with membranes from animals treated chronically with saline, Table 2). Although there was a significant reduction in the number of [^{3}H]5-HT binding sites, no significant changes were observed in the K_d values after chronic treatment with fluoxetine.

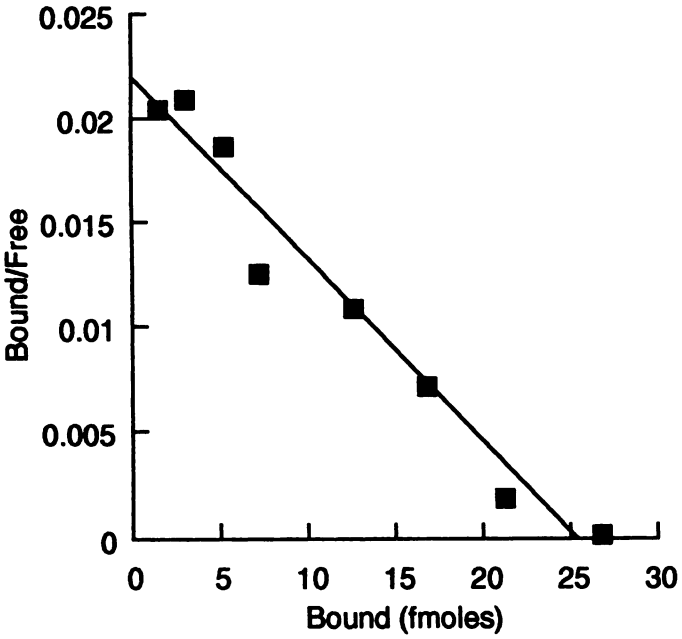

Fig. 2. Representative experiment for the saturation of [^{3}H]5-HT binding sites in a rat hypothalamic membrane preparation (Scatchard-type plot), using a range of [^{3}H]5-HT concentrations (0.06–10 nM). Pindolol (100 nM) was present in all assays, and nonspecific binding was defined by the presence of 10 μM methiothepin.

TABLE 1

Estimated pK_i values for various compounds at the [^{3}H]5-HT binding site

For comparison, the pK_i values for the same compounds at the 5-ht$_7$ receptor binding site are also included (7). All hypothalamic binding assays were performed in the presence of 100 nM pindolol, and nonspecific binding was defined in the presence of 10 μM methiothepin. The data presented here were used to construct the correlation curve shown in Fig. 4.

Compound	pK_i	
	Hypothalamic binding	5-ht$_7$ cell line
5-HT	8.84	8.74
Methiothepin	8.28	8.98
Mesulergine	7.96	8.15
Ritanserin	7.83	7.86
8-OH-DPAT	7.33	7.45
Clozapine	7.31	7.86
Mianserin	6.69	6.68
Ketanserin	6.67	6.95
mCPP	6.42	6.45
Amitriptyline	6.20	6.91
Fluoxetine	5.97	<6.0

Discussion

The purpose of the present study was to determine whether the newly cloned 5-ht$_7$ receptor is expressed in rat brain. High concentrations of the mRNA for the 5-ht$_7$ receptor appear to be present in the hypothalamic regions (5–7) and, therefore, [^{3}H]5-HT binding was attempted in rat hypothalamic membranes. Because [^{3}H]5-HT is a nonspecific radioligand and labels other 5-HT receptor binding sites, including the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor binding sites (12), all binding assays were carried out in the presence of 100 nM pindolol. Pindolol has high affinity for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (9) but has been shown to be inactive at 5-ht$_7$ receptors expressed in cell lines (6, 7).

Under the conditions of the present assay, [^{3}H]5-HT (in the presence of 100 nM pindolol) appeared to label a single population of receptor binding sites, with a K_d of 1.94 nM and a B_{max} of 8.51 fmol/mg of tissue in naive animals. The kinetics of the assay showed that equilibrium was reached within 2 hr at room temperature, and therefore this time was used for incubations in all subsequent experiments. Displacement experiments showed that the affinities of various compounds were similar to those obtained in cell lines transfected with the rat 5-ht$_7$ receptor. Indeed, there was a statistically significant correlation between binding to rat hypothalamic membranes and quoted

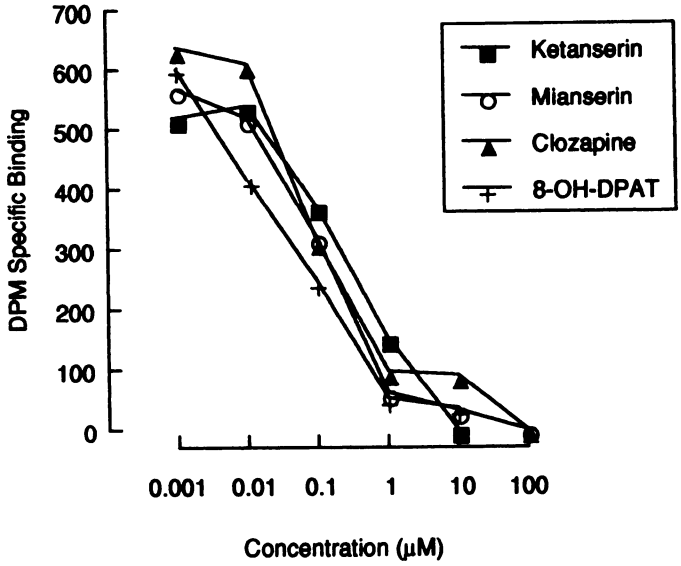

Fig. 3. Representative experiment for the displacement of [^{3}H]5-HT binding from rat hypothalamic binding sites by ketanserin, mianserin, clozapine, and 8-OH-DPAT. Pindolol (100 nM) was present in all assays, and nonspecific binding was defined by the presence of 10 μM methiothepin.

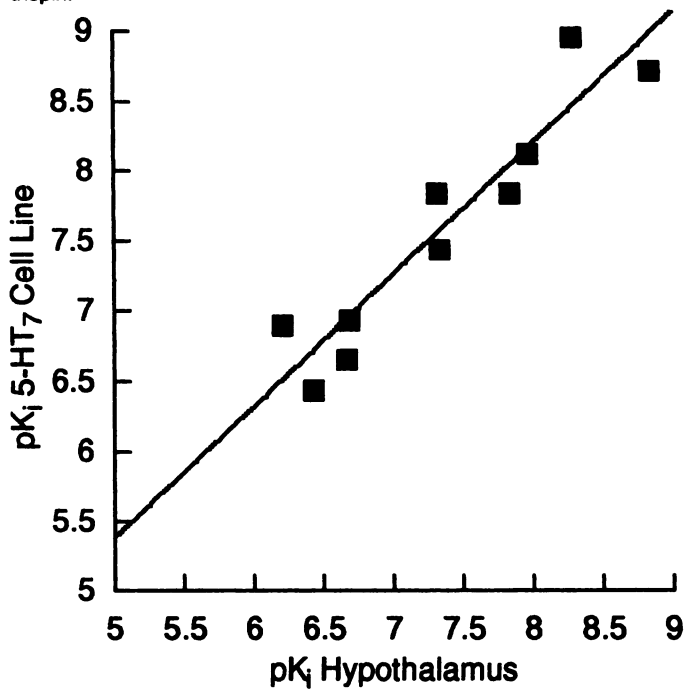

Fig. 4. Correlation of affinities of tested compounds for [^{3}H]5-HT binding sites in the rat hypothalamic membrane preparation with affinities for the recombinant rat 5-ht$_7$ receptor (7). The correlation coefficient was calculated to be 0.921, which was statistically significant ($p < 0.01$).

affinities for the 5-ht$_7$ receptor. No such correlation could be found, however, with any other known 5-HT receptor. The 5-ht$_7$ receptor is characterized as having high affinity for both the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT and the 5-HT$_2$ receptor antagonist ritanserin and, as can be seen from Table 1, this is also the case for the hypothalamic binding site. It should be noted that, because 5-HT is an agonist at the 5-ht$_7$ receptor, it is likely that [^{3}H]5-HT labels only a fraction of the total number of 5-ht$_7$ receptor binding sites (i.e., those that are in their high affinity state, coupled to G proteins). It may be possible to label a greater number of binding sites when a selective antagonist radioligand becomes available.

Certain postsynaptic 5-HT receptors (e.g., 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors) are known to be down-regulated after procedures that chronically increase levels of 5-HT in the synapse, e.g., chronic treatment with some antidepressant drugs (13–18). To determine whether this was also true for the 5-ht$_7$ receptor binding site in the hypothalamus, rats were chronically treated with fluoxetine, an antidepressant that elevates synaptic levels of 5-HT by blocking the removal of 5-HT from the synapse (19). Furthermore, fluoxetine was found to be inactive at the 5-ht$_7$ receptor, both in the present study and in previous reports (7). This treatment caused a significant reduction in the number of 5-ht$_7$ receptor binding sites in the rat hypothalamus but had no effect on the affinity of [^{3}H]5-HT for the receptor (Table 2). It is interesting to speculate that, because the 5-ht$_7$ receptor binding site is affected by chronic treatment with antidepressants, it may have some role in the pathophysiology of some forms of depression. In addition, changes in the hypothalamic-pituitary-adrenal axis have been implicated in depression (20). Therefore, the high level of expression of the mRNA for the 5-ht$_7$ receptor in the hypothalamic regions and the presence of 5-ht$_7$ receptor binding sites could suggest a role for 5-ht$_7$ receptor ligands in the treatment of depression. It would, therefore, be interesting to study any effects on the numbers of 5-ht$_7$ receptor binding sites in the hypothalamus produced by chronic treatment with other classes of antidepressant drugs that have no effect on 5-HT neurotransmission, e.g., maprotiline.

It has also been suggested that the 5-ht$_7$ receptor may be involved in the control of circadian rhythms, by regulating the electrical activity in the suprachiasmatic nucleus (5). Although 8-OH-DPAT appears to be a partial agonist at the 5-ht$_7$ receptor (5), it causes phase shifts in the circadian variation of electrical activity in slices of rat suprachiasmatic nucleus. This effect could not be antagonized by pindolol, which has high affinity for the 5-HT$_{1A}$ receptor but not for the 5-ht$_7$ receptor. The effect could, however, be antagonized by nonselective 5-HT antagonists, including ritanserin, that have high affinity for the 5-ht$_7$ receptor (5, 21). Confirmation that this response is indeed mediated by the 5-ht$_7$ receptor, however, must wait until a selective antagonist for the 5-ht$_7$ receptor is available.

In summary, it appears that the recently cloned 5-ht$_7$ receptor binding site is expressed in rat hypothalamus. In addition, the fact that the receptor is down-regulated after chronic administration of antidepressant drugs suggests that the receptor is functionally expressed in rat brain. Work in our laboratory is presently focused on determining whether the receptor is similarly expressed in the hypothalami of other species. In addition, hypothalamic slices are being studied to determine whether a stimulatory effect of 5-HT on adenylate cyclase activity is present and whether its pharmacology agrees with that of the 5-ht$_7$ receptor.

TABLE 2

Binding parameters (K_d and B_{max} values) calculated from saturation analyses performed on hypothalamic membranes obtained from rats chronically treated with either saline or fluoxetine (5 mg/kg/day, orally)

Rats were treated for 21 days with fluoxetine, and 48 hr after the last administration the animals were killed and the hypothalami were removed. Tissue from five rats within the same treatment group was pooled to obtain one saturation curve, with six separate saturation curves being obtained for each treatment group. Values are given as arithmetic means ± standard errors.

Treatment	K_d	B_{max}
	nM	fmol/mg of tissue
Saline	1.7 ± 0.24	9.36 ± 0.78
Fluoxetine (5 mg/kg/day, orally)	1.9 ± 0.26	6.81 ± 0.66[a]

[a] $p < 0.05$, compared with saline-treated rats.

References

1. Sleight, A. J., P. A. Pierce, A. W. Schmidt, C. R. Hekmatpanah, and S. J. Peroutka. The clinical utility of serotonin receptor active agents in neuropsychiatric disease, in *Serotonin Receptor Subtypes: Basic and Clinical Aspects* (S. J. Peroutka, ed.). Wiley-Liss, New York, 211–228 (1991).
2. Humphrey, P. P. A., P. Hartig, and D. Hoyer. A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol. Sci.* **14**:233–236 (1993).
3. Methes, H., U. Boschert, N. Amlaiky, R. Grailhe, J.-L. Plassat, F. Muscatelli, M.-G. Mattie, and R. Hen. Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. *Mol. Pharmacol.* **43**:313–319 (1993).
4. Monsma, F. J., Y. Shen, R. P. Ward, M. W. Hamblin, and D. R. Sibley. Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Mol. Pharmacol.* **43**:320–327 (1993).
5. Lovenberg, T. W., B. M. Baron, L. de Lecea, J. D. Miller, R. A. Prosser, M. A. Rea, P. E. Foye, M. Racke, A. L. Slone, B. W. Siegel, P. E. Danielson, J. G. Sutcliffe, and M. G. Erlander. A novel adenylyl cyclase-activating serotonin receptor (5-HT$_7$) implicated in the regulation of mammalian circadian rhythms. *Neuron* **11**:449–458 (1993).
6. Ruat, M., E. Traiffort, R. Leurs, J. Tardival-Lacombe, J. Diaz, J.-M. Arrang, and J.-C. Schwartz. Molecular cloning, characterization and localization of a high-affinity serotonin receptor (5-HT$_7$) activating cAMP formation. *Proc. Natl. Acad. Sci. USA* **90**:8547–8551 (1993).
7. Shen, Y., F. J. Monsma, M. A. Metcalf, P. A. Jose, M. W. Hamblin, and D. R. Sibley. Molecular cloning and expression of a 5-hydroxytryptamine$_7$ serotonin receptor subtype. *J. Biol. Chem.* **24**:18200–18204 (1993).
8. Sleight, A. J., F. J. Monsma, E. Borroni, R. H. Austin, and A. Bourson. Effects of altered 5-HT$_6$ expression in the rat: functional studies using antisense oligonucleotides, in *Proceedings of the Third IUPHAR Satellite Meeting on Serotonin.* (1994) in press.
9. Hoyer, D., G. Engel, and H. O. Kalkman. Molecular pharmacology of 5-HT$_1$ and 5-HT$_2$ recognition sites in rat and pig brain membranes: radioligand binding studies with [^{3}H]5-HT, [^{3}H]8-OH-DPAT, (−)[^{125}I]iodocyanopindolol, [^{3}H]mesulergine and [^{3}H]ketanserin. *Eur. J. Pharmacol.* **118**:13–23 (1985).
10. McPherson, G. A. KINETIC, EBDA, LIGAND, LOWRY: a collection of radioligand binding analysis programmes. Biosoft, Cambridge, UK (1985).
11. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–229 (1980).
12. Sleight, A. J., and S. J. Peroutka. 5-HT receptor binding sites in the central nervous system and their distribution, in *Central Serotonin Receptors and Psychotropic Drugs* (C. A. Marsden and D. J. Heal, eds.). Blackwell Scientific Publications, Oxford, UK, 3–15 (1992).
13. Palfreyman, M. G., A. K. Mir, M. Kubina, D. N. Middlemiss, M. Richards, M. D. Tricklebank, and J. R. Fozard. Monoamine receptor sensitivity changes following chronic administration of MDL 72394, a site directed inhibitor of monoamine oxidase. *Eur. J. Pharmacol.* **106**:539–546 (1986).
14. Sleight, A. J., C. A Marsden, M. G. Palfreyman, A. K. Mir, and W. Lovenberg. Chronic MAO A and MAO B inhibition decreases the 5-HT$_{1A}$ receptor mediated inhibition of forskolin-stimulated adenylate cyclase. *Eur. J. Pharmacol.* **154**:255–261 (1988).
15. Peroutka, S. J., and S. H. Snyder. Long-term antidepressant treatment decreases spiroperidol-labeled serotonin receptor binding. *Science (Washington D. C.)* **210**:88–90 (1980).
16. Crews, F. T., J. A. Scott, and N. H. Shorstein. Rapid down-regulation of serotonin$_2$ receptor binding during combined administration of tricyclic antidepressant drugs and *alpha*$_2$ antagonists. *Neuropharmacology* **22**:1203–1209 (1983).
17. Stoltz, J. F., C. A. Marsden, and D. N. Middlemiss. Effect of chronic

antidepressant treatment and subsequent withdrawal on [^{3}H]5-hydroxytryptamine and [^{3}H]spiperone binding in rat frontal cortex and serotonin receptor mediated behaviour. *Psychopharmacology* **80**:150–155 (1983).

18. Kendall, D. A., and S. R. Nahorski. 5-Hydroxytryptamine-stimulated inositol phospholipid hydrolysis in rat cerebral cortex slices: pharmacological characterization and effects of antidepressants. *J. Pharmacol. Exp. Ther.* **233**:473–479 (1985).
19. Perry, K. W., and R. W. Fuller. Effect of fluoxetine on serotonin and dopamine concentration in microdialysis fluid from rat striatum. *Life Sci.* **50**:1683–1690 (1992).
20. File, S. E. Interactions of anxiolytic and antidepressant drugs with hormones of the hypothalamic-pituitary-adrenal axis. *Pharmacol. Ther.* **46**:357–375 (1990).
21. Prosser, R. A., R. R. Dean, D. M. Edgar, H. C. Heller, and J. D. Miller. Serotonin and the mammalian circadian system. I. *In vitro* phase shifts by serotonergic agonists and antagonists. *J. Biol. Rhythms* **8**:1–16 (1993).

Send reprint requests to: A. J. Sleight, Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., 4002 Basel, Switzerland.