0026-895X/92/040627-07$03.00/0

MOLECULAR PHARMACOLOGY, 42:627-633

Identification of Receptor Domains that Modify Ligand Binding to 5-Hydroxytryptamine$_2$ and 5-Hydroxytryptamine$_{1c}$ Serotonin Receptors

M. S. CHOUDHARY, S. CRAIGO, and B. L. ROTH
Department of Psychiatry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106
Received April 13, 1992; Accepted July 24, 1992

SUMMARY

Serotonin [5-hydroxytryptamine (5-HT)] receptors are distinguished pharmacologically by their characteristic affinities for agonists and antagonists. Two serotonin receptors, the 5-HT$_2$ and 5-HT$_{1c}$, share a number of pharmacologic and structural properties while differing in their affinities for certain agonists and antagonists. To identify regions of the 5-HT$_2$ and 5-HT$_{1c}$ receptors important for specifying their unique pharmacology, we constructed six chimeric 5-HT$_2$/5-HT$_{1c}$ receptors in which domains of each receptor were exchanged. The abilities of several drugs to inhibit [^{3}H]mesulergine bound to the chimeric and parent receptors transiently expressed in COS-7 cells were then examined. For spiperone and haloperidol (both butyrophenones), chimeras that exchanged transmembrane (TM) domains I and II or TMs I-III had the greatest effects on binding affinity. The binding affinity of cinanserin (a cinnamanilide) was significantly changed in all the chimeras studied. In contrast, the binding of ketanserin (a 4-fluorobenzoylpiperidine) was strongly influenced by chimeras that exchanged TMs I-III (but not I and II) and by chimeras that exchanged intracellular loop 3 to TM VII. 5-HT binding affinity was greatly altered for chimeras that exchanged domains of intracellular loop 3 to TM VII, with minor effects being noted for chimeras that exchanged TMs I and II and I-III. The affinities of the nonselective drugs mesulergine, mianserin, and *m*-chlorophenylpiperazine were relatively unaffected when domains of the 5-HT$_2$ and 5-HT$_{1c}$ receptors were exchanged. Taken together, these results imply that structurally diverse 5-HT$_2$ antagonists utilize distinct regions of the 5-HT$_2$ receptor for high affinity binding.

Serotonin (5-HT) mediates many physiologic processes including the regulation of the sleep-wake cycle, appetite, mood, perception, aggression, platelet aggregation, and smooth muscle contraction (1). To induce these effects, a large family of serotonergic receptors has evolved. Many members of this family have now been cloned and include the 5-HT$_{1c}$ (2), 5-HT$_{1B}$ (3), 5-HT$_{1c}$ (4), 5-HT$_{1D}$ (5), 5-HT$_2$ (6, 7), and 5-HT$_3$ (8) receptors. Other serotonin receptors have been pharmacologically identified (5-HT$_{1P}$, 5-HT$_4$, and stomach fundus 5-HT receptors) (see Ref. 9 for review) but await verification by structural criteria (e.g., amino acid or cDNA sequence).

The 5-HT$_2$ and 5-HT$_{1c}$ receptors share a number of structural, functional, and pharmacologic similarities. Both receptors have comparable affinities for several pharmacologic agents (10–12a), transduce their signals via activation of phosphoinositide metabolism (13–16), are regulated by exogenous agents in a similar manner (17, 18), show similar developmental profiles (19), and display a relatively high number of structural homologies. In the putative TM regions, the 5-HT$_2$ and 5-HT$_{1c}$ receptors show overall identities of 78% (39 of 176 residues nonidentical) and similarities of 91% (16 of 176 residues nonsimilar), with TM V showing 100% identity. In addition to these similarities, the 5-HT$_2$ and 5-HT$_{1c}$ receptors exhibit a number of pharmacologic differences (20, 21). We recently surveyed typical and atypical neuroleptic binding to the cloned 5-HT$_{1c}$ receptor and found many agents that effectively distinguish between the two receptors (12a). These studies prompted us to determine the structural features required for these interesting pharmacologic differences.

Previous studies of receptor structure-affinity relationships have utilized two major approaches, i.e., site-directed mutagenesis and the construction of chimeric proteins. Site-directed mutagenesis studies have been important for identifying residues that are invariantly important for agonist and/or antagonist binding (22–24). The chimeric approach, on the other hand, has been useful for identifying domains of the catecholamine and muscarinic receptors important for specifying the unique pharmacology of each receptor subtype (25–28).

This work was supported in part by grants from the Scottish Rite Schizophrenia Research Foundation and the Pharmaceutical Manufacturers Association Foundation to B.L.R. B.L.R. is the recipient of a Pharmaceutical Manufacturers Association Foundation Research Starter Grant.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; dsDNA, double-stranded DNA; PCR, polymerase chain reaction; bp, base pair(s); *m*-CPP, *m*-chlorophenylpiperazine; TM, transmembrane; i3, intracellular loop 3.

Because we are interested in domains that specify the pharmacology of each receptor, we chose the chimeric receptor approach for our initial studies.

For the present experiments, we constructed six chimeric receptor proteins in which several domains of the 5-HT_2 and 5-HT_{1c} receptors were exchanged. After verification by dsDNA sequencing, we transiently expressed each receptor in COS-7 cells and then determined the binding profiles for all constructs. For structurally diverse antagonists, different regions of the 5-HT_2 receptor appeared essential for high affinity ligand binding. These results are most consistent with recently published models (29, 30) that predict that ligands utilize several distinct residues located in multiple TM domains to bind with high affinity to 5-HT_2 receptors.

Experimental Procedures

Materials

Tissue culture reagents were from GIBCO/BRL (Gaithersburgh, MD). [^{3}H]Ketanserin (66 Ci/mmol) and α-^{35}S-dATP (1500 Ci/mmol) were from New England Nuclear (Boston, MA). [^{3}H]Mesulergine (70 Ci/mmol) was from Amersham Corporation. Restriction enzymes were from New England Biolabs (Boston, MA), with most other molecular biology reagents being purchased from United States Biochemicals (Cleveland, OH) or Stratagene (Torry Pines, CA). *Taq* polymerase was from Cetus Corporation. COS-7 cells were a gift from B. Kobilka and M. VonZastrow (Stanford University). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Plasmid Constructions

pSVK3-SR2 and pSVK3–5HT1c. Construction and expression of these plasmids have been detailed previously (12a).[1] Conventional techniques of molecular biology were followed (31, 32). In some experiments, GF-6 and A9 cells that stably express the 5-HT_2 and 5-HT_{1c} receptors (gifts of D. Julius and B. Hoffman, respectively) were used, with equivalent results.

Overall strategy for chimeric receptor construction. Before construction of chimeric serotonin receptors, the amino acid sequences of the rat 5-HT_2 (Accession Number M30705) and the rat 5-HT_{1c} cDNAs were aligned using the University of Wisconsin GCG Software command BESTFIT. Regions with appropriate restriction enzyme sites were identified and chimeras were designed that met the following criteria: 1) homologous portions of each receptor were exchanged and 2) deletions, insertions, and point mutations were avoided. In general, chimeras were constructed using PCR technology so that linkers and adaptors (which add extra amino acids) were averted. All PCR-generated inserts were verified by dsDNA sequencing (see Table 1).

Chimer 1. pSVK3-SR2 was digested with *Xho*I (37°; 1 hr) to remove bp +820 to +1740 (see Fig. 1) and was treated with calf intestinal alkaline phosphatase (37°; 30 min), and the 4.8-kilobase fragment was gel purified. The corresponding portion of the 5-HT_{1c} cDNA was then amplified by PCR, using the primers shown in Table 1. Sequences were placed 5′ to the *Xho*I adaptors on both primers because of the tendency of *Taq* polymerase to produce ragged ends, thus ensuring that the *Xho*I adaptors would be synthesized in addition to the amplified sequence. PCR was done using an MJ Research Thermocycler and the following conditions: 95° (5 min; initial denaturation), then 30 cycles of 95° (30 sec), 30° (30 sec), and 72° (1.5 min), and then 7-min terminal extension at 72°; 1 μM each primer, 200 μM deoxynucleoside triphosphates, 0.5 unit of *Taq* polymerase, 1× PCR (1.5 mM $MgCl_2$, 10 mM Tris-Cl, 50 mM KCl, 0.01% gelatin; pH = 8.3) buffer (Cetus Chemical Company); and 100-μl final volume with 50-μl mineral oil overlay. After the reaction, a small aliquot (5 μl) of the PCR product was checked using a 1% TBE (20× TBE = 1 M Tris base, 1 M boric acid, 20 mM disodium EDTA) gel. A single band of approximately 613 bp was visualized by ethidium bromide staining. The rest of the PCR product was purified using a GeneClean kit (Bio-101) and polished with Klenow polymerase [200 μM deoxynucleosidetriphosphates, 1× Klenow buffer (Stratagene) (7 mM Tris-Cl, 7 mM $MgCl_2$, 1 mM dithiothreitol; pH = 7.3), 1 unit of Klenow polymerase] at 25° for 1 hr. The sample was heated to 65° to denature the Klenow polymerase and then digested with *Xho*I for 2 hr at 37°. After repurification using the GeneClean procedure, the *Xho*I insert was ligated to pSVK3-SR2 prepared as described above. After bacterial transformation, minipreps, and large scale plasmid purification (31, 32), the identity of the plasmid was verified by three criteria, 1) digestion with *Xho*I (removes the insert), 2) digestion with *Stu*1 (verifies orientation), and 3) dsDNA sequencing (see below) of the complete insert. Similar techniques were used for the construction of the remaining chimeras.

Chimer 2A. pSVK3-SR2 was digested with *Kpn*I and *Sal*I (removes bp −11 to +416), and the 1400-bp fragment was subcloned into pSVK3 to yield pSVK3-SR2Δ416. The corresponding portion of the 5-HT_{1c} receptor was amplified with the primers shown in Table 1 and was digested with *Kpn*I and chimer 2A was prepared as described above for chimer 1.

Chimer 2B. The *Kpn*I site from pSVK3 was removed by digestion with *Sac*I to produce pSVK3ΔSacI, and the 5-HT_2 cDNA was inserted into the *Eco*RI site. The resulting construct (pSVK3ΔSacI-SR2) was digested with *Kpn*I (removes bp +179 to +416). The corresponding portion of the 5-HT_{1c} sequence was amplified using the primers listed in Table 1.

Chimer 3. Chimer 2B was excised with *Eco*RI, and the insert was ligated to *Eco*RI-digested pSVK3. The resulting plasmid was digested with *Xho*I, and the *Xho*I insert from chimer 1 was inserted in the proper orientation to produce chimer 3.

Chimer 4. pSVK3-SR2 and pSVK3–5HT1c were used to transform the dam$^-$ strain of *Escherichia coli* (JM110). After large scale plasmid preparations, pSVK3–5HT1c was digested with *Bcl*I and *Xba*I (removes bp +694 to +2312), treated with calf intestinal alkaline phosphatase, and gel purified. pSVK3-SR2 was doubly digested with *Bcl*I and *Xba*I, and the fragment (bp +751 to +1829) was gel purified and ligated to pSVK3–5HT1c prepared as described above.

Chimer 5. pSVK3–5HT1c, grown in dam$^-$ *E. coli*, was digested with *Bal*I and *Sna*BI to remove bp +175 to +471. The corresponding homologous portion of the 5-HT_2 cDNA was amplified using the primers in Table 1, to produce chimer 5.

dsDNA sequencing. dsDNA sequencing was performed on the plasmids prepared as described above by using a Sequenase-II kit from United States Biochemicals, which utilizes the dideoxynucleotide (chain termination) method (33). When necessary, primers up- and downstream of the inserted sequences were used to verify the splice sites. Additional sequencing primers were used to verify the entire sequences of the insertions.

Transient Transfection of COS-7 Cells

Transient transfection of COS-7 cells was performed as previously detailed, using the DEAE-dextran technique (12a, 34). In brief, COS-7 cells were seeded at 3 × 10^6 cells/100-mm plate, grown overnight, and exposed to 2 ml of DEAE-dextran/plasmid mixture. After a 10–15-min exposure, 20 ml of complete medium (high-glucose Dulbecco's modified Eagle medium with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin) containing 80 μM chloroquine were added and the incubation was continued for 2.5 hr at 37° in a 5% CO_2 incubator. The mixture was then aspirated, and 10 ml of complete medium containing 10% dimethylsulfoxide were added, with shaking, for 150 sec. After aspiration, 15 ml of complete medium with dialyzed serum were added and the incubation was continued an additional 65 hr.

Cells were harvested with a cell scraper, pelleted at 1000 × g for 10 min, resuspended in 50 mM Tris·HCl (pH 7.40), and centrifuged at

[1] S. Garlow, D. Morilak, B. L. Roth, and R. D. Ciaranello. Production and characterization of an antibody for the 5-HT_2 receptor which labels a subpopulation of rat forebrain neurons. Submitted for publication.

14,000 × *g* for 30 min. The cell pellets were used immediately or stored at −80° until assay, with equivalent results. For all experiments, parallel assays were performed with cells expressing the parent receptor.

Radioligand Binding

Radioligand binding was performed as previously detailed (12a, 12b, 18, 19, 35). In brief, cells were lysed and membranes were harvested by centrifugation and diluted to a total volume of 0.5 ml together with radioligand and unlabeled competitor. After a 90-min incubation at 25°, membranes were harvested onto 0.05% polyethyleneimine-pretreated GF/C filters with a Brandel cell harvester. After drying, filters were quantitated by liquid scintillation counting. Specific binding represented at least 90% of total binding for all the experiments reported here. Experiments were performed and data were analyzed precisely as described by Munson and Rodbard, using the LIGAND program (see Ref. 12b for details); the *F* test was used to distinguish between various binding models at a level of significance of 0.01. For all experiments reported here, the curves represent the theoretical fits obtained with the parameter estimates derived from the LIGAND program. Protein was determined using a Bio-Rad kit (Richmond, CA). Nonspecific binding, a fitted parameter, was determined with either 1 μM mesulergine or 1 μM mianserin and was equivalent regardless of the unlabeled ligand.

Results

Expression of [^{3}H]mesulergine and [^{3}H]ketanserin binding. Fig. 1 shows the structures of the receptor cDNAs used in this study. Table 1 shows the primers used for the construction of the chimeras. In all cases, homologous portions of each receptor (as identified using the program BESTFIT) were used. Additionally, dsDNA sequencing verified that no insertions, deletions, or point mutations occurred during the construction of the chimeric receptors. All receptors, with the exception of chimer 2A, expressed high levels of [^{3}H]mesulergine binding. Because chimer 2A did not express significant [^{3}H]mesulergine or [^{3}H]ketanserin binding, it was not used for subsequent experiments.

Figs. 2–4 show typical isotherms for the inhibition of [^{3}H]mesulergine and [^{3}H]ketanserin binding. Table 2 displays the K_d and B_{max} data for each receptor studied. As is clear, each receptor had relatively high affinities for [^{3}H]mesulergine; in the cases of the 5-HT$_2$ and 5-HT$_{1c}$ receptors, these values were similar to those previously reported for the cloned receptors (12a) as well as for binding identified in brain (35–37). Chimers 1, 2B, 3, 4, and 5 had affinities for [^{3}H]mesulergine similar to values obtained for the 5-HT$_2$ and 5-HT$_{1c}$ receptors.

All of the chimeras (except 2A) and the 5-HT$_2$ receptor bound [^{3}H]ketanserin with high affinities; in the case of chimer 1 this represented a 7.6-fold decrease in affinity, compared with the parent 5-HT$_2$ receptor (see Table 1 for values). For chimer 4 a 3.8-fold increase in affinity was seen, compared with the parent 5-HT$_{1c}$ receptor (see Table 1), whereas chimer 5 showed an 8-fold increase in affinity. For chimer 3 a 9-fold decrease in affinity, compared with the parent 5-HT$_2$ receptor, was found, whereas chimer 2 showed minimal changes (2-fold) in affinity for [^{3}H]ketanserin.

Pharmacologic characterization of chimeric serotonin receptors. We next performed a study of the pharmacology of each of the receptor constructs we created. For these experiments, the chimeric receptor of interest together with its progenitor were studied simultaneously, to limit experiment-to-experiment variability. Additionally, the same stocks of unlabeled ligands were used for all the studies reported here.

Figs. 5 and 6 show typical competition binding isotherms for each receptor using the unlabeled drugs spiperone and haloperidol, respectively. Table 2 presents the K_i values obtained from the LIGAND program for experiments in which the data were fit simultaneously. As can be seen, chimers 1–3 had lower affinities for spiperone, cinanserin, and haloperidol, compared with the predecessor 5-HT$_2$ receptor. For spiperone these ranged from 6- (chimer 1) to 163-fold (chimer 3) lower than the 5-HT$_2$ receptor. Haloperidol had an 8- (chimer 1) to 49-fold (chimer 3) lower affinity, compared with the 5-HT$_2$ receptor. Importantly, the affinities of mesulergine and mianserin were similar for all receptors studied.

Chimers 4 and 5 displayed significantly enhanced affinity for ketanserin and cinanserin (3–8-fold). Chimer 4 showed <3-fold higher affinity for spiperone and no change for haloperidol. Consistent with the results obtained above, chimer 5 (which switches TMs I-III) showed markedly enhanced affinity for spiperone (296-fold) and haloperidol (11.5-fold), compared with the 5-HT$_{1c}$ receptor. Taken together with the results obtained

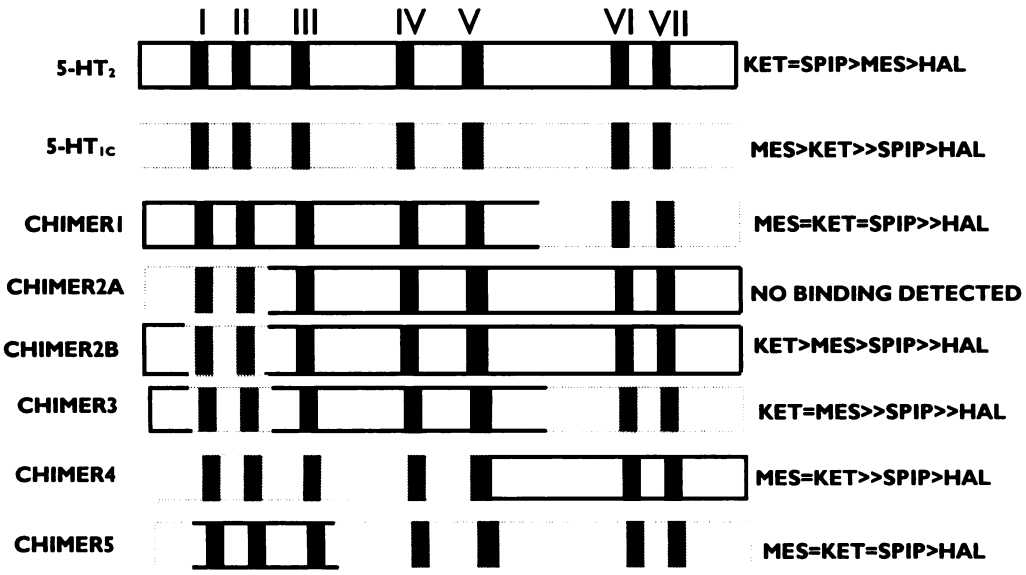

Fig. 1. Structures of receptor cDNAs. Shown are diagrams of the cDNAs used in these experiments. 5-HT$_2$ sequences are shown as *solid*; 5-HT$_{1c}$ sequences are *dotted*. *Roman numerals*, approximate locations of TM domains. Figures are not drawn to scale. Also shown are the rank orders of potencies for the antagonists studied. *MES*, mesulergine; *KET*, ketanserin; *SPIP*, spiperone; *HAL*, haloperidol.

TABLE I

Primers used for construction of chimeric serotonin receptors

Shown are the sequences of the PCR primers used in construction of the chimeric receptors reported in this study. For all chimeras, the homologous portion of the donating receptor was amplified in such a way that no frame-shift, insertion, or deletion mutations were constructed, as is seen by the translations of the splice sites. The sequences of the chimeric receptors were verified by dsDNA sequencing of the entire inserted sequence including the splice site.

Chimera	Upstream PCR primer[a]	Downstream PCR primer	Restriction sites	Upstream splice site	Downstream splice site
Chimer 1	5′-CCACTCGAGAGGAACTGGCTAAT-ATG-3′	5′-CCGCTCGAGTTCGCTTACACACT-ACTA-3′	*XhoI*/*XhoI*	TREELANM	ISSV*
Chimer 2A	5′-GGGGTACCCTGAAGCAATCATG-GTG-3	5′-GCCGGTACCCATAAAGAATAGC-AAGCAG-3′	*KpnI*/*KpnI*	KPEAIMV	LYGYR
Chimer 2B	5′-GGGTACCTCTCCTCCGATGGTGG-ACGCTTG-3′	5′-GCCGGTACCCATAAAGAATAGC-AAGCAG-3′	*KpnI*/*KpnI*	GYLSSDGG	LYGYR
Chimer 3[b]					
Chimer 4	None	None	*BclI*/*BclI*	MVITY	End of plasmid
Chimer 5	5′-AATGGCCAGCTTTATTGACAACT-3′	5′-AATACGTATGGCGACATAGCG-GTC-3′	*BalI*/*Sna*BI	WPALLT	VAIRN

[a] * termination; M, initiation methionine; bold, 5-HT_{1C} sequence; underlined 5-HT_2 sequence.
[b] Chimer 3 is a combination of chimer 2B and chimer 1.

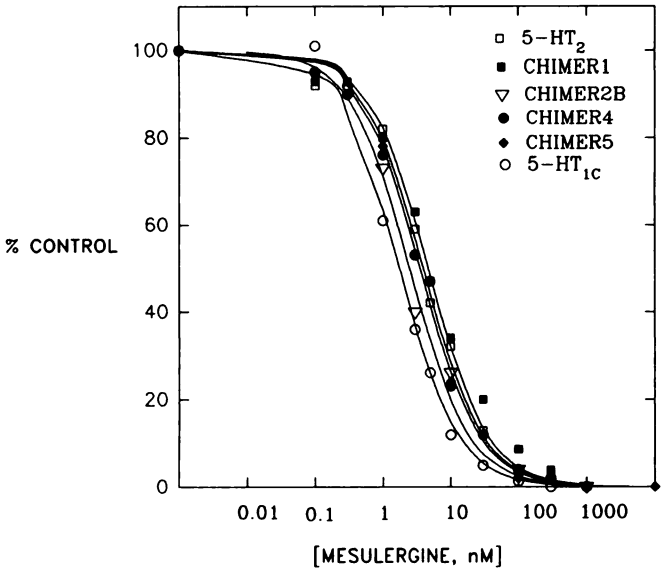

Fig. 2. Mesulergine inhibition isotherms for chimeric and parent receptors. Shown are mean percentages of control specific binding (no unlabeled mesulergine added) for three or four individual experiments, with duplicate determinations at each point, for the inhibition of [^{3}H]mesulergine binding (0.5–1.0 nM) by unlabeled mesulergine. Standard errors are not shown and ranged from 1 to 7% for each value. Specific binding represented at least 90% of total binding. For a typical experiment in which 58,000 counts of [^{3}H]mesulergine were added to each tube, total binding represented 2800 cpm, with nonspecific binding (1 μM mianserin) being 108 cpm.

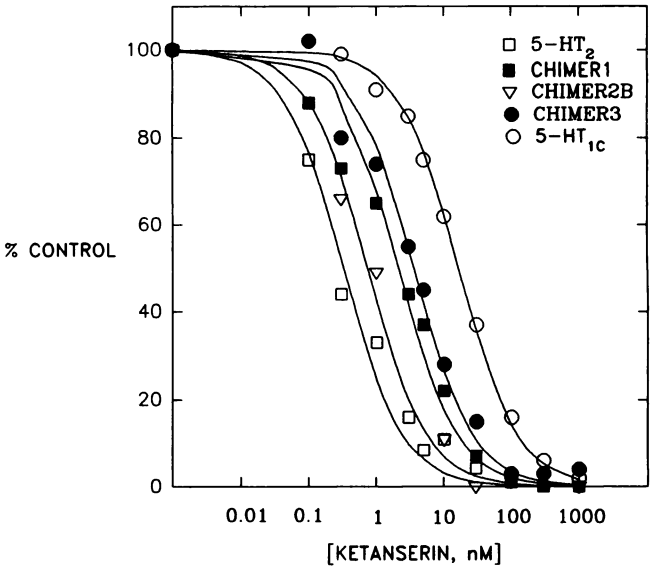

Fig. 3. Ketanserin inhibition isotherms for 5-HT_2-derived chimeric receptors. Shown are mean percentages of control specific binding (no unlabeled ketanserin added) for three separate experiments, with duplicate determinations for each point, for inhibition of [^{3}H]ketanserin (chimer 1, chimer 2B, chimer 3, and 5-HT_2 receptors) binding by unlabeled ketanserin. Specific binding represented at least 90% of total binding.

with chimers 1–3, these findings indicate that the regions encompassed by TMs I-III are essential for high affinity spiperone and haloperidol binding to the 5-HT_2 receptor.

We next studied the agonist profiles of the receptors by evaluating the binding affinities of the full agonist 5-HT and the partial agonist *m*-CPP for the chimeric and parent receptors. Table 2 gives the accumulated data for 5-HT and the partial agonist *m*-CPP. As can be seen, 5-HT had greatly differing affinities for the chimeric receptors we prepared. In general, agonist affinity was shifted toward that seen with donated carboxyl terminus sequences (i3-TMVII). Thus, in the case of chimers 1 and 3, but not chimer 2, 5-HT affinity increased by 6–13-fold, whereas for chimer 4 the affinity of 5-HT was decreased by 70-fold. Chimer 5, which exchanges TMs I-III, shifted the affinity of 5-HT 28-fold. *m*-CPP, which has similar affinities for both the 5-HT_2 and 5-HT_{1c} receptors, showed minor changes in affinities among the various chimeras tested.

Discussion

The major finding of this paper is that multiple structural domains are apparently important for specifying the unique pharmacology of 5-HT_2 and 5-HT_{1c} receptors. We also discovered that structurally distinct 5-HT_2 antagonists were affected to differing degrees by the chimeras we constructed. These results may indicate, as predicted by Leysen (38), that structurally diverse 5-HT_2 antagonists utilize different regions of

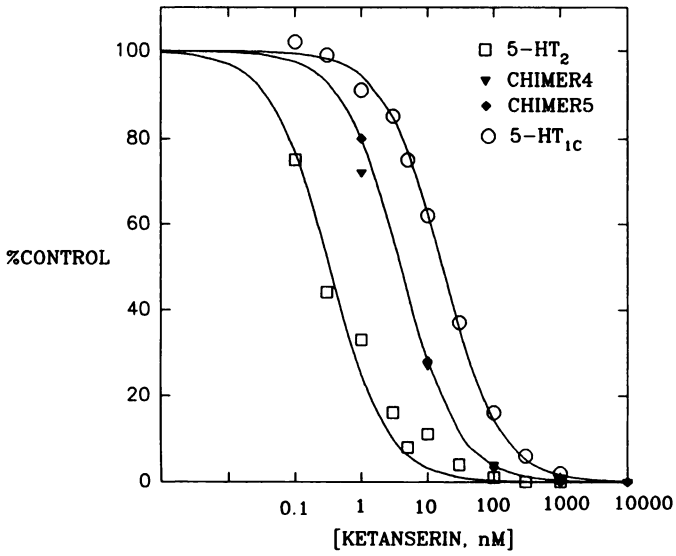

Fig. 4. Ketanserin inhibition isotherms for 5-HT_{1c}-derived chimeric receptors. Shown are mean percentages of control specific binding (no unlabeled ketanserin added) for three separate experiments, with duplicate determinations for each point, for inhibition of [^{3}H]mesulergine (chimer 4, chimer 5, and 5-HT_{1c}) binding by unlabeled ketanserin. Specific binding represented at least 90% of total binding.

the 5-HT_2 receptor for high affinity binding. Our results are also in accord with recent three-dimensional computerized models of ligand binding to 5-HT_2 receptors (29, 30), which predict that amino acids located in multiple TM domains contribute to high affinity agonist and antagonist binding.

Several types of control experiments were performed to verify the construction of each receptor type *in vitro*. Firstly, we measured the affinities for [^{3}H]mesulergine, *m*-CPP, and mianserin because these ligands do not readily distinguish between 5-HT_2 and 5-HT_{1c} receptors. We reasoned that, if functional chimeric receptors were being synthesized and properly inserted into the membranes, they should bind [^{3}H]mesulergine, mianserin, and *m*-CPP equally. Secondly, we evaluated the abilities of several drugs that distinguish between the 5-HT_2 and 5-HT_{1c} receptors to bind to the chimeric receptors. In many instances, the drugs displayed predictable shifts in affinity towards those for the donated sequences. Finally, the sequence of each chimera was verified by dsDNA sequencing using primers up- and downstream of the donated sequences. For the chimeras reported here, no insertions, deletions, or point mutations were found.

A major goal of serotonin receptor molecular pharmacology is the identification of the specific structural features essential for defining the unique pharmacology of each receptor. Before the present findings, few published reports had directly addressed this issue. Buck *et al.* (39) presented preliminary findings, based on deletion mutations, that implied that the TM domains could be important for binding 5-HT. Additionally, Hibert *et al.* (29) presented a theoretical model of 5-HT binding to the 5-HT_2 receptor that emphasized interactions of amino acid residues found in many of the putative TM domains. Dahl's group (30) has recently formulated a model for the binding of agonists and antagonists to 5-HT_2 receptors that differs significantly from that obtained by Hibert. Predictions of the model of Evardsen *et al.* (30) will be discussed below in relationship to the present findings. Finally, Guan *et al.* (40) presented findings suggesting that an asparagine residue found in TM VII of the 5-HT_{1A} receptor may be essential for binding pindolol.

Previous investigators have emphasized the pharmacologic similarities of the 5-HT_2 and 5-HT_{1c} receptors (20, 21, 41). As already mentioned, these receptors also show remarkable resemblances in terms of regulatory properties (17, 42). Clearly, though, the 5-HT_2 and 5-HT_{1c} receptors differ in a number of respects, including regional brain distributions (43, 44) and the affinity profiles of a number of drugs (36). We recently completed a survey of antipsychotic agents and found that these drugs, as a group, prefer the 5-HT_2 receptor (12a). Several drugs preferred the 5-HT_2 receptor by 100-fold or more; we could not identify agents that prefer the 5-HT_{1c} receptor. Those studies suggested to us that certain antipsychotic agents could serve as convenient reference drugs for our structural studies.

We chose for initial investigations the terminal TM domains (see Fig. 1) for the following reasons. Firstly, previous experiments involving adrenergic receptors directly implicated TMs VI–VII in antagonist pharmacology (25, 26). Secondly, more recent studies demonstrated that an asparagine in TM VII was essential for binding a variety of β-adrenergic antagonists (24). Thirdly, Wess *et al.* (27, 28) found that the terminal regions of muscarinic receptors possessed major structural features required for the unique pharmacology of the m2 and m3 muscarinic receptors. Fourthly, Guan *et al.* (40) showed that an asparagine in TM VII is critical for the binding of pindolol to

TABLE 2

Affinities of ligands for 5-HT_2, 5-HT_{1c}, and chimeric receptors expressed *in vitro*

Data represent mean ± standard error of computerized estimates of K_d values (for [^{3}H]mesulergine and [^{3}H]ketanserin) or K_i values of unlabeled ligands for three to five separate experiments.

Receptor	K_d or K_i							
	Ketanserin	Spiperone	Mesulergine	Mianserin	Cinanserin	Haloperidol	5-HT	*m*-CPP
	nM							
5-HT_2	0.38 ± 0.1	0.33 ± 0.12	1.9 ± 0.2	1.0 ± 0.2	2.1 ± 0.2	15.8 ± 7.4	1303 ± 391	163 ± 65
Chimer 1	2.9 ± 1.3[a]	1.9 ± 0.6[a]	2.9 ± 0.9	2.6 ± 0.9	7.5 ± 1.3[a]	127 ± 6.4[a]	217 ± 72[a]	126 ± 38
Chimer 2B	0.68 ± 0.2	19 ± 7[a]	6.7 ± 2.8	ND[b]	12.5 ± 6[a]	579 ± 191[a]	1197 ± 383	144 ± 82
Chimer 3	3 ± 0.7[a]	54 ± 21[a]	6 ± 3	2.4 ± 0.4	11.3 ± 3[a]	776 ± 180[a]	95 ± 10[a]	826 ± 221[a]
5-HT_{1c}	10 ± 2	619 ± 246	1.28 ± 0.3	0.4 ± 0.1	26 ± 8	2050 ± 533	22 ± 8	52 ± 20
Chimer 4	2.6 ± 0.8[c]	271 ± 30[c]	1.9 ± 0.2	0.5 ± 0.05	4.8 ± 0.4[c]	2578 ± 773	1534 ± 506[c]	138 ± 60
Chimer 5	1.3 ± 0.1[c]	2.1 ± 0.6[c]	0.8 ± 0.3	0.6 ± 0.06	6.8 ± 0.6[c]	178 ± 22[c]	625 ± 48[c]	137 ± 10

[a] $p < 0.01$ versus 5-HT_2 K_i value (F test).
[b] ND, not determined.
[c] $p < 0.01$ versus 5-HT_1,[c] K_i value (F test).

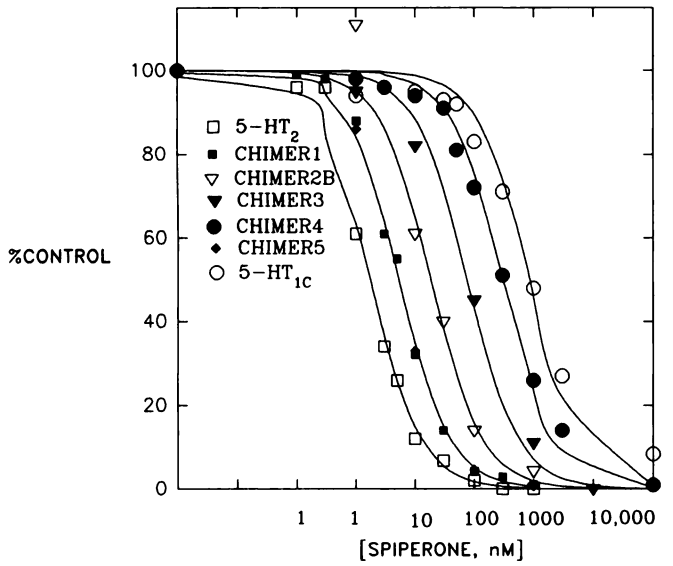

Fig. 5. Spiperone inhibition isotherms for chimeric and parent receptors. Shown are mean percentages of control specific binding (no unlabeled spiperone added) for three separate experiments, with duplicate determinations for each point, for inhibition of [^{3}H]ketanserin (chimer 1 and 5-HT$_2$) or [^{3}H]mesulergine (chimers 2B–5 and 5-HT$_{1c}$) binding.

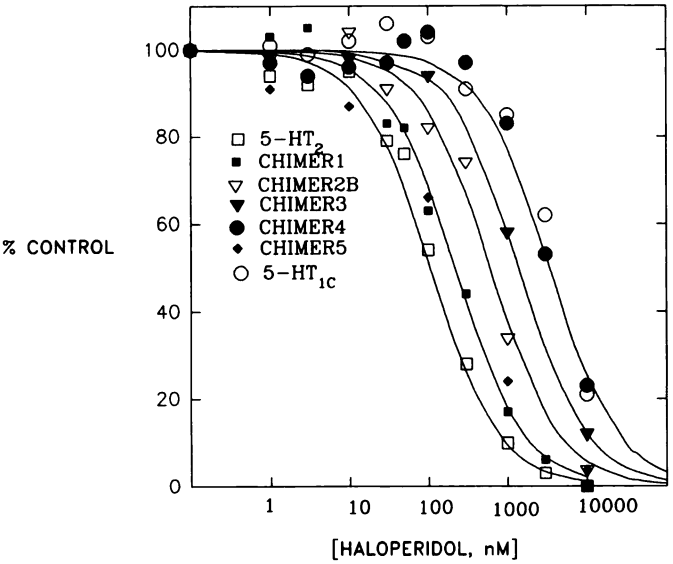

Fig. 6. Haloperidol inhibition isotherms for chimeric and parent receptors. Shown are mean percentages of control specific binding (no unlabeled haloperidol added) for three separate experiments, with duplicate determinations for each point, for inhibition of [^{3}H]ketanserin (chimer 1 and 5-HT$_2$) or [^{3}H]mesulergine (chimers 2B–5 and 5-HT$_{1c}$) binding.

5-HT$_{1c}$ receptors. Coupled with the results from the present studies, we can conclude that the terminal domains, which include TMs VI and VII, are essential for the binding pharmacology of some, but not all, antagonists for adrenergic, muscarinic, and serotonergic receptors. Our results also imply that domains important for specifying high affinity 5-HT binding are also found in the carboxyl terminus of the 5-HT$_2$ and 5-HT$_{1c}$ receptors. Whether these regions reside in the cytoplasmic loop(s) or in the TM domains cannot be answered by the present studies. Computerized models of ligand binding (29, 30) predict that the TM domains are essential for ligand binding, although some prior studies highlight the importance of i3 for agonist affinities (27).

Because of the rather modest shifts in affinity we noted for our initial chimeras (chimer 1 and chimer 4), we constructed three additional chimeras in an effort to pinpoint regions important for specifying antagonist pharmacology. Additionally, the model of Evardsen *et al.* (30) suggested that relatively important interactions for the antagonist ritanserin and ketanserin[2] resided in TMs I, II, and VI (30). In a similar vein, Evardsen *et al.* (30) predicted that TM II, and not TM III as predicted by Hibert *et al.* (29), was essential for agonist pharmacology. We then constructed additional chimeras in which TMs I and II (chimers 2 and 3) and TMs I–III (chimer 5) were exchanged.

Our results are most consistent with the idea that multiple domains are important for specifying the affinities of a variety of antagonists for 5-HT$_2$ and 5-HT$_{1c}$ receptors. These results appear to be in general accord with the model of Evardsen *et al.* (30), which suggests that domains I, II and VI are important for 5-HT binding. Thus, we found that chimeras in which TMs I and II (chimer 2) or I–III (chimer 5) were exchanged showed strikingly altered affinities for spiperone and haloperidol. In all cases, the affinities were shifted toward those for the donated sequences. In the case of chimer 5, the affinity of spiperone was increased 294-fold, whereas the affinity of haloperidol was shifted to a more modest extent (11.5-fold). These results indicate that spiperone and haloperidol (both butyrophenones) may not bind in precisely the same manner to the 5-HT$_2$ receptor.

One advantage of the present approach is that the chimeras were constructed in such a way that "gain of function" alterations were measured. Thus, chimer 5 showed enhanced affinities for antagonists, whereas chimers 1 and 3 showed increased affinities for 5-HT. Site-directed mutagenesis studies, in general, suffer from the inherent problem that loss of function is the usual end-point. Thus, it has frequently been shown that a specific mutation (e.g., conserved aspartate; see Ref. 45) abolishes ligand binding or that agonist binding disappears and antagonist binding is relatively conserved. In general, such "loss of function" studies, although extremely suggestive, cannot demonstrate conclusively that the residues altered are essential for actually binding the ligand.

The chimeric protein approach is not without its disadvantages, however. In particular, it is conceivable that the domains switched have no direct role in ligand binding but merely serve to orient the critical residues. Complementary studies utilizing direct labeling approaches could theoretically overcome these potential difficulties.

In summary, we discovered that major structural determinants important for specifying the unique pharmacology of the 5-HT$_2$ and 5-HT$_{1c}$ receptors reside in multiple domains of each receptor. For drugs like spiperone and haloperidol TMs I–III were essential, whereas ketanserin binding was most affected by domains i3 to TM VII and TM III. Agonists appeared to recognize domains at either end of the receptor, although the carboxyl-terminal region was most essential for specifying the affinity of 5-HT. These results imply that the process of ligand binding is more complicated than previously thought and are in general accord with a recent model published by Evardsen *et al.* (30). These results should prove to be of fundamental

[2] S. G. Dahl, personal communication.

importance for identification of the molecular features essential for ligand recognition by 5-HT_2 and 5-HT_{1c} receptors.

Acknowledgments

We wish to thank Craig Stockmeier, Ph.D., for the cinanserin used in these experiments, David Julius for the GF-6 cells, Brian Kobilka for the COS-7 cells, and Beth Hoffman for the A9 cells. We are indebted to Svein Dahl for helpful comments and for allowing us access to unpublished studies.

References

1. Bradley, P. B., G. Engle, W. Feniuk, J. R. Fozard, P. P. A. Humphrey, D. N. Middlemis, E. J. Lecharane, B. P. Richardson, and P. R. Saxena. Proposals for the classification and nomenclature of functional receptors of 5-hydroxytryptamine. *Neuropharmacology* **25**:563–576 (1986).
2. Kobilka, B. K., T. Frielle, S. Collins, T. Yeng-Feng, T. S. Kobilka, U. Franke, R. J. Lefkowitz, and M. G. Caron. An intron-less gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature (Lond.)* **329**:75–79 (1987).
3. Voight, M. M., D. J. Laurie, P. H. Seeburg, and A. Bach. Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *EMBO J.* **10**:4017–4023 (1991).
4. Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science (Washington D. C.)* **241**:558–564 (1988).
5. Hamblin, M., and M. Metcalf. Primary structure and characterization of a human 5-hydroxytryptamine$_{1D}$ receptor. *Mol. Pharmacol* **40**:143–148 (1991).
6. Pritchett, D. B., A. W. Bach, M. Wozny, O. Taleb, R. DalTaso, J. C. Shih, and P. H. Seeburg. Structure and functional expression of a cloned rat serotonin 5-HT-2 receptor. *EMBO J.* **7**:4135–4140 (1988).
7. Julius, D., K. N. Huang, T. J. Livelli, R. Axel, and T. M. Jessell. The 5HT_2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. *Proc. Natl. Acad. Sci. USA* **87**:928–932 (1990).
8. Maricq, A. V., A. S. Peterson, A. J. Brake, R. M. Myers, and D. A. Julius. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. *Science (Washington D. C.)* **254**:432–437 (1991).
9. Gershon, M. D., P. R. Wade, A. L. Kirchgessner, and H. Tamir. 5-HT receptor subtypes outside the central nervous system. *Neuropsychopharmacology* **3**:385–395 (1990).
10. Pazos, A., D. Hoyer, and J. M. Palacios. The binding of serotonergic ligands to the procine choroid plexus: characterization of a new type of serotonin recognition site. *Eur. J. Pharmacol.* **106**:539–546 (1984).
11. Hoyer, D., G. Engel, and H. D. Kalkman. Molecular pharmacology of 5-HT_1 and 5-HT_2 recognition sites in pig brain membranes: radioligand binding studies with [^{3}H]-5HT, [^{3}H]-8-OH-DPAT, (−)-[^{125}I]-cyanopindolol, [^{3}H]-mesulergine and [^{3}H]-ketanserin. *Eur. J. Pharmacol.* **118**:13–23 (1985).

12a. Roth, B. L., R. D. Ciaranello, and H. Y. Meltzer. Binding of typical and atypical antipsychotic agents to transiently expressed 5-HT_{1c} receptors. *J. Pharmacol. Exp. Ther.* **260**:1361–1366 (1992).

12b. Munson, P., and D. Rodbard. LIGAND: a versatile computerized approach for the analysis of ligand binding data. *Anal. Biochem* **107**:220–239 (1980).

13. Conn, P. J., and E. Sanders-Bush. Selective 5-HT_2 antagonists inhibit serotonin-stimulated phosphatidylinositol metabolism in cerebral cortex. *Neuropharmacology* **23**:993–996 (1984).
14. Roth, B. L., T. Nakaki, D.-M. Chuang, and E. Costa. Aortic recognition sites for serotonin (5-HT) are coupled to phospholipase C and modulate phosphatidylinositol turnover. *Neuropharmacology* **23**:1223–1225 (1984).
15. Roth, B. L., and D.-M. Chuang. Minireview: multiple mechanisms of serotonergic signal transduction. *Life Sci.* **41**:1051–1064 (1987).
16. Conn, P. J., E. Sanders-Bush, B. J. Hoffman, and P. R. Hartig. A unique serotonin receptor in choroid plexus is linked to phosphatidylinositol turnover. *Proc. Natl. Acad. Sci. USA* **83**:4086–4088 (1986).
17. Sanders-Bush, E. Adaptive regulation of central serotonin receptors linked to phosphoinositide hydrolysis. *Neuropsychopharmacology* **3**:411–416 (1990).
18. Roth, B. L., and R. D. Ciaranello. Miaserin decreases 5-HT_2 radioligand binding without altering 5-HT_2 receptor mRNA levels. *Eur. J. Pharmacol.* **207**:169–172 (1991).
19. Roth, B. L., M. W. Hamblin, and R. D. Ciarnello. Developmental regulation of 5-HT_2 and 5-HT_{1c} mRNA and receptor levels. *Dev. Brain Res.* **58**:51–58 (1991).
20. Conn, P. J., and E. Sanders-Bush. Relative efficacies of piperazines at the phosphoinositide hydrolysis-linked serotonergic (5-HT_2 and 5-HT_{1c}) receptors. *J. Pharmacol. Exp. Ther.* **242**:552–557 (1987).
21. Sahin-Erdemli, I., P. Schoeffter, and D. Hoyer. Competitive antagonism by recognised 5-HT_2 receptor antagonists at 5-HT_{1c} receptors in pig choroid plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **344**:137–142 (1991).
22. Strader, C. D., I. S. Sigal, M. R. Candelore, E. Rands, W. S. Hill, and R. A. F. Dixon. Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol. Chem.* **263**:10267–10271 (1988).
23. Dixon, R. A. F., C. D. Strader, and I. S. Sigal. *Annual Reports in Medicinal Chemistry*. Academic Press, New York (1988).
24. Suryanarayana, S., D. A. Daunt, M. Von Zastrow, and B. K. Kobilka. A point mutation in the seventh hydrophobic domain of the α_2-adrenergic receptor increases its affinity for a family of β-receptor antagonists. *J. Biol. Chem.* **266**:15488–15492 (1991).
25. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Chimeric α_2-/β_2-adrenergic receptors: deliniation of domains involved in effector coupling and ligand binding specificity. *Science (Washington D. C.)* **240**:1310–1316 (1988).
26. Frielle, T., K. W. Daniel, M. G. Caron, and R. J. Lefkowitz. Structural basis of β-adrenergic receptor subtype specificity studied with chimeric β1/β2-adrenergic receptors. *Proc. Natl. Acad. Sci. USA* **85**:9494–9498 (1988).
27. Wess, J., T. I. Bonner, F. Dorje, and M. R. Brann. Delineation of muscarinic receptor domains conferring selectivity of coupling to guanine nucleotide-binding proteins and second messengers. *Mol. Pharmacol.* **38**:517–523 (1990).
28. Wess, J., T. I. Bonner, and M. R. Brann. Chimeric m2/m3 muscarinic receptors: role of carboxyl terminal domains in selectivity of ligand binding and coupling to phosphoinositide hydrolysis. *Mol. Pharmacol.* **38**:872–877 (1991).
29. Hibert, M. F., S. Trumpp-Kallmeyer, A. Bruinvels, and J. Hoflack. Three-dimensional models of neurotransmitter guanine nucleotide-binding protein-coupled receptors. *Mol. Pharmacol.* **40**:8–15 (1991).
30. Evardsen, O., I. Sylte, and S. G. Dahl. Molecular dynamics of serotonin and ritanserin interacting with the 5-HT_2 receptor. *Mol. Brain Res.* **14**:166–178 (1992).
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
32. Davis, L. G., M. D. Dibner, and J. F. Battey. *Basic Methods of Molecular Biology*. Elsiver, New York (1986).
33. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5466 (1977).
34. Cullen, B. R. Use of eukaryotic expression technology in the functional analysis of cloned genes, in *Guide to Molecular Cloning Techniques* (eds.). Academic Press, New York, 684–705 (1987).
35. Roth, B. L., S. McLean, X.-Z. Zhu, and D.-M. Chuang. Characterization of two [^{3}H]-ketanserin recognition sites in rat striatum. *J. Neurochem.* **49**:1833–1838 (1987).
36. Hoyer, D. Functional correlates to 5-HTI recognition sites. *J. Recept. Res.* **8**:59–81 (1988).
37. Leysen, J. E., C. J. E. Niemegeers, J. M. Van Nueten, and P. M. Laduron. [^{3}H]-Ketanserin (R 41 468), a selective ^{3}H-ligand for serotonin$_2$ receptor binding sites. *Mol. Pharmacol.* **21**:301–314 (1982).
38. Leysen, J. E. Gaps and peculiarities in 5-HT_2 receptor studies. *Neuropsychopharmacology* **3**:361–370 (1990).
39. Buck, F., W. Meyerhof, H. Werr, and D. Richter. Characterization of N- and C-terminal deletion mutants of the rat serotonin 5HT_2 receptor in *Xenopus laevis* oocytes. *Biochem. Biophys. Res. Commun.* **178**:1421–1428 (1991).
40. Guan, X.-M., S. J. Peroutka, and B. K. Kobilka. Identification of a single amino acid residue responsible for the binding of a class of β-adrenergic receptor antagonists to 5-hydroxytryptamine$_{1A}$ receptors. *Mol. Pharmacol.* **41**:695–698 (1992).
41. Hoyer, D., C. Waeber, P. Schoeffter, J. M. Palacios, and A. Dravid. 5-HT_{1c} receptor-mediated stimulation of inositol phosphate production in pig choroid plexus: a pharmacological characterization. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**:252–258 (1989).
42. Roth, B. L., M. W. Hamblin, and R. D. Ciaranello. Regulation of 5-HT_2 and 5-HT_{1c} serotonin receptor levels: methodology and mechanisms. *Neuropsychopharmacology* **3**:427–433 (1990).
43. Pazos, A., and J. M. Palacios. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res.* **346**:231–249 (1985).
44. Pazos, A., R. Cortes, and J. M. Palacios. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. II. Serotonin-2 receptors. *Brain Res.* **346**:231–249 (1985).
45. Strader, C. D., I. S. Sigal, and R. A. F. Dixon. Structural basis of *beta*-adrenergic receptor function. *FASEB J.* **3**:1825–1831 (1989).

Send reprint requests to: Bryan L. Roth, M. D., Ph.D., Department of Psychiatry, 2040 Abington Road, Cleveland, OH 44106.