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Identification of the Ligand-Binding Subunit of the Human 5-Hydroxytryptamine$_{1A}$ Receptor with *N*-(*p*-Azido-*m*-[^{125}I] iodophenethyl)spiperone, a High Affinity Radioiodinated Photoaffinity Probe

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SUMMARY

The ligand-binding subunit of the human 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor transiently expressed in COS-7 cells and of the native human 5-HT$_{1A}$ receptor derived from hippocampus and frontal cortex were identified by photoaffinity labeling with *N*-(*p*-azido-*m*-[^{125}I]iodophenethyl)spiperone ([^{125}I]N_3-NAPS), previously characterized as a high affinity radioiodinated D_2-dopamine receptor probe. The identity of the ligand-binding subunit was confirmed by immunoprecipitation with an antipeptide rabbit antiserum, JWR21, raised against a synthetic peptide derived from the predicted amino acid sequence of the putative third intracellular loop of the human 5-HT$_{1A}$ receptor. In transiently transfected COS-7 cells expressing 14 ± 3 pmol/mg of protein human 5-HT$_{1A}$ receptors, a single broad 75-kDa band was photoaffinity labeled by [^{125}I]N_3-NAPS. This band displayed the expected pharmacology of the 5-HT$_{1A}$ receptor, as evidenced by the ability of a series of competing ligands to block [^{125}I]N_3-NAPS photoincorporation. Moreover, antiserum JWR21 specifically and quantitatively immunoprecipitated the 75-kDa photoaffinity-labeled band from a soluble extract of the transfected COS-7 cell membranes, further confirming its identity. Finally, utilizing a combination of photoaffinity labeling and immunoprecipitation, the native ligand-binding subunit of 62–64 kDa was identified in human hippocampus and frontal cortex. The availability of the high specific activity, high affinity, photoaffinity ligand [^{125}I]N_3-NAPS and of a potent immunoprecipitating antiserum (JWR21) should greatly facilitate the biochemical characterization of the human 5-HT$_{1A}$ receptor.

At least five subtypes of G protein-coupled 5-HT receptors have been characterized by pharmacological and physiological methods (1). The recent cloning and expression of the human 5-HT$_{1A}$ (2, 3) and rat 5-HT$_{1C}$ (4) and 5-HT$_2$ (5) receptors have revealed remarkable sequence and/or structural similarity to other G protein-coupled receptors, such as the various adrenergic (6) and muscarinic receptors (7), the substance K receptor (8), and the opsin light receptor group (9), as well as the *mas* oncogene (10). Further insight into the structure of these receptors has been gained by the use of photoaffinity labels. This approach, coupled with purification by affinity chromatography, has proven highly successful in the case of the adrenergic receptors (11–17). Unfortunately, efforts thus far to purify 5-HT receptors have met with only modest success, due to the lack of specific high affinity resins and photoaffinity labels. Two groups have recently designed, synthesized, and characterized photoaffinity ligands for the 5-HT$_{1A}$ receptor (18, 19). These probes, *p*-azido-[^{3}H]PAPP (18) and [^{3}H]8-methoxy-2-[*N*-*n*-propyl-*N*-3-(2-nitro-4-azidophenyl)aminopropyl]aminotetralin (19), suffer from the drawbacks of low specific activity (50–100 mCi/mmol) and limited commercial availability. Another approach that has been utilized to characterize receptor proteins involves the use of antibodies to specifically immuno-

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; 5-HT, 5-hydroxytryptamine (serotonin); [^{3}H]*p*-azido-PAPP, [^{3}H]1-[2-(4-azidophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine; [^{125}I]N_3-NAPS, *N*-(*p*-azido-*m*-[^{125}I]iodophenethyl)spiperone; NPA, (*R*-(−)-propylnorapomorphine hydrochloride; 8-OH-DPAT, 8-OH-2-(di-*n*-propylamino)-1,2,3,4-tetrahydronaphthalene; mCPP, 1-(3-chlorophenyl)piperazine; TFMPP, *m*-trifluoromethylphenylpiperazine; PAPP (LY-165,163), *p*-aminophenylethyl-*m*-trifluoromethylphenyl piperazine; MDL-72222, 3-tropanyl-3,5-dichlorobenzoate; ICS 205931, 3-tropanylindole-3-carboxylate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate).

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precipitate or immunoblot the protein of interest. This approach has yielded only modest success with respect to G protein-coupled receptors, particularly the β-adrenergic receptors (20–22). In this manuscript, we describe the identification of the human 5-HT_{1A} receptor binding subunit by photoaffinity labeling with [^{125}I]N_3-NAPS (Fig. 1) (23). Its identity is confirmed by immunoprecipitation with an antipeptide antibody.

Experimental Procedures

Materials

[^{125}I]N_3-NAPS (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA); [^{3}H]8-OH-DPAT (110 Ci/mmol) from Research Products International (Mt. Prospect, IL); dopamine hydrochloride, 5-HT hydrochloride, (−)-epinephrine, histamine dihydrochloride, sulpiride, and protein A-Sepharose 6MB from Sigma Chemical Co. (St. Louis, MO); and NPA, (±)-8-OH-DPAT hydrobromide, spiperone, ketanserin, spiroxatrine, mCPP, TFMPP, PAPP, MDL-72222, ICS205930, (+)-butaclamol, and (−)-butaclamol from Research Biochemicals Incorporated (Natick, MA). Keyhole limpet hemocyanin was from Calbiochem (La Jolla, CA). Ritanserin was a gift from Janssen, and mesulergine was a gift from Farmitalia. All other compounds were of the highest possible grade from commercial sources.

Methods

Transfection of COS-7 cells. The expression vector pSPXB (2), containing the *Xba*I-*Bam*HI fragment of the 5-HT_{1A} human genomic clone, was digested with *Hind*III in the pSP64 polylinker and *Bam*HI. The resulting 1.7 kilobase *Hind*III-*Bam*HI fragment was inserted between unique *Bam*HI and *Hind*III sites of the expression vector pBC12BI (24). The COS-7 cells were transfected by the DEAE-dextran method (25) and harvested 72 hr after transfection.

Membrane preparation. COS-7 cell membranes were prepared as previously described (3), by lysis on ice in 10 mM Tris·HCl, pH 7.4, 5 mM EDTA that contained 5 μg/ml concentrations each of leupeptin, benzamidine, pepstatin, and soybean trypsin inhibitor. The membranes were centrifuged at 1000 × *g* for 10 min to remove whole cells and nuclear debris, and the supernatant was centrifuged at 37,000 × *g* for 20 min. The resulting pellet was washed twice by resuspension in lysis buffer followed by centrifugation. The membranes were resuspended at a final protein concentration of ≈1 mg/ml in 50 mM Tris·HCl, 2.5 mM $MgCl_2$, pH 7.4, frozen by immersion in liquid nitrogen, and stored at −80°. Human hippocampus and frontal cortex, obtained 6 hr *post mortem* (from a patient without neurological or psychiatric symptoms or disease), was minced with a razor blade and then prepared in the same manner as membranes from COS-7 cells.

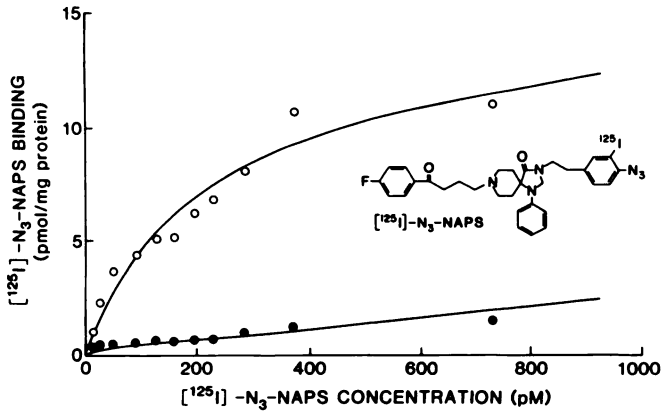

Fig. 1. Saturation analysis of [^{125}I]N_3-NAPS binding in membranes prepared from COS-7 cells transfected with the human 5-HT_{1A} DNA. Transfection, membrane preparation, and binding assays were performed as described in Experimental Procedures. Binding was determined in the presence (●) or absence (○) of 10 μm 5-HT. The data presented, fitted to a single site (44), are derived from a single experiment performed in triplicate (representative of three experiments). There was no significant difference between the fits for one or two sites.

Radioligand binding assays. [^{3}H]8-OH-DPAT and [^{125}I]N_3-NAPS binding assays were performed essentially as previously described (3, 23). Membranes (≈150 μg of protein/ml) were resuspended in binding buffer (50 mM Tris·HCl, pH 7.4, 2.5 mM $MgCl_2$) and incubated with 0.5 nM [^{3}H]8-OH-DPAT in the presence and absence of various concentrations of competing ligand. Incubations (30 min at 30°) were terminated by the addition of 5 ml of ice-cold binding buffer and rapid vacuum filtration through Whatman GF/C filters, followed by two 5-ml washes. The filters were counted by liquid scintillation (≈45% efficiency).

Nonspecific adsorption of [^{125}I]N_3-NAPS to the glass fiber filters in the presence of denatured (boiled) membranes was initially problematical, approaching ≈50% of total ligand. The addition of 0.01% Triton X-100 to the incubation and wash buffers and of 0.1% bovine serum albumin to the wash buffer and reduction of the filtration surface area decreased the nonspecific adsorption to filters to ≈1–2% of the total ligand. Under these conditions, no binding was displaced by 10 μM 5-HT in the absence or presence of boiled membranes. Samples (15–25 μg of protein/ml; 150 or 200 μl incubation volume) were incubated for 30 min at 22° in the dark. Assays were terminated by vacuum filtration through Whatman GF/C filters (presoaked in wash buffer) that were mounted on a Schleicher and Schuell minifold at 4°; filters were washed four times with 500 μl of ice-cold wash buffer (binding buffer containing 0.1% bovine serum albumin and 0.01% Triton X-100), followed by determination of γ radioactivity (Packard Autogamma 800). For saturation curve analysis, nonspecific binding was determined in the presence of 10 μM 5-HT.

Photoaffinity labeling. Membranes were used fresh or stored at −80° for up to 2 months. Approximately 50 μl (50 μg) of membranes were resuspended in ice-cold binding buffer in a final volume of 500 μl, with 500–700 pM [^{125}I]N_3-NAPS and an appropriate concentration of competing ligand, and were incubated on ice for 2 hr in the dark. Competing ligands were made fresh as 1 mM stock solutions dissolved in 70% ethanol, except MDL72222 (dimethylsulfoxide), ritanserin (dimethylformamide), TFMPP, 5-HT, and 8-OH-DPAT (water). Solvent blanks were tested and did not affect photoaffinity labeling. The assays were transferred to a Corning 24-well polystyrene cell culture dish on ice and were irradiated for 30 sec with a hand-held Mineralight UVG-54 UV light source (Ultra-Violet Products Inc., San Gabriel, CA) from a distance of 6 cm. The suspension was then layered over 200 μl of incubation buffer supplemented with 30% (v/v) glycerol and was centrifuged at ≈500,000 × *g* for 15 min in a Beckman TL-100 ultracentrifuge. The supernatant was discarded, and the pellet was resuspended in SDS-PAGE sample buffer (50 mM Tris·HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-pmercaptoethanol, 0.003% bromphenol blue) and allowed to stand for 30–45 min at room temperature. The samples were electrophoresed on 10% SDS-polyacrylamide gels according to the method of Laemmli (26), dried, and autoradiographed with Kodak XAR film that was exposed at −80° for 3–48 hr. The photoaffinity label was specifically incorporated into ≈1% of receptors, as determined from [^{3}H]8-OH-DPAT binding.

Protein determinations. Protein concentrations were determined by the method of Bradford (27), using bovine serum albumin as the standard.

Antibody production. Synthetic peptides derived from the predicted amino acid sequence of the putative third intracellular loop of the human 5-HT_{1A} receptor, GASPAPQPKKSVNGESGSRNWRLGVE (residues 242–267) and SKAGGALCANGAVRFGGAALEVIE (residues 268–293), were assembled by an automated solid-phase method (28) on an Applied Biosystems 430A peptide synthesizer with the standard *N-tert*-butoxycarbonyl technique, using the manufacturer's cycles, followed by deprotection of the amino acid side chains and HF cleavage. Purity (>95%) was assessed by high pressure liquid

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chromatography and amino acid analysis. Peptide 242–267 was coupled to keyhole limpet hemocyanin with glutaraldehyde (29).

Rabbits were immunized with 1 mg of coupled peptide that was dissolved in 1.0 ml each of PBS and Freund's complete adjuvant, followed by monthly booster injections in Freund's incomplete adjuvant. Rabbits were bled by ear vein puncture 10 days after the third booster injection. Antisera were assayed for their ability to interact specifically with unconjugated peptide by a solid phase radioimmunoassay. Antiserum JWR21 was subsequently demonstrated to immunoprecipitate [^{3}H]8-OH-DPAT-labeled 5-HT$_{1A}$ receptors that resulted from solubilization of plasma membranes from transfected COS-7 cells with 10 mM CHAPS, 200 mM NaCl, 50 mM Tris·ClH, pH 7.4 (3).

Immunoprecipitation. After membranes from transfected COS-7 cells (≈1 mg of protein) or human hippocampus or frontal cortex (≈2 mg) were photolabeled, the resulting pellet was resuspended in 1 ml of PBS, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, pH 7.4, and was stirred on ice for 1 hr. The solution was centrifuged at 500,000 × *g* for 30 min in a Beckman TL-100 ultracentrifuge, and the supernatant was harvested. The supernatant was divided into 100-μl aliquots, diluted to 460 μl with PBS, pH 7.4, and incubated with 40 μl of crude antiserum that was preincubated with equal amounts of PBS or PBS containing 20 μM corresponding or noncorresponding peptide. After incubation overnight at 4°, 65 μl of 50% (w/v) preswollen Protein-A-Sepharose-6MB beads in PBS were added to each condition, and the suspensions were rotated for 90 min at 22°. The beads were centrifuged at 1,000 × *g* for 5 min, and washed three times with 1 ml of PBS, 0.1% Triton X-100, 0.01% SDS, pH 7.4. Supernatants were saved, dialyzed against 5 mM Tris·HCl, pH 7.4, lyophilized, and resuspended in sample buffer for SDS-PAGE analysis. The beads were vortexed in SDS-PAGE sample buffer, boiled for 3 min, cooled, and centrifuged at 1,000 × *g* for 5 min. The sample buffer was loaded onto 10% polyacrylamide gels, which were run under reducing conditions.

Results

Binding properties of [^{125}I]N$_3$-NAPS. Because photoaffinity ligands for the 5-HT$_{1A}$ receptor are of low specific activity and limited availability (18, 19), we sought to adapt a commercially available high specific activity photoaffinity label as a probe for the human 5-HT$_{1A}$ receptor expressed in COS-7 cells. Because amino-NAPS has a moderate affinity for 5-HT$_{1A}$ receptors (8.0 ± 3.0 nM; Table 1), we reasoned that its radioiodinated azido-derivative might be utilized as a photoaffinity probe for 5-HT$_{1A}$ receptors. Experiments with transfected COS-7 cell membranes, performed in the dark, demonstrated that [^{125}I]N$_3$-NAPS binding was saturable (B_{max} = 11.1 ± 1.2 pmol/mg of protein) (Fig. 1). Binding in the dark was reversible and of high affinity (K_d = 148 ± 27 pM). Specific binding comprised ≈85–90% of total binding near the K_d. Typical values at 200 pM [^{125}I]N$_3$-NAPS were ≈20,000 total and ≈2,000 nonspecific cpm. The maximal binding density of [^{125}I]N$_3$-NAPS binding sites is in good agreement with the density of [^{3}H]8-OH-DPAT binding sites in the same membranes (14 ± 3 pmol/mg of protein) (3). Competition assays with various ligands demonstrated that the binding site for [^{125}I]N$_3$-NAPS possesses a typical 5-HT$_{1A}$ pharmacology (Fig. 2; Table 1). The specific 5-HT$_{1A}$ ligand 8-OH-DPAT (K_i = 9 ± 5.5 nM), as well as spiroxatrine (2.2 ± 0.1 nM) and 5-HT (6.7 ± 0.4 nM), possesses a high affinity for the site, whereas the typical dopaminergic ligands NPA (905 ± 295 nM) and dopamine (≈2 μM) exhibit much lower affinities. These affinities correlate well with those obtained in competition assays utilizing [^{3}H]8-OH-DPAT (Table 1). Additionally, the binding site demonstrated stereospecificity, inasmuch as the affinity of (+)-butaclamol (9.5 ± 1.9 nM) was several orders of magnitude higher than that of (−)-butaclamol (≈3 μM).

TABLE 1

Comparison of binding properties of [^{3}H]8-OH-DPAT and [^{125}I]N$_3$-NAPS in membranes prepared from COS-7 cells transfected with the human 5-HT$_{1A}$ DNA

	[^{3}H]8-OH-DPAT[a]		[^{125}I]N$_3$-NAPS	Inhibition of Photoincorporation of [^{125}I]N$_3$-NAPS[b]
	K_H[c]	K_L	K_i	
	nM			%
Serotonin agonists				
8-OH-DPAT[d]	0.06 ± 0.01	22 ± 6.4	9.0 ± 5.5	91–99
Ipsapirone[d]	0.24 ± 0.08	31 ± 2.7	2.0 ± 2.0	86–93
5-HT[d]	0.27 ± 0.07	63 ± 5.5	6.7 ± 0.4	85–95
PAPP	0.55 ± 0.05	76 ± 9.0	13.0 ± 2.0	75–85
Buspirone[d]	4.0 ± 1.6	97 ± 25	74.1 ± 26.2	80
TFMPP	32.1 ± 9.7	737 ± 249		46–51
mCPP	54.5 ± 32.5	1397 ± 988		28–42

	[^{3}H]8-OH-DPAT K_i	[^{125}I]N$_3$-NAPS K_i	Inhibition of Photoincorporation of [^{125}I]N$_3$-NAPS
	nM		%
Serotonin antagonists			
Spiroxatrine[d]	1.8 ± 0.7	2.2 ± 0.1	87–96
Spiperone[d]	63 ± 28	66.2 ± 31	59–81
Mesulergine[d]	400 ± 62	1008 ± 566	43–51
Sulpiride	589 ± 256		38–46
Ritanserin	892 ± 48		5–14
ICS 205930	1788 ± 473		7–22
MDL 72222	2041 ± 505		8–29
Ketanserin[d]	2100 ± 450		4–11
Miscellaneous compounds			
(+)-Butaclamol	6.6 ± 3.7	9.5 ± 1.9	83–95
Amino-NAPS	8.0 ± 3.0	17.3 ± 2.5	
NPA	845 ± 132	905 ± 295	15–29
Dopamine[d]	2053 ± 52	2088 ± 55	12–17
(−)-Butaclamol	2245 ± 107	3099 ± 278	2–6
Epinephrine[d]	5672 ± 1102		0–3
Histamine[d]	6011 ± 867		9–18

[a] All data for competition studies represent the means ± standard errors of three to five experiments performed in duplicate or triplicate.

[b] Values depicted represent the range of values determined by scanning densitometry in two to five separate experiments. All experiments were performed at a competing ligand concentration of 500 or 1000 nM. The value for buspirone was obtained from a single experiment.

[c] Values for putative 5-HT$_{1A}$ agonists were modeled to two sites against the agonist radioligand [^{3}H]8-OH-DPAT, because saturation analysis had previously revealed two binding sites with affinities of 0.06 and 14.5 nM (3). Values for putative antagonists and other compounds were modeled to one site. Values for all compounds determined with [^{125}I]N$_3$-NAPS were modeled to one site, because the data could not be fitted to two sites. Because it has been previously shown that the high affinity state in these membranes represents approximately 3–5% of [^{3}H]8-OH-DPAT binding activity (3), the values for [^{125}I]N$_3$-NAPS reported as K_i values probably most closely approximate K_L values.

[d] Values for [^{3}H]8-OH-DPAT binding have been previously published (3).

Photoaffinity labeling of the 5-HT$_{1A}$ binding subunit. Fig. 3 clearly demonstrates specific labeling of a single broad band of M_r ≈75,000 in membranes from 5-HT$_{1A}$-transfected COS-7 cell membranes. There was no specific labeling in membranes prepared from nontransfected COS-7 cells or COS-7 cells transfected with the DNA of the α_2-C4 (100 μg; ≈2 pmol/mg of protein) or the α_2-C10 (100 μg; ≈10 pmol/mg of protein) adrenergic receptors (30) (data not shown). Furthermore, the ability of various ligands to block photoincorporation demonstrated the appropriate 5-HT$_{1A}$ pharmacology for both agonists and antagonists. Among the agonists, at concentrations of 500 nM, photoincorporation was potently blocked by 5-HT and the highly specific 5-HT$_{1A}$ agonist 8-OH-DPAT, moderately blocked by the 5-HT$_{1A}$ agonist PAPP, and partially blocked by the nonspecific 5-HT agonists TFMPP and mCPP. At a con-

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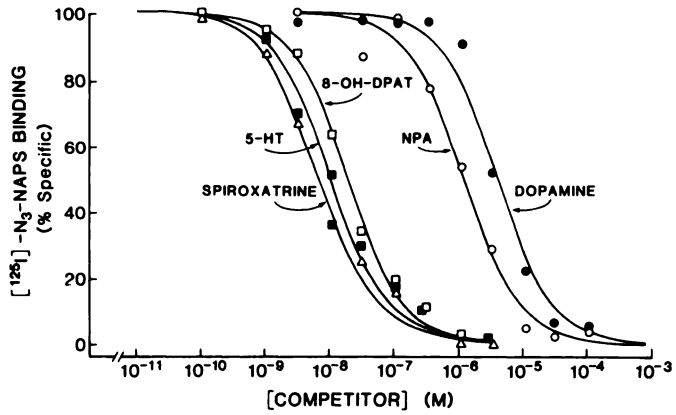

Fig. 2. Competition analyses versus specific [^{125}I]N_3-NAPS binding in membranes prepared from COS-7 cells transfected with the human 5-HT_{1A} DNA. Data presented are from typical experiments performed in duplicate or triplicate (representative of two to five experiments). The data were fitted to a single site (see Table 1). Drugs analyzed are spiroxatrine (△), 5-HT (■), 8-OH-DPAT (□), NPA (○), and dopamine (●).

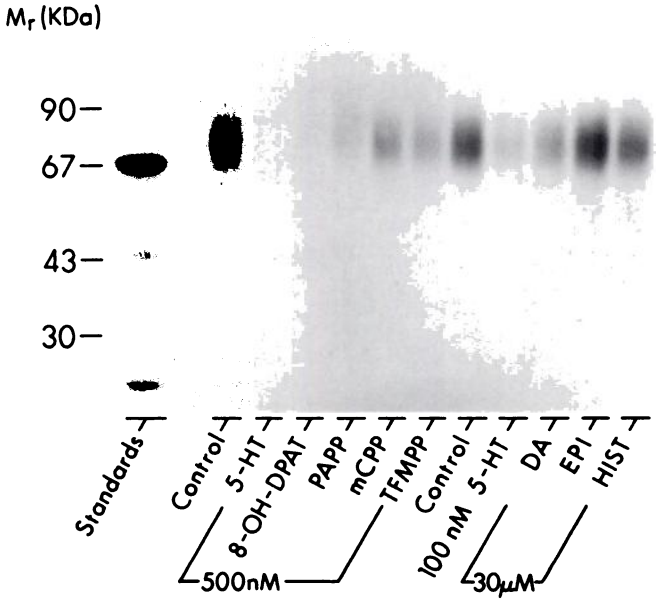

Fig. 3. Effects of various agonists on photoincorporation of [^{125}I]N_3-NAPS in membranes prepared from COS-7 cells transfected with the human 5-HT_{1A} DNA. Photoaffinity labeling was carried out as described in Experimental Procedures, followed by 10% SDS-PAGE electrophoresis and autoradiography. This experiment is representative of two to five separate experiments performed with the various compounds. This autoradiograph was exposed for 12 hr at −80°. *DA*, dopamine; *EPI*, epinephrine; *HIST*, histamine.

centration of 30 μM, dopamine slightly blocked photoincorporation, and epinephrine and histamine were essentially inert. The D_2-dopamine agonist NPA only weakly inhibited photoincorporation (Table 1). Furthermore, the 5-HT_{1A} agonists ipsapirone and buspirone (500 nM) nearly completely blocked photoincorporation (Table 1). As shown in Fig. 4, antagonists also demonstrated typical 5-HT_{1A} pharmacology. At 1 μM, spiroxatrine, which is potent at the 5-HT_{1A} receptor, completely blocked photoincorporation, whereas the 5-HT_2 antagonists ritanserin and ketanserin, the 5-HT_2 and 5-HT_{1C} antagonist mesulergine, and the 5-HT_3 antagonists MDL72222 and ICS205930 were nearly inert. Furthermore, the potent D_2-dopamine receptor antagonist sulpiride was weakly active.

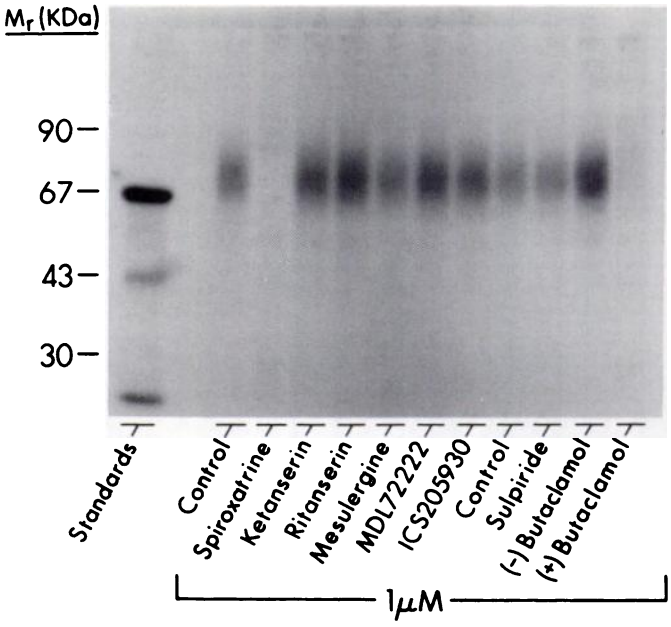

Fig. 4. Effects of various antagonists on photoincorporation of [^{125}I]N_3-NAPS in membranes prepared from COS-7 cells transfected with the human 5-HT_{1A} DNA. This experiment is representative of two to five separate experiments performed with the various competing ligands. The autoradiograph was exposed for 20 hr at −80°.

Further supportive pharmacological evidence that the labeled band represents the 5-HT_{1A} receptor is presented in Table 1. For the various ligands studied, the rank order of potency and magnitude of inhibition of photoincorporation of [^{125}I]N_3-NAPS in membranes prepared from COS-7 cells that were transfected with the 5-HT_{1A} receptor DNA correlated very well with the K_i (or K_H and K_L) values obtained for both [^{3}H]8-OH-DPAT and [^{125}I]N_3-NAPS in reversible binding assays in the same membranes.

Immunoprecipitation of the photoaffinity-labeled 5-HT_{1A} receptor by antipeptide antiserum JWR21. Because [^{125}I]N_3-NAPS had been previously characterized as a specific ligand for the D_2-dopamine receptor, we felt that a more rigorous proof of the identity of the specifically labeled band as the 5-HT_{1A} receptor was desirable. Therefore, the antipeptide antiserum JWR21, raised to residues 242–267 (see Experimental Procedures) from the putative third intracellular loop of the 5-HT_{1A} receptor, was utilized to specifically and quantitatively immunoprecipitate the 75-kDa photoaffinity-labeled band. Fig. 5 demonstrates that the antiserum JWR21 specifically immunoprecipitated the [^{125}I]N_3-NAPS-labeled band from 5-HT_{1A} receptor-transfected COS-7 cell membranes. Significantly, the immunoprecipitation of the 75-kDa band was quantitative. The preimmune serum from the JWR21 rabbit and JWR21 serum preblocked with corresponding antigenic peptide were unable to immunoprecipitate the photolabeled band (Fig. 5). Conversely, a noncorresponding peptide from the 5-HT_{1A} sequence (residues 268–293) and the carrier protein keyhole limpet hemocyanin were not able to block immunoprecipitation of the photolabeled 5-HT_{1A} receptor from the same membranes (data not shown).

Although attempts to specifically label the native 5-HT_{1A} receptor from crude membrane preparations of human hippocampus and frontal cortex were unsuccessful due to numerous prominent nonspecific bands, we were able to demonstrate that

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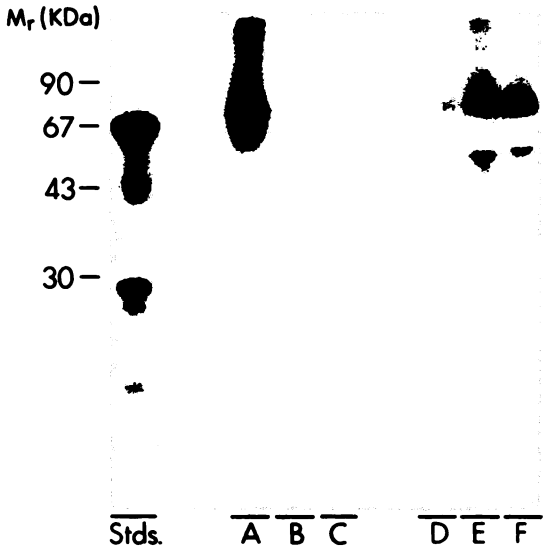

Fig. 5. Immunoprecipitation of photolabeled 5-HT$_{1A}$ receptors derived from transfected COS-7 cells. All procedures were performed as described in Experimental Procedures. Each *lane* represents ≈100 μg of protein of photolabeled membranes. After photolabeling with 700 pM [^{125}I]N$_3$-NAPS, membrane pellets were solubilized and immunoprecipitated. *Lanes A–C* represent the immunoprecipitates of antiserum JWR21 (*lane A*), JWR21 preblocked by its corresponding peptide (*lane B*), and preimmune serum from the same rabbit (*lane C*). *Lanes D–F* represent 25% of the respective supernatants remaining after immunoprecipitation and lyophilization. The photolabeled 75-kDa band is precipitated by JWR21 (*lane A*); the same band is greatly diminished in the corresponding supernatant (*lane D*). Approximately 90–95% of the specifically incorporated radioactivity (determined by scanning densitometry and measurement of γ-radiation) was precipitated by JWR21. The bands in the supernatants appear compressed due to the presence of serum proteins.

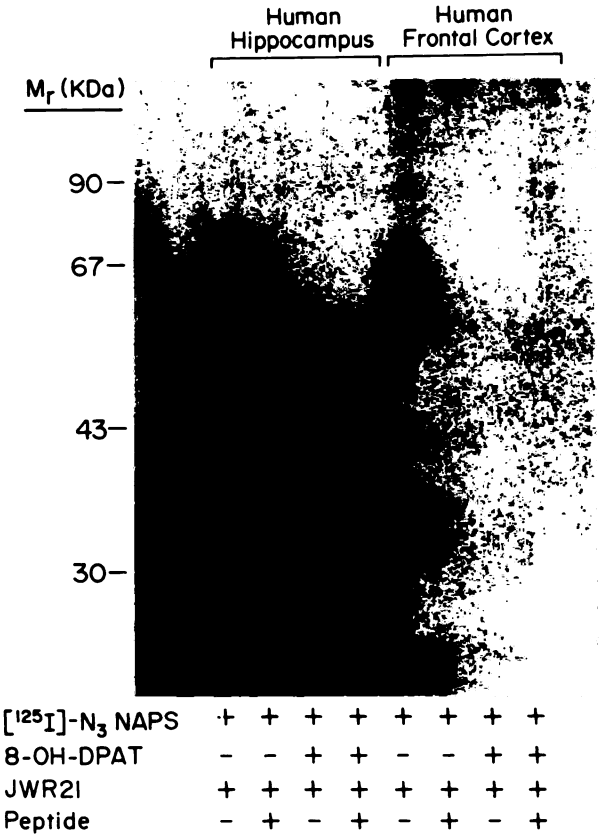

Fig. 6. Immunoprecipitation of the native 5-HT$_{1A}$ receptor binding subunit from human hippocampus and frontal cortex. All procedures were performed as described in Experimental Procedures and the legend to Fig. 5. Because numerous nonspecific bands were photolabeled, the pellets were solubilized and immunoprecipitated with antiserum JWR21. For each condition, ≈200 μg protein were photolabeled in the presence or absence of 10 μM 8-OH-DPAT. Immunoprecipitations were performed with JWR21 in the absence or presence of 10 μM corresponding peptide. After SDS-PAGE gel electrophoresis (10%), the gel was autoradiographed for 12 days at −80°.

[^{125}I]N$_3$-NAPS labels the binding subunit of the native human 5-HT$_{1A}$ receptor in human hippocampus and frontal cortex, by utilizing photoaffinity labeling followed sequentially by solubilization and immunoprecipitation (Fig. 6). The size of the 64- and 62-kDa bands labeled in human hippocampal and cortical preparations, respectively, is in good agreement with that reported for the rat brain 5-HT$_{1A}$ receptor (18, 19, 31). The fact that the 5-HT$_{1A}$ receptor expressed in COS-7 cells migrates with an M_r of 75,000 is discussed below.

Discussion

The results presented here demonstrate that the previously characterized D$_2$-dopamine photoaffinity probe [^{125}I]N$_3$-NAPS can be effectively utilized to irreversibly label 5-HT$_{1A}$ receptors in membranes prepared from COS-7 cells that were transfected with the DNA encoding the 5-HT$_{1A}$ receptor or from human brain tissues. In transfected COS-7 cell membranes, this ligand specifically labels a broad band with a molecular mass of ≈75 kDa, which has the appropriate pharmacology of the 5-HT$_{1A}$ receptor. The 75-kDa band is larger than that reported by two other groups, in rat brain membranes (≈60 kDa), using tritiated photoaffinity labels followed by scintillation counting of SDS-PAGE gel slices (18, 19) or radiation inactivation (31) rather than autoradiography. It is also larger than the apparent size of the native human brain 5-HT$_{1A}$ receptor (≈62–64 kDa), which more closely correlates with that reported for the rat brain 5-HT$_{1A}$ receptor. The apparent differences in size of these labeled bands may be due to differential processing or to degradation of the native binding subunit. With respect to the differences in M_r between the human 5-HT$_{1A}$ receptor binding subunit in membranes derived from human brain and from transfected COS-7 cells, it is known that various G protein-coupled receptors can migrate with larger M_r values when expressed in transfected cell lines, as opposed to native tissues. In the case of the human β_2-receptor expressed in CHW cells, the larger M_r has been shown to be due to different states of glycosylation (32); treatment with endoglycosidases yielded core proteins of the same M_r. Recently, a similar phenomenon has been observed with bovine rhodopsin (33). When expressed in COS-1 cells, the rhodopsin glycoprotein demonstrated a more complex pattern of glycosylation, with some species migrating with larger apparent M_r than rhodopsin purified from bovine rod outer segments. Similarly, the α_2-C4-receptor expressed in COS-7 cells migrates with an M_r of ≈75,000 (30), considerably larger than the M_r of ≈44,000 reported for a putative endogenous α_2-C4-receptor derived from neonatal rat lung (34); the potential mechanisms responsible for this difference in M_r have not yet been rigorously investigated for the α_2-C4-receptor. It is likely that the difference in M_r of the human 5-HT$_{1A}$ receptor expressed in COS-7 cells and of that native to the hippocampus and frontal cortex is due primarily to different glycosylation states, although this remains to be shown. Even

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though protease inhibitors were utilized in this study, a role for degradation of the native receptor has not been ruled out at this time.

The high specific activity of [^{125}I]N_3-NAPS allows rapid autoradiographic visualization of the specifically labeled receptor binding subunit. Additionally, [^{125}I]N_3-NAPS is readily available from a commercial source. The labeling technique is simple and identifies the 5-HT_{1A} receptor binding subunit as a single broad 75-kDa band in transfected COS-7 cell plasma membranes. When combined with the antiserum JWR21, the native 5-HT_{1A} receptor binding subunit from human brain membranes can be identified. Unlike in the adrenergic receptor field, where protein purification typically preceeded the sequencing and cloning of the various receptors (11–17, 20, 30, 35–38), three 5-HT receptors (2–5) have been cloned in the absence of complete purification of any of the 5-HT receptors. The purification of the 5-HT_{1A} receptor has lagged behind, due to difficulties with solubilization of the receptor and with labeling of the solubilized receptor (39). The use of [^{125}I]N_3-NAPS may ameliorate problems with the labeling, particularly when utilized in cell lines that express a high density of 5-HT_{1A} receptors.

It is not surprising that a ligand "specific" for the D_2-dopamine receptor is able to interact with high affinity with 5-HT_{1A} receptors that are expressed in high density of COS-7 cells, a cell line bereft of D_2-dopamine receptors. [^{125}I]N_3-NAPS has a high affinity for both receptors, but its affinity for the 5-HT_{1A} receptor expressed in COS-7 cells (K_d = 148 ± 27 pM) is actually higher than that for the native D_2-dopamine receptor (1.6 ± 0.05 nM) (23). In fact, several ligands such as (+)-butaclamol, amino-NAPS, lysergic acid diethylamide, lisuride, spiroxatrine, metergoline, and buspirone share affinities of ≈1–50 nM for both receptor subtypes (40) (Table 1). This overlapping pharmacology suggests that these two receptor subtypes may share common structural features important for ligand binding. Such a structure-function relationship has already been demonstrated for the α_2-, β_1-, and β_2-adrenergic receptors. Using a series of α_2,β_2- (41) and β_1,β_2- (42) chimeric receptors, the sixth and seventh membrane-spanning domains were shown to be most important in determining antagonist binding specificities. Because of the overlapping pharmacology of the 5-HT_{1A} and D_2-dopamine receptors, such a model might predict that these two receptors share particular sequence identities in certain transmembrane domains. Such sequence identity has already been demonstrated between the sixth and seventh transmembrane domains of the human 5-HT_{1A} and β_2-adrenergic receptors, perhaps explaining the ability of the 5-HT_{1A} receptor to bind β-adrenergic antagonist ligands (3). The human 5-HT_{1A} receptor and a recently cloned rat brain D_2-dopamine receptor share the highest sequence identity (≈50%) in the second, third, sixth, and seventh transmembrane domains (43). Clearly, further studies utilizing mutant or chimeric receptors will be necessary to fully elucidate the relationship of structure to ligand binding in these receptors.

The data presented here clearly demonstrate that [^{125}I]N_3-NAPS can be utilized as a high specific activity photoaffinity label for the 5-HT_{1A} receptor in transfected cell systems. Additionally, the combination of [^{125}I]N_3-NAPS and the potent immunoprecipitating antiserum JWR21 can be utilized to identify the native human 5-HT_{1A} receptor derived from brain tissues. The use of these tools should facilitate the biochemical characterization of the 5-HT_{1A} receptor.

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References

1. Hoyer, D. 5-Hydroxytryptamine receptors and effector coupling mechanisms in peripheral tissues, in *Peripheral Actions of 5-HT* (J. R. Fozard, ed.). Oxford University Press, Oxford, in press.
2. Kobilka, B. K., T. Frielle, S. Collins, T. Yang-Feng, T. S. Kobilka, U. Francke, R. J. Lefkowitz, and M. G. Caron. An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature (Lond.)* **329**:75–79 (1987).
3. Fargin, A., J. R. Raymond, M. J. Lohse, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. The genomic clone G-21, which resembles a β-adrenergic receptor sequence, encodes the 5-HT_{1A} receptor. *Nature (Lond.)* **335**:358–360 (1988).
4. Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science (Wash. D. C.)* **242**:558–564 (1988).
5. Pritchett, D. B., A. W. J. Bach, M. Wozny, O. Taleb, R. Dal Toso, J. C. Shih, and P.H. Seeburg. Structure and functional expression of cloned rat serotonin 5-HT-2 receptor. *EMBO J.* **7**:4135–4140 (1988).
6. Dohlman, H. G., M. G. Caron, and R. J. Lefkowitz. A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* **26**:2657–2664 (1987).
7. Kubo, T., K. Fukuda, A. Mikami, A. Maeda, H. Takahashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kanawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature (Lond.)* **323**:411–416 (1986).
8. Masu, Y., K. Nakayama, Y. Hanada, M. Kuno, and S. Nakanashi. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature (Lond.)* **329**:836–838 (1987).
9. Nathans, J. A. Molecular biology of visual pigments. *Annu. Rev. Neurosci.* **10**:163–194 (1987).
10. Jackson, T. R., L. A. C. Blair, J. Marshall, M. Goedeit, and M. R. Hanley. The *mas* oncogene encodes an angiotensin receptor. *Nature (Lond.)* **335**:437–440 (1988).
11. Benovic, J. L., R. G. L. Shorr, M. G. Caron, and R. J. Lefkowitz. The mammalian β_2-adrenergic receptor: purification and characterization. *Biochemistry* **23**:4510–4518 (1984).
12. Regan, J. W., H. Nakata, R. M. DeMarinis, M. G. Caron, and R. J. Lefkowitz. Purification and characterization of the human platelet α_2-adrenergic receptor. *J. Biol. Chem.* **261**:3894–4000 (1986).
13. Lomasney, J. W., L. M. F. Leeb-Lundberg, S. Cotecchia, J. W. Regan, J. F. DeBernardis, M. G. Caron, and R. J. Lefkowitz. Mammalian α_1-adrenergic receptor: purification and characterization of the native receptor ligand binding subunit. *J. Biol. Chem.* **261**:7710–7716 (1986).
14. Yarden, Y., H. Rodriguez, S. K. F. Wong, D. R. Brandt, D. C. May, J. Burnier, R. N. Harkins, E. Y. Chen, J. Ramachandran, A. Ullrich, and E. M. Ross. The avian β-adrenergic receptor: primary structure and membrane topology. *Proc. Natl. Acad. Sci. USA* **83**:6795–6799 (1986).
15. Lavin, T. N., P. Nambi, S. L. Heald, P. W. Jeffs, R. J. Lefkowitz, and M. G. Caron. ^{125}I]labeled *p*-azidobenzylcarazolol, a photoaffinity label for the β-adrenergic receptor. *J. Biol. Chem.* **257**:12332–12341 (1982).
16. Leeb-Lundberg, L. M. F., K. E. J. Dickinson, S. L. Heald, J. E. S. Wikberg, P. O. Hagen, J. F. DeBernardis, M. Winn, D. L. Arendsen, R. J. Lefkowitz, and M. G. Caron. Photoaffinity labeling of mammalian α_1-adrenergic receptors: identification of the ligand binding subunit with a high affinity radioiodinated probe. *J. Biol. Chem.* **259**:2579–2587 (1984).
17. Regan, J. W., J. R. Raymond, R. J. Lefkowitz, and R. M. DeMarinis. Photoaffinity labeling of human platelet and rabbit kidney α_2-adrenoceptors with [^{3}H]SKF 102229. *Biochem. Biophys. Res. Commun.* **137**:606–613 (1986).
18. Ransom, R. W., K. B. Asarch, and J. C. Shih. Photoaffinity labeling of the 5-hydroxytryptamine 1A receptor in rat hippocampus. *J. Neurochem.* **47**:1066–1072 (1986).
19. Emerit, M. B., S. El Mestikawy, H. Gozlan, J. M. Cossery, R. Besselievre, A. Marquet, and M. Hamon. Identification of the 5-HT_{1A} receptor binding subunit in rat brain membranes using the photoaffinity probe [^{3}H]8-methoxy-2[*N*-*n*-propyl]-*N*-3-(2-nitro-4-azidophenyl)aminopropyl]aminotetralin. *J. Neurochem.* **49**:373–380 (1987).
20. Dixon, R. A. F., B. K. Kobilka, D. J. Strader, J. L. Benovic, H. G. Dohlman, T. Freille, M. A. Bolanowski, C. D. Bennett, E. Rands, R. E. Diehl, R. A. Mumford, e. E. Slater, I. S. Sigal, M. G. Caron, R. J. Lefkowitz, and C. D. Strader. Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. *Nature (Lond.)* **321**:75–79 (1986).
21. Kaveri, S. V., P. Cervantes-Olivier, C. Delavier-Klutchko, and A. D. Strosberg. Monoclonal antibodies directed against the human A431 β_2-adrenergic receptor recognize two major polypeptide chains. *Eur. J. Biochem.* **167**:449–456 (1987).

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22. Rubinstein, R. C., S. K.-F. Wong, and E. M. Ross. The hydrophobic tryptic core of the β-adrenergic receptor retains G_s regulatory activity in response to agonists and thiols. *J. Biol. Chem.* **262**:16655–16662 (1987).
23. Amlaiky, N., and M. G. Caron. Photoaffinity labeling of the D_2-dopamine receptor using a novel high affinity radioiodinated probe. *J. Biol. Chem.* **260**:1983–1986 (1985).
24. Cullen, B. R. Use of eukaryotic expression techniques in the functional analysis of cloned genes. *Methods Enzymol.* **152**:684–704 (1987).
25. Lopata, M., D. W. Cleveland, and B. Sollner-Webb. High-level expression of a chloramphenicol acetyltransferase gene by DEAE-dextran-mediated DNA transfection coupled with dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**:5707–5717 (1984).
26. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **228**:680–685 (1970).
27. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Anal. Biochem.* **72**:248–254 (1976).
28. Merrifield, R. B. Automated synthesis of peptides. *Science (Wash., D. C.)* **150**:178–185 (1965).
29. Avrameas, S., and T. Ternynck. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry* **6**:53–66 (1969).
30. Regan, J. W., T. S. Kobilka, T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. USA* **85**:6301–6305 (1988).
31. Gozlan, H., M. B. Emerit, M. D. Hall, M. Nielsen, and M. Hamon. *In situ* molecular sizes of the various types of 5-HT binding sites in the rat brain. *Biochem. Pharmacol.* **35**:1891–1897 (1987).
32. Bouvier, M., M. Hnatowich, S. Collins, B. K. Kobilka, A. DeBlasi, R. J. Lefkowitz, and M. G. Caron. Expression of a human cDNA encoding the β_2-adrenergic receptor in Chinese hamster fibroblasts (CHW): functionality and regulation of the expressed receptors. *Mol. Pharmacol.* **33**:133–139 (1988).
33. Karnik, S. S., T. P. Sakmar, H.-B. Chen, and H. G. Khorana. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc. Natl. Acad. Sci. USA* **85**:8459–8563 (1988).
34. Lanier, S. M., C. J. Homcy, C. Patenaude, and R. M. Graham. Identification of structurally distinct α_2-adrenergic receptors. *J. Biol. Chem.* **263**:14491–14496 (1988).
35. Kobilka, B. K., R. A. F. Dixon, T. Frielle, H. G. Dohlman, M. A. Bolanowski, I. S. Sigal, T. L. Yang-Feng, U. Franke, M. G. Caron, and R. J. Lefkowitz. cDNA for the human β_2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* **84**:46–40 (1987).
36. Frielle, T., S. Collins, K. W. Daniel, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning of the cDNA for the human β_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:7920–7927 (1987).
37. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science (Wash. D. C.)* **268**:650–656 (1987).
38. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **85**:7159–7163 (1988).
39. Gozlan, H., M. B. Emerit, S. El Mestikawy, J. M. Cossery, A. Marquet, R. Besselievre, and M. Hamon. Photoaffinity labeling and solubilization of the central 5-HT_{1A} receptor binding site. *J. Recept. Res.* **7**:195–221 (1987).
40. Leysen, J. E. The use of 5-HT receptor agonists and antagonists for the characterization of their respective receptor sites, in *Neuromethods, Neuropharmacology II: Drugs as Tools in Neurotransmitter Research* (A. A. Boulton, G. B. Baker, and A. V. Juorio, eds.). Humana Press, Clifton, NJ, in press.
41. 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: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Wash. D. C.)* **241**:1350–1316 (1988).
42. Frielle, T., K. W. Daniel, M. G. Caron, and R. J. Lefkowitz. Structural basis of β-adrenergic receptor subtype specificity studied with chimeric β_1-, β_2-adrenergic receptors. *Proc. Natl. Acad. Sci. USA*, in press
43. Bunzow, J. R., H. H. M. Van Tol, D. K. Grandy, P. Albert, J. Salon, M. Christie, C. A. Machida, K. A. Neve, and O. Civelli. Cloning and expression of a rat D_2-dopamine receptor cDNA. *Nature (Lond.)* **336**:783–787 (1988).
44. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).

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