Biochemical Pharmacology, Vol. 59, pp. 1117–1121, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00416-5

SHORT COMMUNICATION

Induction of a High-Affinity Ketanserin Binding Site at the 5-Hydroxytryptamine$_{1B}$ Receptor by Modification of Its Carboxy-Terminal Intracellular Portion

*Thierry Wurch, Christiane Palmier and Petrus J. Pauwels**

Department of Cellular and Molecular Biology, Centre De Recherche Pierre Fabre, 81106 Castres, France

ABSTRACT. Two chimeric 5-hydroxytryptamine (5-HT) receptors were constructed by exchanging the C-terminal portion of the human (h) 5-HT$_{1B}$ receptor with the equivalent domain of the h 5-HT$_{2A}$ receptor (5-HT$_{1B/2A}$) or with this domain truncated from its last 44 amino acids (5-HT$_{1B/2A\Delta44}$). The equilibrium dissociation constant of the radioligand [^{3}H]GR 125743 was similar for both chimera compared to the wild-type (wt) h 5-HT$_{1B}$ receptor upon transient expression in COS-7 cells. Ketanserin binding affinity was 21-fold increased from pK_i: 5.79 (wt h 5-HT$_{1B}$ receptor) to pK_i: 7.11 at the 5-HT$_{1B/2A}$ chimeric receptor, this latter value being close to that of the wt h 5-HT$_{1D}$ receptor (pK_i: 7.62). This enhanced ketanserin binding affinity was lost when the last 44 C-terminal amino acids of the 5-HT$_{2A}$ receptor were deleted in the chimera 5-HT$_{1B/2A\Delta44}$ (pK_i: 5.80). The binding affinities of the 5-HT antagonists ritanserin, GR 125743, and SB-224289 were not modified at either chimeric 5-HT receptor. The agonists F 11356, 5-HT, zolmitriptan, and sumatriptan yielded slightly increased (2- to 6-fold) binding affinities at both chimera as compared to the wt h 5-HT$_{1B}$ receptor. The present data suggest a role for the C-terminal intracellular receptor domain in modifying ketanserin/5-HT$_{1B}$ receptor interactions. BIOCHEM PHARMACOL **59**;9:1117–1121, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. ketanserin; chimeric receptor; ligand binding; recombinant 5-HT$_{1B}$; 5-HT$_{2A}$ receptors

The serotonin (5-HT†) antagonist ketanserin was initially described as a selective 5-HT$_2$ receptor ligand. Amongst the 5-HT$_2$ receptor subtypes, it displays selectivity for the 5-HT$_{2A}$ receptor (pK_i: 8.50; [1]) compared to its 60- and 200-fold lower binding affinity for the 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors, respectively [1]. More recently, ketanserin has also been shown to display binding affinity for human (h) 5-HT$_{1D}$ (pK_i: 7.40; [2]), rat 5-HT$_{1D}$ (pK_i: 7.98 to 8.20; [3, 4]), rabbit 5-HT$_{1D}$ (pK_i: 7.66; [3]), and guinea pig 5-HT$_{1D}$ receptors (pK_i: 6.86 to 7.79; [3, 4]), silent antagonism at 5-HT$_{1D}$ receptors of guinea pig and rat (pK_b: 7.51 to 7.92; [4]), and inverse agonism at h 5-HT$_{1D}$ receptors (pIC_{50}: 7.90; [5]). Otherwise, ketanserin binds poorly to the canine 5-HT$_{1D}$ receptor [6] and does not recognize 5-HT$_{1B}$ receptors of different species reported so far [7]. The construction of chimeric 5-HT$_{1D}$/5-HT$_{1B}$ receptors allowed the delineation of a ketanserin binding site to the 5-HT$_{1D}$ receptor: the exchange of a domain encompassing the second extracellular loop and the fifth TMD of the h 5-HT$_{1D}$ receptor with an equivalent domain of the h 5-HT$_{1B}$ receptor decreased the ketanserin binding affinity, and reciprocally [7]. This led us to postulate a ketanserin binding site at the second extracellular loop and/or near the exofacial surface of the fifth TMD of the h 5-HT$_{1D}$ receptor [7].

In this study, we report on the binding affinities of chimeric h 5-HT$_{1B}$ receptors for which the C-terminal intracellular portion was replaced by the equivalent domain of the h 5-HT$_{2A}$ receptor or by this domain deleted of its last 44 amino acids. Both chimera were transiently expressed in COS-7 cells and analyzed for inhibition of the binding of [^{3}H] GR 125743, a selective 5-HT$_{1B/1D}$ receptor ligand, by a series of 5-HT ligands. Data were compared to the ligand binding profiles of the h 5-HT$_{1D}$ receptor and of the parental h 5-HT$_{1B}$ and h 5-HT$_{2A}$ receptors. The 5-HT$_{1B}$ receptor chimera carrying the 5-HT$_{2A}$ receptor-derived C-terminal portion yielded an increased ketanserin binding affinity without significant modifications of the binding affinities for the other ligands being investigated.

MATERIALS AND METHODS

Construction of Chimeric 5-HT$_{1B/2A}$ Receptors

Chimeric receptors were constructed by exchanging the C-terminal intracellular portion of the h 5-HT$_{1B}$ receptor

* Corresponding author: Dr. Peter Pauwels, Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, 17, av. Jean Moulin, 81106 Castres cédex, France. Tel. 0033-5.63.71.42.65; FAX 0033-5.63.71.43.63; E-mail: peter.pauwels@pierre-fabre.com

† *Abbreviations:* h, human; wt, wild-type; 5-HT, 5-hydroxytryptamine, serotonin; and TMD, transmembrane domain.

Received 22 June 1999; accepted 11 October 1999.

(Asn^{373} to Ser^{390}; RC: 2.1.5-HT.01B; GeneBank accession number: M89478) with the entire homologous domain of the h 5-HT$_{2A}$ receptor (Asn^{384} to Val^{471}; RC: 2.1.5-HT.02A, GeneBank accession number: M86841; 5-HT$_{1B/2A}$) or by a domain deleted from its last 44 amino acids (Asn^{384} to Gly^{437}; 5-HT$_{1B/2A\Delta44}$). They were amplified by a polymerase chain reaction-based modified overlap extension technique as described [8]. The chimeric receptors were cloned into the expression vector pCR3.1 and fully sequenced.

Expression of 5-HT Receptors and Radioligand Binding Experiments

COS-7 cells (5×10^6 cells) were transfected with 10 μg of either plasmid pcDNA$_3$/h 5-HT$_{1B}$ [9], pCR3.1/5-HT$_{1B/2A}$, or pCR3.1/5-HT$_{1B/2A\Delta44}$ by electroporation as previously described [9]. A human embryonic kidney 293 cell line stably expressing the h 5-HT$_{2A}$ receptor was grown in complete Dulbecco's modified Eagle's medium and selected on 1.25 mg/mL geneticin as described [9]. Binding assays were performed with either 1.0 nM [^{3}H] *N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridil)-benzamide (GR 125743) for 5-HT$_{1B}$ receptor binding or 1.0 nM [^{3}H]ketanserin for 5-HT$_{2A}$ receptor binding. Incubation mixtures consisted of 0.4 mL of cell membrane, 0.05 mL of radioligand, and 0.05 mL of compound for inhibition or 10 μM 5-HT to determine non-specific binding. The reactions were performed as described [9]. Data were analyzed graphically with inhibition curves and IC_{50} values were derived as the concentration of the compound producing 50% inhibition of specific radioligand binding. Inhibition constants K_i were calculated according to the equation $K_i = IC_{50}/(1 + C/K_d)$, with C the concentration and K_d the equilibrium dissociation constant of the radioligand. The K_d values were obtained from saturation binding experiments performed as described [9]. Membrane protein levels were estimated with a dye-binding assay using the BioRad protein assay kit and BSA as a standard [10].

Materials

The pCR3.1 vector was from Invitrogen. The COS-7 cell line was obtained from ATCC. [^{3}H]GR 125743 (80 Ci/mmol) and [^{3}H]ketanserin (66.4 Ci/mmol) were from Amersham and New England Nuclear, respectively. 5-HT, ketanserin, ritanserin, and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) were from RBI-Sigma. 4-[4-[2-(2-aminoethyl)-1*H*-indol-5-yloxyl]-acetyl]-piperazinyl-1-yl] benzonitrile (F 11356), zolmitriptan, sumatriptan, GR 125743, and 1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4′-piperidine] (SB-224289) were synthesized internally.

TABLE 1. K_d and B_{max} values for [^{3}H]GR125743 or [^{3}H]ketanserin binding to wild-type h 5-HT$_{1B}$, h 5-HT$_{2A}$, and chimeric 5-HT$_{1B/2A}$ and 5-HT$_{1B/2A\Delta44}$ receptors

	K_d (nM)	B_{max} (pmol/mg protein)
[^{3}H]GR 125743		
h 5-HT$_{1B}$	0.61 ± 0.09	8.1 ± 2.1
5-HT$_{1B/2A}$	0.67 ± 0.06	26.3 ± 8.8
5-HT$_{1B/2A\Delta44}$	0.53 ± 0.08	32.3 ± 9.8
[^{3}H]ketanserin		
h 5-HT$_{2A}$	0.28–0.30	2.7–2.8

The equilibrium dissociation constants (K_d) and the maximal binding capacity (B_{max}) were determined for each of the wt and chimeric 5-HT receptors on cellular membranes as described in Methods. Results are expressed as mean values ± SEM from 3 independent experiments or as the mean from 2 independent experiments, each one performed in duplicate.

RESULTS AND DISCUSSION

Saturation binding experiments were performed with [^{3}H]GR 125743, a selective 5-HT$_{1B/1D}$ ligand, to membrane preparations of COS-7 cells transiently expressing either the wt h 5-HT$_{1B}$ or the chimeric 5-HT$_{1B/2A}$ and 5-HT$_{1B/2A\Delta44}$ receptors, and with [^{3}H]ketanserin to membranes of human embryonic kidney 293 cells stably expressing the wt h 5-HT$_{2A}$ receptor. The [^{3}H]GR 125743 equilibrium dissociation constants of both chimera were similar to that of the wt h 5-HT$_{1B}$ receptor (Table 1). The maximal binding capacity of the wt h 5-HT$_{1B}$ receptor was 3- to 4-fold lower than that of the chimeric 5-HT$_{1B/2A}$ and 5-HT$_{1B/2A\Delta44}$ receptors and 3-fold higher than that of the wt h 5-HT$_{2A}$ receptor (Table 1). A series of nine 5-HT ligands was tested for inhibition of [^{3}H]GR 125743 binding to wt h 5-HT$_{1B}$, 5-HT$_{1B/2A}$, and 5-HT$_{1B/2A\Delta44}$ receptors and compared to their binding affinities for the wt h 5-HT$_{1D}$ and h 5-HT$_{2A}$ receptors (Table 2). The chimeric 5-HT$_{1B/2A}$ receptor yielded a 21-fold increased binding affinity for the 5-HT$_2$ antagonist ketanserin as compared to the wt h 5-HT$_{1B}$ receptor. This binding affinity is close (3-fold lower) to what is observed for the wt h 5-HT$_{1D}$ receptor but 87-fold lower than for the wt h 5-HT$_{2A}$ receptor (Table 2). The structurally related piperidine derivative ritanserin yielded an almost similar binding affinity as compared to the wt h 5-HT$_{1B}$ receptor, this value being 3- and 47-fold lower than for the wt h 5-HT$_{1D}$ and h 5-HT$_{2A}$ receptors, respectively. This ketanserin binding feature was lost when the last 44 amino acids of the 5-HT$_{2A}$ receptor-derived C-terminal portion were truncated in the chimeric 5-HT$_{1B/2A\Delta44}$ receptor. The binding affinities of the 5-HT antagonists GR 125743 and SB-224289 were not modified in either chimeric 5-HT receptor as compared to the parental h 5-HT$_{1B}$ receptor. The binding affinities of the agonists F 11356, zolmitriptan, 5-HT, and sumatriptan were slightly increased (1.6- to 6-fold) at both chimeric receptors as compared to the wt h 5-HT$_{1B}$ receptor. The 5-HT$_2$ agonist DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) was inactive at either the wt h 5-HT$_{1B}$, h 5-HT$_{1D}$, or chimeric 5-HT$_{1B}$ receptor.

TABLE 2. pK_i values of 5-HT receptor ligands for inhibition of [^{3}H]GR 125743 or [^{3}H]ketanserin binding to wt h 5-HT_{1B}, h 5-HT_{1D}, h 5-HT_{2A}, and chimeric 5-$HT_{1B/2A}$ and 5-$HT_{1B/2A\Delta44}$ receptors

Radioligand	[^{3}H]GR 125743				[^{3}H]Ketanserin
Receptor	wt h 5-HT_{1B}	5-$HT_{1B/2A}$	5-$HT_{1B/2A\delta44}$	wt h 5-HT_{1D}*	wt h 5-HT_{2A}
Agonists					
F 11356	8.73 ± 0.08	9.29 ± 0.12	9.09 ± 0.06	8.51 ± 0.05	6.88 ± 0.02
Zolmitriptan	7.66 ± 0.07	7.99 ± 0.13	7.94 ± 0.15	9.09 ± 0.02	<5
5-HT	7.47 ± 0.07	8.22 ± 0.17	7.86 ± 0.09	8.25 ± 0.05	7.41 ± 0.03
Sumatriptan	7.16 ± 0.04	7.37 ± 0.04	7.62 ± 0.09	8.22 ± 0.17	<5
DOI	<5	<5	<5	<5	8.24 ± 0.05
Antagonists					
GR 125743	8.85 ± 0.06	8.77 ± 0.05	8.88 ± 0.06	8.31 ± 0.14	6.74 ± 0.17
SB 224289	8.24 ± 0.05	7.93 ± 0.01	7.99 ± 0.11	6.63 ± 0.17	6.26 ± 0.06
Ritanserin	6.94 ± 0.05	7.28 ± 0.10	6.98 ± 0.08	7.77 ± 0.09	8.97 ± 0.11
Ketanserin	5.79 ± 0.09	7.11 ± 0.05	5.80 ± 0.13	7.62 ± 0.09	9.05 ± 0.05

Radioligand binding was performed with 1.0 nM [^{3}H]GR 125743 or 1.0 nM [^{3}H]ketanserin as described in Methods. Results (pK_i) are expressed as mean values ± SEM from 3 to 10 independent experiments, each one performed in duplicate. DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.

*Values were taken from Wurch *et al.* [7] except for F 11356.

Our data demonstrate the selective increase in ketanserin binding affinity at the h 5-HT_{1B} receptor when its C-terminal intracellular portion is exchanged with the equivalent domain of the h 5-HT_{2A} receptor. Remarkably, this binding affinity is close to the ketanserin value for the wt h 5-HT_{1D} receptor. Whereas we postulated that the second extracellular loop and the fifth TMD may be involved in the high-affinity ketanserin binding for the wt h 5-HT_{1D} receptor [7], this is unlikely to be the case for the chimeric 5-$HT_{1B/2A}$ receptor. The C-terminal intracellular portion beside TMD VI and VII of the wt h 5-HT_{1D} receptor does not seem to be important for ketanserin binding to this receptor [7]. The present study suggests an alternative hypothesis for the observed high-affinity site for ketanserin by which the structure of the chimeric 5-$HT_{1B/2A}$ receptor might be affected by the exchange of the C-terminal intracellular portion. The binding affinities of ritanserin, a structurally related piperidine derivative of ketanserin, as well as the other investigated 5-HT antagonists, were not affected. Slightly improved binding affinities were observed with the 5-HT agonists. The h 5-HT_{1B} and h 5-HT_{2A} receptors share a low (44%) overall homology at the amino acid level. Hence, it is unlikely that the ketanserin/5-$HT_{1B/2A}$ receptor contact points are similar to the ketanserin binding sites at the h 5-HT_{2A} receptor. TMD prediction does not envisage the existence of an additional TMD located in the C-terminal portion or a modification of the membrane junctions of the seven classical TMD (Fig. 1). An eighth hydrophobic domain has been postulated at the N-terminal extracellular portion of the 5-HT_{2C} receptor [11], but this feature is absent in both 5-HT_{1B} and 5-HT_{2A} receptors [11]. Interestingly, a basic BBXXB motif (where B is a basic amino acid and X a non-basic residue) is located in the C-terminal portion of the h 5-HT_{2A} receptor (Lys^{429}-Lys-Glu-Asn-Lys) and is removed by the 44-amino-acid deletion in the chimera 5-$HT_{1B/2A\Delta44}$. Similar domains are also present at the distal end of the third intracellular loop (Lys^{310}-Lys-Ala-Thr-Lys) and in the C-terminal portion close to TMD VII (His^{381}-Lys-Leu-Ile-Arg) of the h 5-HT_{1B} receptor and may be involved in G protein interactions [12]. The presence of a BBXXB motif in the 5-HT_{2A} receptor C-terminal intracellular portion, distal to TMD VII as compared to the wt h 5-HT_{1B} receptor, may modify the chimeric 5-$HT_{1B/2A}$ receptor:G protein interactions as compared to the wt h 5-HT_{1B} receptor and alter the chimera's ligand binding pocket to facilitate the binding of ketanserin. Several molecular models of ketanserin/5-HT_{2A} receptor interactions have been proposed, but none envisage a direct interaction of ketanserin with either the C-terminal intracellular portion or any of the extramembrane domains [13, 14]. Most probably, the 5-HT_{2A} receptor-derived full-length C-terminal portion may interact either directly with the 5-HT_{1B} receptor's intracellular domains or indirectly via a G protein, thereby modifying the positioning of the h 5-HT_{1B} TMD and favoring the binding of ketanserin. The shortening of the 5-HT_{2A} receptor-derived C-terminal portion in the chimera 5-$HT_{1B/2A\Delta44}$ may release structural TMD constraints generated by the last 44 5-HT_{2A} receptor C-terminal amino acids. This domain is particularly charged (8 basic and 8 acidic amino acid residues) and hydrophilic (Fig. 1, B and C); it may generate ionic bonds with the intracellular domains of the h 5-HT_{1B} receptor.

In conclusion, we report here on the modulation of ketanserin binding affinity at the h 5-HT_{1B} receptor by exchanging its C-terminal intracellular portion with that of the h 5-HT_{2A} receptor. These data suggest the possible importance of extramembrane domains in ligand binding for the 5-HT_{1B} receptor. This concept may advance the development of tridimensional models of G protein-coupled receptors.

We sincerely thank Fabrice Lestienne and Fréderic Finana for technical expertise and Stéphanie Cecco for secretarial assistance.

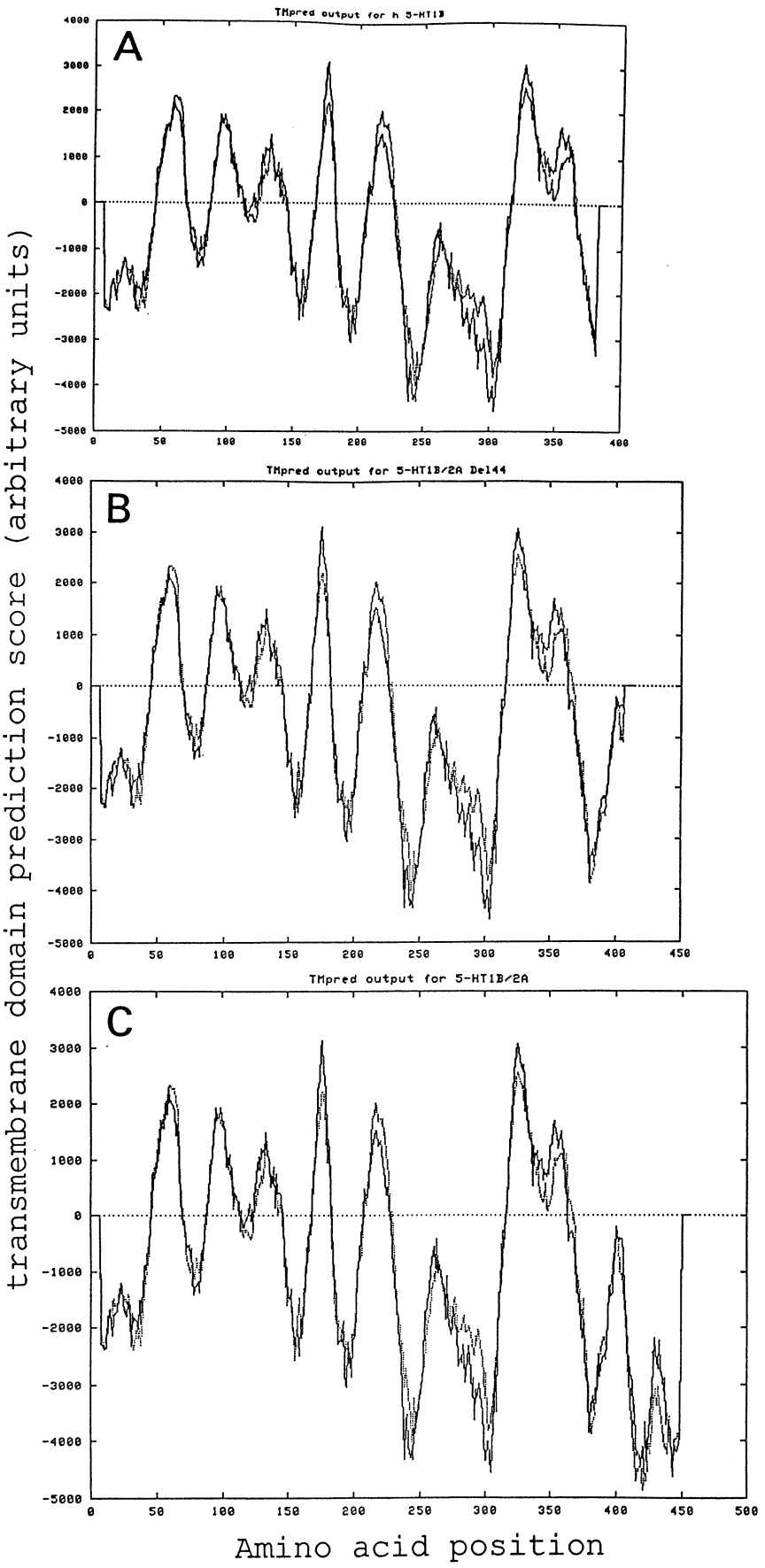

FIG. 1. Transmembrane domain prediction for wt h 5-HT_{1B} (A), chimeric 5-$HT_{1B/2A\Delta44}$ (B), and 5-$HT_{1B/2A}$ (C) receptors. The calculation algorithm is based on the statistical analysis of the transmembrane protein database TMbase as developed by Hofmann and Stoffel [15], and the resulting scores are plotted on the vertical axis. Positive scores above 500 are considered as significant for TMD prediction (TMpred).

References

1. Bonhaus DW, Bach C, DeSouza A, Salazar FH, Matsuoka BD, Zuppan P, Chan HW and Eglen RM, The pharmacology and distribution of human 5-hydroxytryptamine$_{2B}$ (5-HT_{2B}) receptor gene products: Comparison with 5-HT_{2A} and 5-HT_{2C} receptors. *Br J Pharmacol* **115:** 622–628, 1995.
2. Pauwels PJ, Reihsaus E, Palmier C, Journot L, Lyons J and Colpaert FC, Ketanserin differentiates between cloned human 5-$HT_{1D\alpha}$ receptors and cloned human 5-$HT_{1D\beta}$, sheep and bovine caudate nucleus 5-HT_{1D} receptor sites. *Cell Pharmacol* **2:** 183–191, 1995.
3. Zgombick JM, Bard JA, Kucharewicz SA, Urquhart DA, Weinshank RL and Branchek TA, Molecular cloning and pharmacological characterization of guinea pig 5-HT_{1B} and 5-HT_{1D} receptors. *Neuropharmacology* **36:** 513–524, 1997.
4. Wurch T, Palmier C, Colpaert FC and Pauwels PJ, Sequence and functional analysis of cloned guinea pig and rat serotonin 5-HT_{1D} receptors: Common pharmacological features within the 5-HT_{1D} receptor subfamily. *J Neurochem* **68:** 410–418, 1997.
5. Pauwels PJ, Tardif S, Palmier C, Wurch T and Colpaert FC, How efficacious are 5-$HT_{1B/D}$ receptor ligands: An answer from GTPγS binding studies with stably transfected C6-glial cell lines. *Neuropharmacology* **36:** 499–512, 1997.
6. Zgombick JM, Weinshank RL, Macchi M, Schechter LE, Branchek TA and Hartig PR, Expression and pharmacological characterization of a canine 5-hydroxytryptamine$_{1D}$ receptor subtype. *Mol Pharmacol* **40:** 1036–1042, 1991.
7. Wurch T, Colpaert FC and Pauwels PJ, Chimeric receptor analysis of the ketanserin binding site in the human 5-hydroxytryptamine$_{1D}$ receptor: Importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. *Mol Pharmacol* **54:** 1088–1096, 1998.
8. Wurch T, Lestienne F and Pauwels PJ, A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes. *Biotechnol Techniques* **12:** 653–657, 1998.
9. Pauwels PJ, Palmier C, Wurch T and Colpaert FC, Pharmacology of cloned human 5-HT_{1D} receptor-mediated functional responses in stably transfected rat C6-glial cell lines: Further evidence differentiating human 5-HT_{1D}, and 5-HT_{1B} receptors. *Naunyn Schmiedebergs Arch Pharmacol* **353:** 144–156, 1996.
10. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
11. Hurley JH, Bloem LJ, Pavalko F, Liu J, Tian M, Simon JR and Yu L, Structure–function studies of the eighth hydrophobic domain of a serotonin receptor. *J Neurochem* **72:** 413–421, 1999.
12. Okamoto T and Nishimoto I, Detection of G protein-activator regions in M4 subtype muscarinic, cholinergic, and α_2-adrenergic receptors based upon characteristics in primary structure. *J Biol Chem* **267:** 8342–8346, 1992.
13. Westkaemper RB and Glennon RA, Molecular graphics models of members of the 5-HT_2 subfamily: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. *Med Chem Res* **3:** 317–334, 1993.
14. Kristiansen K and Dahl SG, Molecular modeling of serotonin, ketanserin, ritanserin and their 5-HT_{2C} receptor interactions. *Eur J Pharmacol* **306:** 195–210, 1996.
15. Hofmann K and Stoffel W, TMbase—A database of membrane-spanning protein segments. *J Biol Chem* **374:** 166–, 1993.