British Journal of Pharmacology (1998) 123, 51–62

Pharmacological analysis of G-protein activation mediated by guinea-pig recombinant 5-HT_{1B} receptors in C6-glial cells: similarities with the human 5-HT_{1B} receptor

[1]Petrus J. Pauwels, Thierry Wurch, Christiane Palmier & Francis C. Colpaert

Centre de Recherche Pierre Fabre, Department of Cellular & Molecular Biology, 17, avenue Jean Moulin, 81106 Castres Cédex, France

1 The guinea-pig recombinant 5-hydroxytryptamine$_{1B}$ (gp 5-HT_{1B}) receptor stably transfected in rat C6-glial cells was characterized by monitoring G-protein activation in a membrane preparation with agonist-stimulated [^{35}S]-GTPγS binding. The intrinsic activity of 5-HT receptor ligands was compared with that determined previously at the human recombinant 5-HT_{1B} (h 5-HT_{1B}) receptor under similar experimental conditions.

2 Membrane preparations of C6-glial/gp 5-HT_{1B} cells exhibited [^{3}H]-5-carboxamidotryptamine (5-CT) and [^{3}H]-N-[4-methoxy-3,4-methylpiperazin-1-yl) phenyl]-3-methyl-4-(4-pyridinyl)benzamide (GR 125743) binding sites with a pK_d of 9.62 to 9.85 and a B_{max} between 2.1 to 6.4 fmol mg^{-1} protein. The binding affinities of a series of 5-HT receptor ligands determined with [^{3}H]-5-CT and [^{3}H]-GR 125743 were similar. Ligand affinities were comparable to and correlated (r^2: 0.74, $P<0.001$) with those determined at the recombinant h 5-HT_{1B} receptor.

3 [^{35}S]-GTPγS binding to membrane preparations of C6-glial/gp 5-HT_{1B} cells was stimulated by the 5-HT receptor agonists that were being investigated. The maximal responses of naratriptan, zolmitriptan, sumatriptan, N-methyl-3-[pyrrolidin-2(**R**)-ylmethyl]-1*H*-indol-5-ylmethylsulphonamide (CP122638), rizatriptan and dihydroergotamine were between 0.76 and 0.85 compared to 5-HT. The potency of these agonists showed a positive correlation (r^2: 0.72, $P=0.015$) with their potency at the recombinant h 5-HT^{1B} receptor. 1-naphthylpiperazine, (±)-cyanopindolol and (2′-methyl-4′-(5-methyl[1,2,4] oxadiazole-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide (GR 127935) elicited an even smaller response (E_{max}: 0.32 to 0.63).

4 The ligands 1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazole-3-yl) biphenyl-4-carbonyl)-2,3,6,7-tetrahydrospiro [furo[2,3-f]indole-3-spiro-4′-piperidine] (SB224289), methiothepin and ritanserin displayed inhibition of basal [^{35}S]-GTPγS binding at concentrations relevant to their binding affinity for the gp 5-HT_{1B} receptor. Methiothepin and SB224289 behaved as competitive antagonists at gp 5-HT_{1B} receptors; pA_2 values were 9.74 and 8.73, respectively when 5-HT was used as an agonist. These estimates accorded with the potencies measured in antagonism of zolmitriptan-mediated inhibition of forskolin-stimulated cyclic AMP formation. Ketanserin acted as a weak antagonist (pK_B: 5.87) at gp 5-HT_{1B} receptors.

5 In conclusion, the recombinant gp 5-HT_{1B} receptor shares important pharmacological similarities with the recombinant h 5-HT_{1B} receptor. The finding that negative activity occurs at these receptors further suggests that SB224289, methiothepin and ritanserin are likely to be inverse agonists.

Keywords: Guinea-pig recombinant 5-HT_{1B} receptor; human 5-HT_{1B} receptor; [^{35}S]-GTPγS binding response; agonist/inverse agonist; rat C6-glial cell line

Introduction

5-Hydroxytryptamine (5-HT) elicits diverse physiological responses as a neurotransmitter or neuromodulator in the mammalian central nervous system through distinct multiple receptor subtypes (Hoyer *et al*., 1994). Of these, 5-HT_{1B} receptors account for a large percentage of 5-HT_1 binding sites in the brain, and have been shown to be involved in mediating 5-HT actions in a number of physiological processes, such as locomotor activity, nociception, vasomotor tone and mood (Jacobs & Azmitzia, 1992; Zifa & Fillion, 1992; Hoyer *et al*., 1994; Voight, 1997). One of the first functional actions described for 5-HT_{1B} receptor activation was the attenuation of 5-HT release from brain slices, demonstrating that it acted as a terminal autoreceptor for this neurotransmitter (see Zifa & Fillion, 1992). Fink *et al*. (1995) found that the 5-HT_{1B} receptor acts as an autoreceptor in human cerebral cortex. Other studies have shown that the 5-HT_{1B} receptor can also control the release of other neurotransmitters, such as acetylcholine in the hippocampus and γ-aminobutyric acid (GABA) in the substantia nigra. The data available thus support the contention that this receptor acts as a presynaptic heteroreceptor on neurones that are postsynaptic targets for 5-HT (Johnson *et al*., 1992; Zifa & Fillion, 1992; Hoyer *et al*., 1994). It is also thought that this receptor can modulate 5-HT biosynthesis (Hjorth *et al*., 1995). Behavioral studies with 5-HT_{1B} 'knock-out' mice point to the role of 5-HT_{1B} receptors in motor activity and have provided evidence that they may also be involved in aggression (Saudou *et al*., 1994).

The initial cloning of a member of the 5-$HT_{1B/D}$ subfamily occurred with the isolation of an orphan receptor from dog, designated RDC-4 (Libert *et al*., 1989). This cDNA remained unidentified until a human gene homologue was pharmacologically characterized as being a h 5-HT_{1D} receptor subtype (Hamblin & Metcalf, 1991). Molecular cloning then identified two related proteins in rat, defined as r 5-HT_{1B} and r 5-HT_{1D} receptors based upon binding properties (Voight *et al*., 1991;

[1] Author for correspondence.

Hamblin *et al*., 1992; Adham *et al*., 1992; Bach *et al*., 1993). The presence of two genes in the rat suggested that a second gene was present in other species as well. It was found to be the homologue of the r 5-HT$_{1B}$ in man (h 5-HT$_{1B}$, Weinshank *et al*., 1992), rabbit (rb 5-HT$_{1B}$, Harwood *et al*., 1995; Wurch *et al*., 1996) and dog (ca 5-HT$_{1B}$, Branchek *et al*., 1995); on the basis of their pharmacological properties they differ strikingly from the r 5-HT$_{1B}$ receptor. Results from site-directed mutagenesis experiments (Metcalf *et al*., 1992; Oksenberg *et al*., 1992; Parker *et al*., 1993) demonstrated that the pharmacological disparity between the r 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors can be attributed to a single amino acid in the putative transmembrane domain VII; in the rat this amino acid is aspargine (Asn351) while it is a threonine (Thr355) in the human. A threonine is also present in the rb 5-HT$_{1B}$ and ca 5-HT$_{1B}$ receptor sequence (Branchek *et al*., 1995; Harwood *et al*., 1995; Wurch *et al*., 1996). Recently, the guinea-pig 5-HT$_{1B}$ (gp 5-HT$_{1B}$) receptor has been cloned (Luyten *et al*., 1996; Zgombick *et al*., 1996); its sequence contains a conserved threonine residue in transmembrane domain VII, a structural feature imparting non-rodent 5-HT$_{1B}$ pharmacology.

The aim of the present study was to analyse the pharmacological properties of the recombinant gp 5-HT$_{1B}$ receptor under similar experimental conditions as previously described for the recombinant h 5-HT$_{1B}$ receptor (Pauwels *et al*., 1997). Stably transfected rat C6 glial cells were used to determine the intrinsic activity of a series of 5-HT receptor ligands by monitoring G-protein activation with agonist-stimulated [^{35}S]-GTPγS binding. [^{35}S]-GTPγS binding responses to membrane preparations containing h 5-HT$_{1B}$ receptors have been shown (Pauwels *et al*., 1997) to differentiate between slight variations in agonist efficacy, and to discriminate between neutral antagonists and inverse agonists. The sequence as well as the pharmacological features of the gp 5-HT$_{1B}$ receptor are discussed with regard to those known for other 5-HT$_{1B}$ receptors.

Methods

Cloning of a guinea-pig 5-HT$_{1B}$ receptor gene

Cloning was performed by PCR with primers (sense primer: 5′GCCGCCACCATGGGGAACCCTGAGGC; reverse primer: 5′TGACTCAGGTTGTGCACTTAAA) designed according to a gp 5-HT$_{1B}$ receptor nucleotide sequence communicated by Luyten *et al*. (1996). PCR mixtures (each 50 μl) contained 1 μg of guinea-pig genomic DNA, 25 μM of each dNTP, 400 nM of each primer and 1.25 u Taq DNA polymerase in 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). The PCR amplification programme consisted of 30 repetitive cycles with a strand separation step at 94°C for 1 min, an annealing step at 60°C for 1 min and an elongation step at 72°C for 1.5 min. The PCR product of about 1200 bp was separated by agorose gel electrophoresis, purified with a Geneclean II kit and ligated into 50 ng of pCR3.1 plasmid. Sequencing was performed on denaturated double-stranded plasmid DNA with a Sequenase quick denature kit. The nucleotide and amino acid sequences were analysed with a WISCONSIN software package version 9.0.

Construction of a stable rat C6-glial cell line expressing gp 5-HT$_{1B}$ receptors

C6-glial cells stably transfected with a pCR3.1/gp 5-HT$_{1B}$ plasmid were prepared as a monoclonal cell line and cultured as previously described (Pauwels *et al*., 1996), and used for reverse-transcription PCR, radioligand binding experiments, [^{35}S]-GTPγS binding responses and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) measurements.

RNA isolation and reverse-transcription PCR

Total RNA was extracted from nontransfected C6-glial cells and C6-glial/gp 5-HT$_{1B}$ cells with a Trizol reagent. The total RNA was treated with RNase-free DNase (1 u 2 μg^{-1} RNA) for 1 h at 37°C and reverse-transcribed into single-stranded cDNA by use of random primers (20 pmol) and Superscript RNase H$^-$ reverse transcriptase (200 u/1 μg RNA) for 1 h at 42°C. Reverse transcription (RT)-PCR was performed with the above mentioned primers and conditions on cDNA of nontransfected and transfected cells.

5-HT$_{1B}$ receptor binding

Membrane preparations of the stably transfected C6-glial cell line expressing gp 5-HT$_{1B}$ receptors were prepared in 50 mM Tris-HCl pH 7.7 containing 4 mM $CaCl_2$, 10 μM pargyline and 0.1% ascorbic acid as previously described (Pauwels *et al*., 1996). Binding assays were performed with 0.5 nM [^{3}H]-5-carboxamidotryptamine (5-CT), or 0.3 nM [^{3}H]-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide (GR125743). Incubation mixtures consisted of 0.4 ml cell membranes (4 to 12 μg protein), 0.05 ml radioligand and 0.05 ml compound for inhibition or 10 μM 5-HT to determine nonspecific binding. The reactions were stopped after a 30 min incubation at 25°C by adding 3.0 ml ice-cold 50 mM Tris-HCl, pH 7.7, and rapid filtration over Whatman GF/B glass fibre filters with a Brandel harvester, washed and counted as previously described (Pauwels *et al*., 1996). In the case of [^{3}H]-GR125743, 50 mM Tris-HCl pH 7.7 was used and filtration was performed over 0.2% polyethyleneimine-treated Whatman GF/B glass fibre filters. Data were analysed graphically with inhibition curves and IC_{50}-values (concentration of the compound producing 50% inhibition of specific binding) were derived. K_i values 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 ^{3}H ligand. pK_d values were obtained from saturation binding studies performed as previously described (Pauwels *et al*., 1996).

[^{35}]-GTPγS binding responses to membrane preparations of C6-glial cells stably expressing gp 5-HT$_{1B}$ receptors

Cells were collected in phosphate-buffered-saline (pH 7.4) and centrifuged for 20 min at 48,000 *g*. The pellet was homogenized with a Polytron in 20 mM HEPES containing 10 mM EDTA (pH 7.4) and recentrifuged for 10 min at 48,000*g*. The resulting pellet was washed twice in 20 mM Hepes containing 0.1 mM EDTA (pH 7.4) and homogenized with a Polytron. The pellet was stored at −80°C in fractions of 400 μg protein. For [^{35}S]-GTPγS binding assays, the pellet was thawed, diluted 150 times in 20 mM HEPES (pH 7.4) supplemented with 0.3 μM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ascorbic acid. Incubation mixtures were prepared in glass tubes and consisted of 0.4 ml of membrane preparation (5 μg of protein) and 0.05 ml of compound in either the absence or presence of antagonist. After an incubation period of 30 min at 25°C, 0.05 ml [^{35}S]-GTPγS (500 pM) was added for an additional period of 30 min. The reactions were stopped by adding 3 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ and rapid filtration

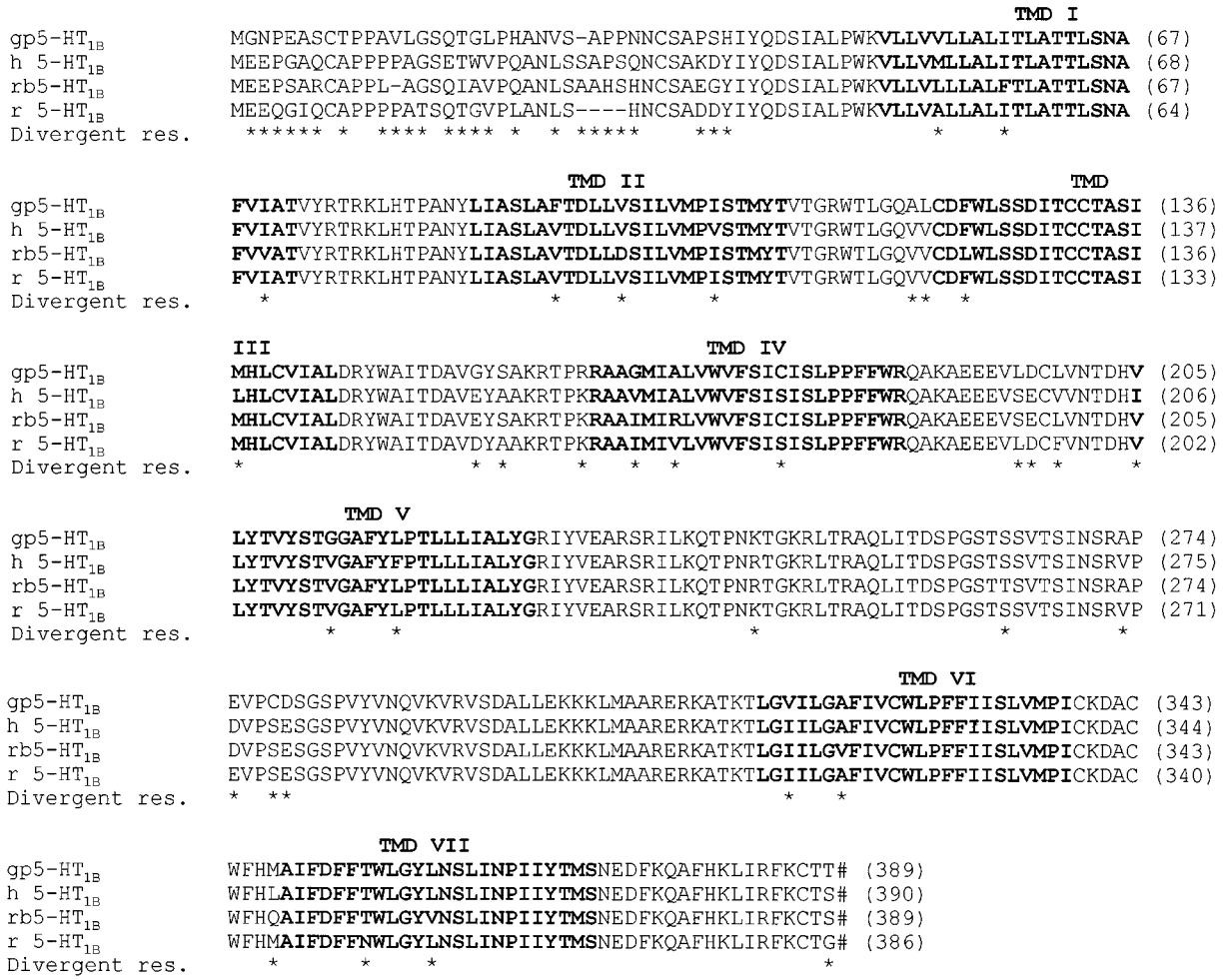

Figure 1 Alignment of the amino acid sequences of gp 5-HT$_{1B}$, h 5-HT$_{1B}$, rb 5-HT$_{1B}$ and r 5-HT$_{1B}$ receptors. An asterisk indicates the presence of one or more divergent residues within the four presented 5-HT$_{1B}$ receptor sequences. Putative transmembrane domains (TMD) are highlighted in boldface. The # corresponds to the termination codon of the nucleotide sequence (res.: residues).

Table 1 Percentages of overall and partial (transmembrane (TMD) and N-terminal extracellular (N-term.) domain) amino acid identity between gp 5-HT$_{1B}$, h 5-HT$_{1B}$, rb 5-HT$_{1B}$ and r 5-HT$_{1B}$ receptors

	gp 5-HT$_{1B}$	*h 5-HT$_{1B}$*	*rb 5-HT$_{1B}$*	*r 5-HT$_{1B}$*
gp 5-HT$_{1B}$	100	89	88	90
h 5-HT$_{1B}$		100	93	93
rb 5-HT$_{1B}$			100	91
r 5-HT$_{1B}$				100
gp 5-HT$_{1B}$ (TMD)	100	94	93	94
gp 5-HT$_{1B}$ (N-term.)	100	62	58	56

The percentages were calculated over the sequences that are represented in Figure 1.

over Whatmann GF/B glass fibre fiters with a Brandel harvester. The filters were rinsed three additional times with 3 ml HEPES buffer, placed in scintillation vials and the radioactivity was extracted in 4 ml of Emulsifier-Safe. Nonspecific binding was determined in the presence of 10 μM unlabelled GTPS. Maximal stimulation of [^{35}S]-GTPγS binding was defined in the presence of 10 μM 5-HT. E_{max} values were expressed as a percentage of the maximal response obtained with 10 μM 5-HT. EC_{50} values were defined as the concentration of compound at which 50% of its own maximal stimulation was obtained. IC_{50} values represent the concentration of the compound that showed 50% of its inhibition of basal [^{35}S]-GTPγS binding obtained at 1 μM. In antagonist experiments, the putative antagonist was co-incubated with 5-HT. Concentration-ratios were calculated and used to obtain estimates of pA_2 values using the following equation: $pA_2 = \log(\text{concentration ratio} - 1) - \log(\text{antagonist concentration})$.

5-HT$_{1B}$ receptor-mediated inhibition of stimulated cyclic AMP formation

Inhibition of forskolin (100 μM)-stimulated cyclic AMP formation in the C6-glial/gp 5-HT$_{1B}$ cell line was measured as previously described (Pauwels *et al*., 1996). Cultures were incubated for 5 min at 37°C with 1.0 ml controlled salt solution containing 1 mM isobutylmethylxanthine in the presence of 100 μM forskolin either in the absence or presence of 1 μM 5-HT or zolmitriptan to determine maximal cyclic AMP inhibition. The reaction was stopped by the addition of 0.1 ml ice-cold HCl_4 to a final concentration of 0.04 M and afterwards neutralized. The cellular cyclic AMP content was assayed using a radioimmunoassay kit. Inhibition of forskolin-induced cyclic AMP formation was calculated as the percentage of that obtained with 1 μM 5-HT. EC_{50} values (concentration of the compound yielding 50% inhibition induced by 1 μM zolmitriptan) were derived. Antagonists were given 15 min before the agonists. pK_B values were calculated as

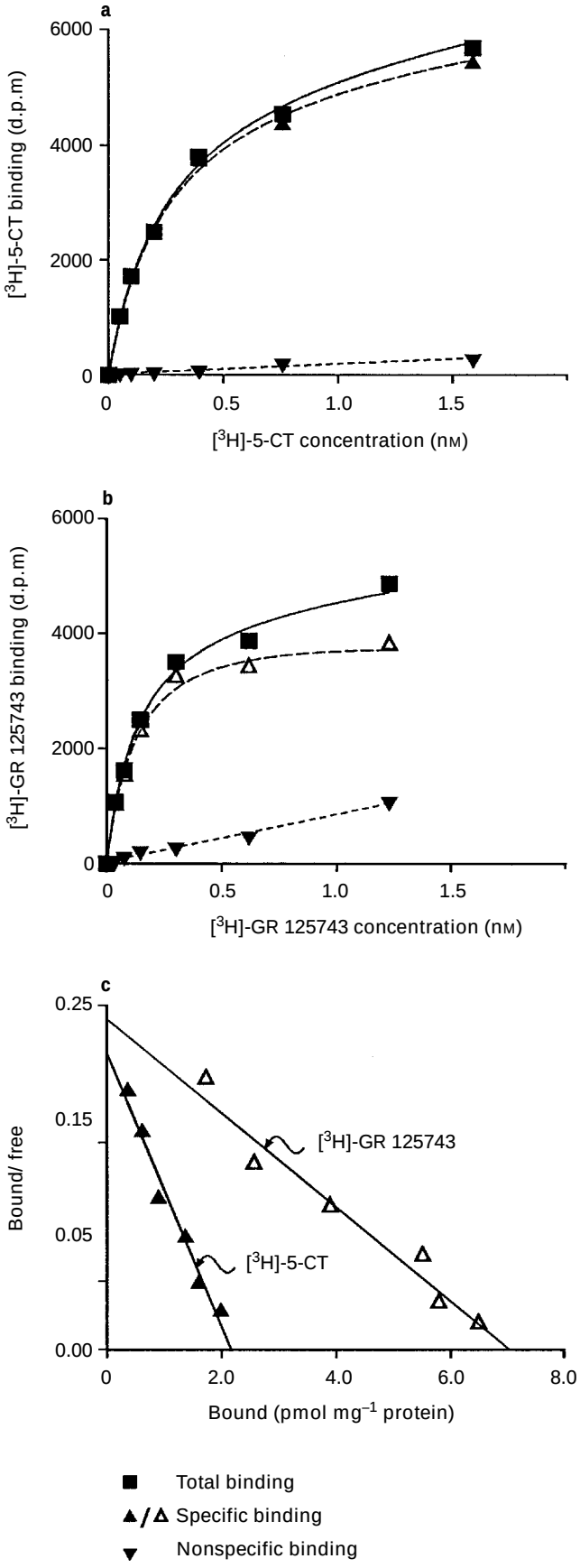

Figure 2 Saturation binding curves and Scatchard plots of [^{3}H]-5-CT and [^{3}H]-GR 125743 binding to a membrane preparation of C6-glial/gp 5-HT$_{1B}$ cells. (a) [^{3}H]-5-CT binding, (b), [^{3}H]-GR125743 binding, (c), Scatchard plots of [^{3}H]-5-CT and [^{3}H]-GR 125743 binding. The free ligand concentration was calculated as the counted ligand concentration minus the totally bound ligand concentration; specific binding was calculated as the total binding minus the nonspecific binding. Curves were constructed with mean values of one representative experiment out of three independent experiments, each one performed in duplicate.

$K_B = (B)/(A'/A)-1$ where B is the concentration of the antagonist, and A and A′ are the EC_{50} values of agonist concentration measured in the absence and presence of antagonist.

Protein content

Membrane protein levels were estimated with the dye-binding assay by use of the Bio-Rad kit (Bradford, 1976). Bovine serum albumin was used as a standard.

Materials

C6-glial cells were obtained from ATCC (Rockville, U.S.A.). Cell culture media, foetal calf serum, culture plates were obtained from Gibco Biocult. Laboratories (Paisley, U.K.). The Geneclean II kit was purchased from Bio101 Inc. (La Jolla, U.S.A.). The pCR3.1 vector and TA cloning kit were from Invitrogen (San Diego, U.S.A.). The quick denature sequencing kit was from Amersham (Les Ulis, France). RNase-free DNase was from Stratagene (La Jolla, U.S.A.). Taq DNA polymerase and Superscript RNase H$^-$ reverse transcriptase was from Gibco Life Technology (Gaitersburg, USA). The cyclic AMP radioimmunoassay kit was from Immunotech (Marseille, France). The Emulsifier-Safe was obtained from Packard (Warrenville, PA, U.S.A.). [^{3}H]-5-CT (51.3 Ci mmol^{-1}), [N-methyl-^{3}H]- GR125743 (69 Ci mmol^{-1}), and [^{35}S]-GTPγS (guanosine-5′-(γ-thiotriphosphate); 1100 Ci mmol^{-1}) were obtained from New Engand Nuclear (Les Ulis, France) or Amersham (Les Ulis, France). Zolmitriptan (4(S)-[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-ylmethyl]oxazolidin-2-one), sumatriptan, naratriptan [N-methyl-2-[3-(1-methylpiperidin-4-yl)-1*H*-indol-5-yl]ethanesulphonamide), N-methyl-3-[pyrrolidin-2(**R**)-ylmethyl]-1*H*-indol-5-ylmethyl-sulphonamide (CP12 2638), rizatriptan (N,N-dimethyl-2-[5-(1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethylamine). 2′-Methyl-4′-(5-methyl[1,2,4]oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]amide (GR127935), 1-naphthylpiperazine and 1′-methyl-5-(2′-methyl-4′-(5-methyl-1,2,4-oxadiazole-3-yl) biphenyl-4-carbonyl)2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3-spiro-4′-piperidine] (SB224289) were prepared at the Centre de Recherche Pierre Fabre. Methiothepin and 5-CT were obtained from Tocris Cookson (Bristol, U.K.). 5-HT and dihydroergotamine were from Sigma (St Louis, U.S.A.). Ketanserin, ritanserin, (±)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and its enantiomers were from RBI (Natick, U.S.A.). (±)-cyanopindolol was from Tebu (Le Perray en Yvelines, France). Stock solutions (10^{-3} M) of compounds were prepared in water, ethanol or dimethylsulphoxide.

Results

Cloning, sequence analysis and stable expression of a gp 5-HT$_{1B}$ receptor gene

Cloning of a gp 5-HT$_{1B}$ receptor gene was performed by PCR. Three independent PCR products were sequenced in order to eliminate errors due to random nucleotide misincorporations by the Taq DNA polymerase. An intronless, single open

Table 2 E_{max} and pEC_{50} values of 5-HT receptors ligands for stimulation of [^{35}S]-GTPγS binding to membrane preparations of C6-glial cell lines expressing either recombinant gp 5-HT$_{1B}$ or h 5-HT$_{1B}$ receptors, and their corresponding pK_i values

Receptor subtype	*[^{35}S]-GTPγS binding responses*				*Binding affinities*		
	gp 5-HT$_{1B}$		*h 5-HT$_{1B}$*		*gp 5-HT$_{1B}$*		*h 5-HT$_{1B}$*
	E_{max} (%)	*pEC_{50}/pIC_{50} (95% CL)*	*E_{max} (%)*	*pEC_{50}*	*[^{3}H]-5-CT pK_i (95% CL)*	*[^{3}H]-GR125743 pK_i (95% CL)*	*[^{3}H]-5-CT pK_i (95% CL)*
Compound:							
5-HT	100	7.74 (0.24)	100	7.04	8.89 (0.14)	7.67 (0.01)	9.04
Naratriptan	85±2	7.14 (0.21)	80	7.64	8.26 (0.24)		9.33
R(+)-8-OH-DPAT	83±3	6.16 (0.55)			7.04 (0.17)		7.12[a]
Zolmitriptan	81±5	7.25 (0.10)	99	7.22	8.75 (0.27)		9.08
Sumatriptan	79±2	6.49 (0.33)	96	6.63	7.84 (0.29)		8.50
CP122638	78±3	6.85 (0.18)	72	6.52	7.88 (0.16)		8.60
Dihydroergotamine	77±2	8.45 (0.45)	47	8.69	8.65 (0.13)		9.10
Rizatriptan	76±3	6.92 (0.24)	75	6.63	8.12 (0.42)		8.14
S(−)-8-OH-DPAT	67±9	4.89 (0.52)			5.70 (0.07)		5.76[a]
1-Naphthylpiperazine	63±10	7.68 (0.04)	15	<5[b]	8.90 (0.25)		8.40[c]
(±)-Cyanopindolol	39±9	6.32 (0.39)			7.10 (0.14)		6.97 (0.14)[d]
GR127935	32±5	8.45 (0.54)	10	<5	8.87 (0.03)	8.66 (0.14)	9.56
Ketanserin	−3±2	<5	−2	<5	5.43 (0.05)	6.41 (0.00)	<5
Ritanserin	−19±4	6.59	−16	7.72[b]	6.94 (0.16)	7.54 (0.19)	6.09[c]
SB224289	−20±4	8.05			8.42 (0.01)	8.63 (0.13)	8.27 (0.58)[d]
Methiothepin	−24±7	8.66	−14	8.52	8.80 (0.08)	9.53 (0.16)	7.69

Binding was performed with 500 pM [^{35}S]-GTPγS to membrane preparations of stably transfected C6-glial cell lines as described in Methods. Mean E_{max} values±s.e.mean are expressed versus the stimulation obtained with 10 μM 5-HT. pEC_{50} values are defined as the concentration at which 50% of the maximal stimulation was obtained for each individual compound. pIC_{50} values are underscored and calculated versus the inhibition obtained at 1 μM for each individual compound. pK_i values were obtained from [^{3}H]-5-CT and [^{3}H]-GR125743 binding experiments. Data for the C6-glial/h 5-HT$_{1B}$ receptor were taken from Pauwels *et al.* (1997), except for [a]Pauwels & Colpaert. (1996); [b]Pauwels (1997b); [c]Pauwels *et al.* (1996). [d]Data were obtained in Cos-7 instead of C6-glial cells.

reading frame of 1170 base pairs encoding a polypeptide of 389 amino acids was revealed. Its amino acid sequence is illustrated in Figure 1 and compared to the h 5-HT$_{1B}$ (Weinshank *et al.*, 1992), rb 5-HT$_{1B}$ (Wurch *et al.*, 1996) and r 5-HT$_{1B}$ (Voigt *et al.*, 1991) receptor sequences. These four 5-HT$_{1B}$ receptors share between 88 and 93% overall amino acid sequence identity (Table 1); 59 amino acids are different in one or more of the presented sequences (Figure 1). The rb 5-HT$_{1B}$ and gp 5-HT$_{1B}$ receptors are slightly more divergent (88%) than the h 5-HT$_{1B}$ and rb 5-HT$_{1B}$ receptor sequences (93%). The amino acid identity increases to 94% when the 7 transmembrane domains (TMD) are taken into account. Sixteen of the 18 TMD amino acid differences are conservative; on the other hand Asp97 in TMD II and Arg170 in TMD IV of the rb 5-HT$_{1B}$ receptor sequence are substituted by aliphatic residues (Val or Ala) in the gp 5-HT$_{1B}$, h 5-HT$_{1B}$ and r 5-HT$_{1B}$ receptor sequences. A Thr354 residue is present in TMD VII of the gp 5-HT1B receptor as is the case for h 5-HT$_{1B}$ and rb 5-HT$_{1B}$ receptors, opposed to an Asn residue in the r 5-HT$_{1B}$ receptor. The extra-membrane loops account for 15 amino acid differences between these four 5-HT$_{1B}$ receptors, 10 of them are conservative substitutions. The aliphatic Gly155 and Cys278 residues of the gp 5-HT$_{1B}$ receptor are replaced respectively by acidic (Asp or Glu) or hydroxylated (Ser) amino acids in h 5-HT$_{1B}$, rb 5-HT$_{1B}$ and r 5-HT$_{1B}$ receptors. The Ser and Gln residues (Ser157 in gp 5-HT$_{1B}$ and rb 5-HT$_{1B}$, Ser197 in h 5-HT$_{1B}$ and rb 5-HT$_{1B}$, and Gln347 in rb 5-HT$_{1B}$ receptors) are exchanged for an aliphatic Ala, Leu or Met residue. All 18 amino acids of the C-terminal intracellular end are identical, except the last one, which corresponds to a basic residue (Thr or Ser) in gp 5-HT$_{1B}$, h 5-HT$_{1B}$ and rb 5-HT$_{1B}$ receptors and to a glycine residue for the r 5-HT$_{1B}$ receptor. The N-terminal extracellular domain of the gp 5-HT$_{1B}$ receptor is the most different part of the 5-HT$_{1B}$ receptor (Table 1): 25 of the 45 to 49 amino acids of this domain are divergent between the gp 5-HT$_{1B}$ and the three presented species corresponding to 56 to 62% identity (Table 1). A Glu-Glu doublet following the initiator Met residue, conserved in h 5-HT$_{1B}$, rb 5-HT$_{1B}$ and r 5-HT$_{1B}$ receptors, is replaced by a Gly-Asn doublet in the gp 5-HT$_{1B}$ receptor (Figure 1). Whereas the h 5-HT$_{1B}$ receptor possesses one extra-residue (Pro14 and Ser27 compared to the rb 5-HT$_{1B}$ and gp 5-HT1B receptor sequences, respectively), four amino acids are deleted in the r 5-HT$_{1B}$ compared to the h 5-HT$_{1B}$ receptor sequence. In spite of these differences, two putative N-glycosylation sites located in the N-terminal region are conserved amongst these 5-HT$_{1B}$ receptors; Asn24 and Asn31 for the gp 5-HT$_{1B}$ receptor (Figure 1).

A rat C6-glial cell line stably expressing a gp 5-HT$_{1B}$ receptor was constructed. The presence of corresponding gp 5-HT$_{1B}$ receptor mRNA was confirmed by RT-PCR. The amplification of 1 μg guinea-pig genomic DNA and 50 ng C6-glial/gp 5-HT$_{1B}$ RT, DNase-treated RNA yielded a similar amplification product of about 1200 base pairs as for 1 ng of a pCR3.1 vector containing the gp 5-HT$_{1B}$ receptor coding sequence. Partial sequencing of the PCR product obtained with C6-glial/gp 5-HT$_{1B}$ RT, DNase-treated RNA confirmed its nucleotide specificity. No amplification was obtained in RNA samples (500 ng) of transfected cells without reverse-transcription and RT, DNase-treated RNA (500 ng) from nontransfected C6-glial cells.

Binding properties of 5-HT receptor ligands at recombinant gp 5-HT$_{1B}$ receptors

Saturation binding experiments with [^{3}H]-5-CT and [^{3}H]-GR125743 on a membrane preparation of C6-glial cells stably transfected with a gp 5-HT$_{1B}$ receptor gene indicated the presence of a single high affinity binding site with a similar affinity for [^{3}H]-5-CT (pK_d: 9.62, 95% CL: 0.07) and [^{3}H] GR125743 (pK_d: 9.85, 95% CL: 0.20). The maximal binding capacity for both radioligands was between 2.13 ± 0.10 ([^{3}H]-5-CT) and 6.40±0.06 ([^{3}H]-GR125743) pmol mg^{-1} protein (Figure 2). Binding with [^{3}H]-5-CT to gp 5-HT$_{1B}$ receptors on

intact C6-glial cells showed a 12 fold lower affinity (pK_d: 8.52, 95% CL: 0.09) and B_{max} of 0.83 ± 0.11 pmol mg^{-1} protein (not shown). This supports that only a fraction (39%) of the total 5-HT$_{1B}$ receptor population in transfected C6-glial cells binds [^{3}H]-5-CT at the plasma membrane, in line with previous observations for rb 5-HT$_{1B}$ receptors (Wurch *et al*., 1997a). It can be assumed that 5-CT, being chemically very similar to 5-HT, does not penetrate the plasma membrane. Therefore, 5-CT is likely to label only functionally active gp 5-HT$_{1B}$ receptors on intact C6-glial cells.

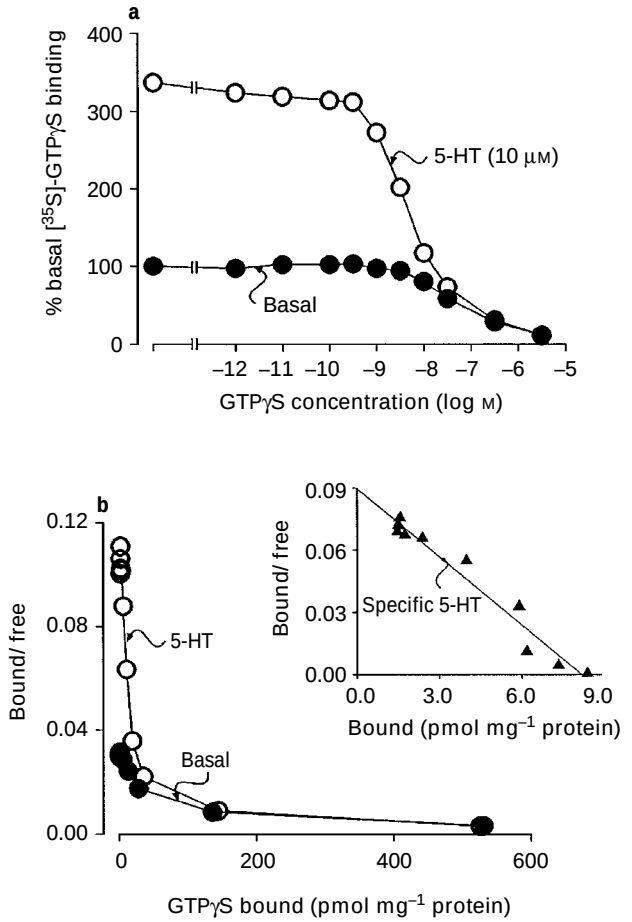

Figure 3 Homologous displacement and Scatchard analysis of [^{35}S]-GTPγS binding to C6-glial/gp 5-HT$_{1B}$ cell membranes. Membranes were incubated with 500 pM [^{35}S]-GTPγS, 0.3 μM GDP and either without or with 1 pM to 3 μM unlabelled GTPγS in the absence or presence of 10 μM 5-HT. (a) Data shown are from a representative experiment as percentage of basal [^{35}S]-GTPγS binding in the absence of unlabelled GTPγS, (b) Scatchard analysis of basal and 5-HT-stimulated [^{35}S]-GTPγS binding. Inset, Scatchard analysis of 5-HT-specific-stimulated [^{35}S]-GTPγS binding. Basal [^{35}S]-GTPγS binding was 658 fmol mg^{-1} protein. pK_d and B_{max} values from Scatchard analysis are given in Table 3.

A series of sixteen 5-HT receptor ligands was tested for inhibition of [^{3}H]-5-CT and/or [^{3}H]-GR125743 binding to gp 5-HT$_{1B}$ receptors in a transfected C6-glial membrane preparation (Table 2). Whereas the binding affinity of 5-HT was higher with [^{3}H]-5-CT, the other ligands tested showed either a similar or higher affinity with [^{3}H]-GR 125743 compared to [^{3}H]-5-CT. The gp 5-HT$_{1B}$ receptor binding data showed the following features: the triptan-like molecules bound with similar binding affinity; **R**(+)-8-OH-DPAT and **S**(−)-8-OH-DPAT displayed a 40 fold difference in their affinity; (±)-cyanopindolol and ketanserin showed submicromolar and micromolar affinity, respectively; and GR127935, SB224289 and methiothepin recognized this binding site with nanomolar affinities. Ligand affinities were comparable to those determined at the recombinant h 5-HT$_{1B}$ receptor (r^2: 0.74, $P < 0.001$) although slight binding differences were apparent with ritanserin, methiothepin and naratriptan. Ritanserin and methiothepin displayed higher affinity for the gp 5-HT$_{1B}$ receptor whereas naratriptan yielded a 12 fold lower affinity compared to the h 5-HT$_{1B}$ receptor (Table 2).

[^{35}]GTPγS binding responses to membrane preparations containing gp 5-HT$_{1B}$ receptors

In contrast to nontransfected C6-glial cell membranes, 5-HT (10 μM) stimulated [^{35}S]-GTPγS binding to membranes containing gp 5-HT$_{1B}$ receptors. The 5-HT stimulation was dependent on the GDP concentration (not shown) and the present data were obtained with 0.3 μM GDP. Scatchard analysis of basal and 5-HT-stimulated [^{35}S]-GTPγS binding to membranes is shown in Figure 3. Relatively little high affinity [^{35}S]-GTPγS binding in the absence of agonist was observed, as demonstrated by both the homologous displacement of [^{35}S]-GTPγS binding by increasing concentrations of GTPγS and Scatchard analysis of these data. The addition of 5-HT (10 μM) produced stimulation of [^{35}S]-GTPγS binding above basal levels. This stimulation resulted in a biphasic Scatchard plot with an increase in high affinity binding and almost no change in low affinity binding. Scatchard analysis of 5-HT-specific-stimulated [^{35}S]-GTPγS binding indicated a single class of high affinity [^{35}S]-GTPγS binding sites (Figure 3, inset). The corresponding pK_d and B_{max} values for each of these conditions are summarized in Table 3.

A comparison between the concentration-effect curve for 5-HT and other 5-HT receptor ligands that yielded stimulation of [^{35}S]-GTPγS binding is shown in Figure 4. The corresponding E_{max} and pEC_{50} values are summarized in Table 2, and compared with the data obtained at the h 5-HT$_{1B}$ receptor stably expressed in C6-glial cells. The maximal responses of rizatriptan, dihydroergotamine, CP122638, sumatriptan, zolmitriptan and naratriptan were between 0.76 and 0.85 compared to that of 5-HT, whereas sumatriptan and zolmitriptan showed the same maximal response as 5-HT at

Table 3 Scatchard analysis of basal and 5-HT-stimulated [^{35}S]-GTPγS binding to C6-glial/gp 5-HT$_{1B}$ membranes

	High affinity [^{35}S]-GTPγS binding		*Low affinity [^{35}S]-GTPγS binding*	
	pK_d (95% CL)	B_{max} (pmol mg^{-1} protein)	pK_d (95% CL)	B_{max} (pmol mg^{-1} protein)
Basal	7.54 (0.14)	27.52 ± 6.09	5.69 (0.02)	672 ± 107
5-HT 10 μM	8.30 (0.16)	17.87 ± 2.10	5.86 (0.14)	584 ± 105
Specific 5-HT	8.59 (0.12)	8.13 ± 1.80		

Data were obtained from 3 independent experiments as described in the legend to Figure 3. pK_d and B_{max} (± s.e.mean) values are given from biphasic Scatchard analysis of basal and 5-HT-mediated [^{35}S]-GTPγS binding, and from monophasic Scatchard analysis of 5-HT-specific-stimulated [^{35}S]-GTPγS binding.

the h 5-HT$_{1B}$ receptor. The enantiomers of 8-OH-DPAT also stimulated [^{35}S]-GTPγS binding with a 20 fold difference in their potency. 1-Naphthylpiperazine, (±)-cyanopindolol as well as GR127935 induced a partial [^{35}S]-GTPγS binding response; the maximal responses of 1-naphthylpiperazine and GR127935 were more pronounced at the gp 5-HT$_{1B}$ than h 5-HT$_{1B}$ receptor. Similarly, the maximal response of dihydroergotamine was more pronounced at the gp 5-HT$_{1B}$ than h 5-HT$_{1B}$ receptor. The agonist potencies fitted well with their corresponding pK_i values for the gp 5-HT$_{1B}$ receptor (r^2: 0.86, $P<0.001$). Correlation analysis performed between the agonist potencies at the recombinant gp 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptor subtype shows a positive significant correlation (r^2: 0.72, $p=0.015$).

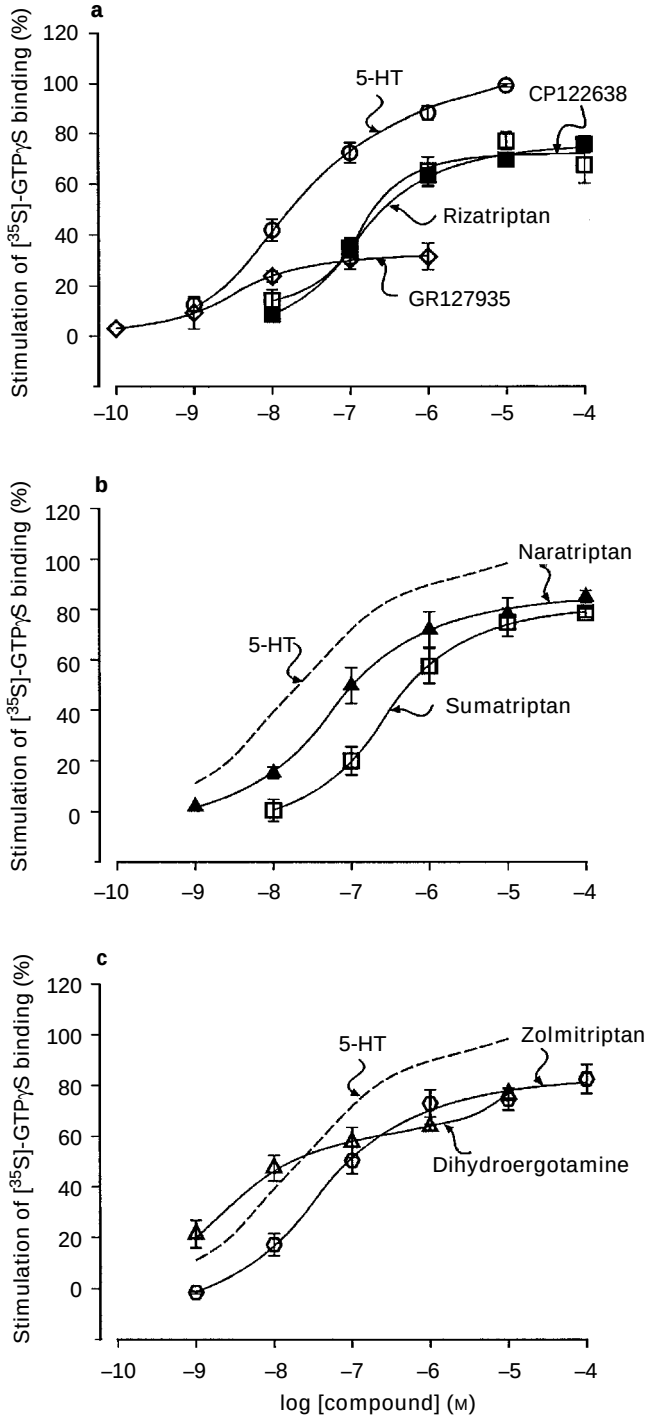

Figure 4 Concentration-effect curves of 5-HT receptor ligands for stimulation of [^{35}S]-GTPγS binding to C6-glial cellular membranes containing gp 5-HT$_{1B}$ receptors. Stimulation of [^{35}S]-GTPγS binding is expressed as a percentage of that obtained with 10 μM 5-HT. Curves were constructed with mean values from 11 (5-HT) and 4 to 6 independent experiments for the other compounds, each one performed in triplicate; vertical lines show s.e.mean. Mean E_{max} and pEC$_{50}$ values are summarized in Table 2. Basal and 5-HT (10 μM) [^{35}S]-GTPγS binding values were 853 ± 75 and 2047 ± 116 pmol mg^{-1} protein, respectively.

The ligands methiothepin, SB224289, ritanserin and ketanserin inhibited basal [^{35}S]-GTPγS binding. Their concentration-effect curves were with the exception of ketanserin biphasic (Figure 5). The inhibition of basal [^{35}S]-GTPγS binding by SB224289 was reversed at micromolar and higher concentrations, whereas the inhibition was accentuated for the other compounds. Analysis of the concentration-effect curves in the submicromolar range indicated pIC$_{50}$ values close to their pK_i values (Table 2). Methiothepin, SB224289 and GR127935 potently antagonized the 5-HT-stimulated [^{35}S]-GTPγS binding response. Figure 6 shows the concentration-dependent antagonism of 5-HT-induced stimulation of [^{35}S]-GTPS binding. Parallel displacement of the concentration-effect curve of 5-HT was observed for methiothepin and SB224289. This was also apparent for GR127935 if the intrinsic activity of GR127935 was taken into account. The pA_2 values derived from Schild analysis (Figure 6) are in a similar concentration range as that obtained by measuring antagonism of zolmitriptan-mediated inhibition of 100 μM forskolin-induced cyclic AMP formation (Table 4). Ketanserin was a weak antagonist (pK_B: 5.87) of the 5-HT-induced [^{35}S]-GTPγS binding response at gp 5-HT$_{1B}$ receptors.

Discussion

The present study demonstrated the stable and functional expression of recombinant gp 5-HT$_{1B}$ receptors in rat C6-glial cells. A pharmacological comparison between the gp 5-HT$_{1B}$ receptor and h 5-HT$_{1B}$ receptor under the same experimental conditions revealed important similarities with regard to binding properties and functional responses of the 5-HT ligands being investigated. The [^{35}S]-GTPγS binding responses showed that these 5-HT ligands can be classified into efficacious agonists, more partial agonists and inverse agonists. Slight differences between gp 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors were observed in their maximal responses to zolmitriptan, sumatriptan and dihydroergotamine. The C6-glial/gp 5-HT$_{1B}$ cell line appears also to be more sensitive to intrinsic activity of 1-naphthylpiperazine and GR127935 in comparison with the C6-glial/h 5-HT$_{1B}$ cell line. It is not clear whether this is a consequence either of the recombinant C6-glial cell line properties or of species differences. The [^{35}S]-GTPγS binding responses mediated by the gp 5-HT$_{1B}$ receptor showed that not any of the agonists investigated produced a maximal response similar to that of 5-HT, whereas zolmitriptan and sumatriptan showed a maximal response in the C6-glial/h 5-HT$_{1B}$ cell line. Therefore, most of the 5-HT$_{1B}$ receptor agonists investigated are partial agonists. This observation underlines the notion that very few ligands are highly efficacious at gp 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors. It also emphasizes the point that the failure to observe a difference in apparent ligand efficacy in a particular model system does not necessarily preclude the absence of such a difference, only that the system was inadequate to make it observable. There was apparently no ligand that could be classified as a neutral antagonist at the gp 5-HT$_{1B}$ receptor. This implies that none of the antagonists studied binds with equal affinity to both the G-protein-

uncoupled and G-protein-coupled forms of this receptor. The ligands SB224289, methiothepin and ritanserin inhibited basal [^{35}S]-GTPγS binding to C6-glial/gp 5-HT$_{1B}$ and C6-glial/h 5-HT$_{1B}$ membranes and can therefore be described as inverse agonists at gp 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptors. The ability to detect negative intrinsic activity mainly depends on a measurable constitutive or agonist-independent activity. Moreover, the ability of a ligand to act as an inverse agonist may also be cell specific (Chiu *et al*., 1996). This may explain why some of these ligands are often described as antagonists rather than as inverse agonists.

Sequence analysis of the gp 5-HT$_{1B}$ receptor with the genes encoding h 5-HT$_{1B}$ and rb 5-HT$_{1B}$ receptors shows a high (88 to 89%) overall amino acid identity. Its homology with the gp 5-HT$_{1D}$ receptor (Wurch *et al*., 1997b) is much lower (63%), as it is for the homology between the h 5-HT$_{1B}$/5-HT$_{1D}$ (63%, Weinshank *et al*., 1992), rb 5-HT$_{1B}$/5-HT$_{1D}$ (62%, Hamblin *et al*., 1992), and r 5-HT$_{1B}$/5-HT$_{1D}$ receptor subtypes (61%, Harwood *et al*., 1995). The presence of a Thr354 in transmembrane domain VII (Luyten *et al*., 1996; Zgombick *et al*., 1996; this study) together with a moderate binding affinity of (±)-cyanopindolol for the gp 5-HT$_{1B}$ receptor strongly suggest that the pharmacology of the gp 5-HT$_{1B}$ receptor is more related to the h 5-HT$_{1B}$ (Weinshank *et al*., 1992) and rb 5-HT$_{1B}$ (Wurch *et al*., 1997a) receptor subtypes than to the rodent 5-HT$_{1B}$ receptor subtypes (Guan *et al*., 1992; Oksenberg *et al*., 1992; Adham *et al*., 1994). Some amino acid changes are obvious, in particular in the N-terminal region of the 5-HT$_{1B}$ receptors, where amino acids are deleted in gp 5-HT$_{1B}$, r 5-HT$_{1B}$ and rb 5-HT$_{1B}$ receptors compared to the h 5-HT$_{1B}$ receptor. It is less clear what the significance is of these amino acid changes in relation to 5-HT$_{1B}$ receptor binding and/or functional properties. On the basis of its affinity for ketanserin the gp 5-HT$_{1B}$ receptor is likely to resemble more the h 5-HT$_{1B}$ than the rb 5-HT$_{1B}$ receptor. The rb 5-HT$_{1B}$ receptor displays a higher potency (pK_B: 7.0; Wurch *et al*., 1997a) for the antagonist ketanserin than h 5-HT$_{1B}$ and gp 5-HT$_{1B}$ receptors. Ketanserin shares various affinities for 5-HT$_{1B}$/5-HT$_{1D}$ receptors depending on the species. Ketanserin is unable to differentiate between the ca 5-HT$_{1D}$ and ca 5-HT$_{1B}$ receptor subtypes, as it has a low affinity for them (pK_i of 5.5 and 5.3, respectively; Branchek *et al*., 1995). In contrast, ketanserin has binding affinity for and shows competitive antagonist properties at recombinant h 5-HT$_{1D}$ (pK_B: 7.28 to 7.76), gp 5-HT$_{1D}$ (pK_B: 7.51) and r 5-HT$_{1D}$ (pK_B: 7.92) receptors (Zgombick *et al*., 1995; Pauwels *et al*., 1996; Wurch *et al*., 1997b). Its low affinity for h 5-HT$_{1B}$, gp 5-HT$_{1B}$ and r 5-HT$_{1B}$ (pK_i: <5; Van Wijngaarden *et al*., 1990) receptors demonstrates ketanserin to differentiate between 5-HT$_{1D}$ and 5-HT$_{1B}$

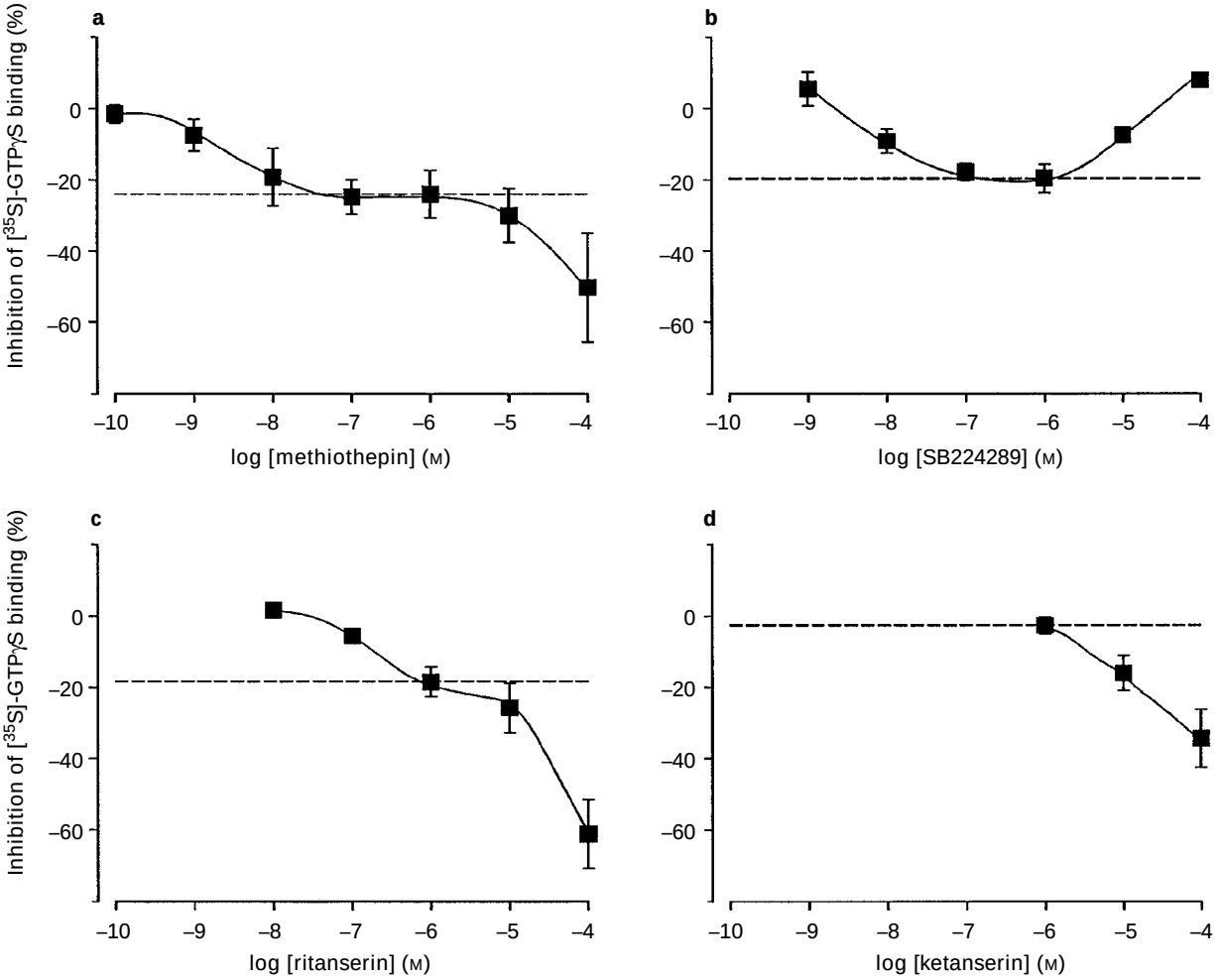

Figure 5 Inhibition of basal [^{35}S]-GTPγS binding to C6-glial cellular membranes containing gp 5-HT$_{1B}$ receptors by (a) methiothepin, (b) SB224289, (c) ritanserin and (d) ketanserin. [^{35}S]-GTPγS binding was measured as described in Methods. [^{35}S]-GTPγS binding is expressed as a percentage of that obtained with 10 μM 5-HT. Curves were constructed with mean values from 3 to 6 independent experiments, each one performed in triplicate; vertical lines show s.e.mean. Values for inhibition of [^{35}S]-GTPγS binding at 1 μM and pIC$_{50}$ values are summarized in Table 2. The dotted line represents the inhibition obtained with 1 μM of each of the compounds.

receptors from man, guinea-pig and rat. Very few ligands differentiate between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Besides ketanserin, BRL15572 is also selective for h 5-HT$_{1D}$ receptors (Price *et al*., 1996), whereas SB216641 and SB224289 are selective for 5-HT$_{1B}$ receptors (Roberts *et al*., 1997b). The ligand binding profile of gp 5-HT$_{1B}$ and gp 5-HT$_{1D}$ (Wurch *et al*., 1997b) receptors is similar (r^2: 0.80, $p < 0.001$) with the exception of ketanserin and SB224289. Whereas ketanserin preferentially binds to the gp 5-HT$_{1D}$ receptor (Wurch *et al*., 1997b), SB224289 selectively binds to the gp 5-HT$_{1B}$ receptor (pK_i gp 5-HT$_{1D}$: 6.18, unpublished results). The observed binding affinities of 5-HT compounds for 5-HT$_{1B/D}$ binding

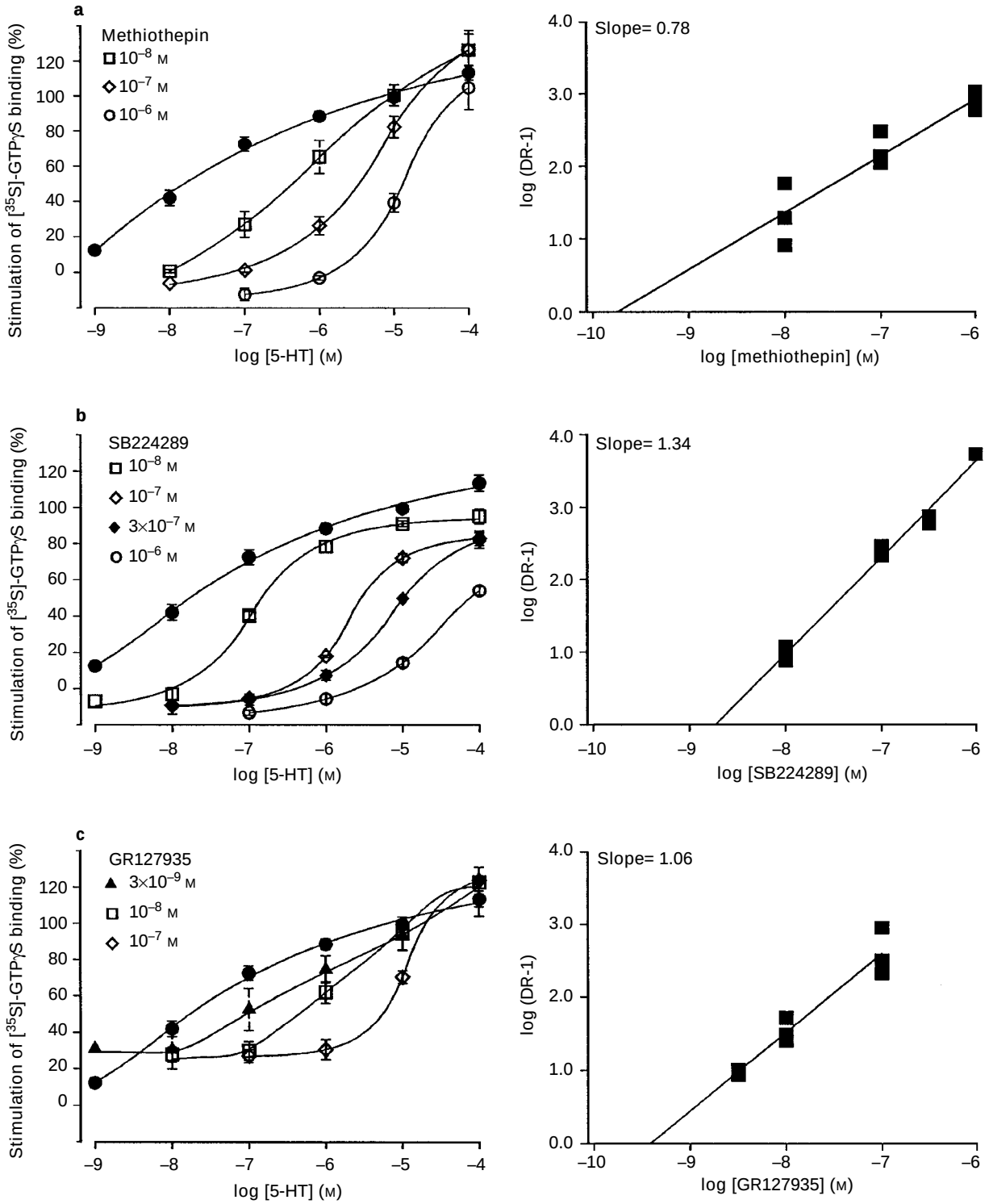

Figure 6 Antagonist effects of (a) methiothepin, (b) SB224269 and (c) GR127935 on 5-HT-mediated stimulation of [^{35}S]-GTPγS binding to C6-glial cellular membranes containing gp 5-HT$_{1B}$ receptors. Membranes were preincubated with the indicated concentrations of 5-HT in either the absence or presence of 10 to 1000 nM methiothepin (a), 10 to 1000 nM SB224289 (b), and 3 to 100 nM GR127935 (c) for 30 min before [^{35}S]-GTPγS binding was performed. Stimulation of [^{35}S]-GTPγS binding is expressed as a percentage of that obtained with 10 μM 5-HT. Concentration binding curves were constructed with mean values from 3 independent experiments, each one performed in triplicate; vertical lines show s.e.mean. Schild plots were constructed with individual EC$_{50}$ values. The gradient of the best-fit straight line was determined by linear regression. In the case of GR127935, its intrinsic activity was subtracted to calculate EC$_{50}$ values. pA$_2$ values are summarized in Table 4.

Table 4 Potencies of methiothepin, SB224289, GR127935 and ketanserin for antagonism of gp 5-HT$_{1B}$ receptor-mediated stimulation of [^{35}S]-GTPγS binding and corresponding potencies obtained by measuring antagonism of gp 5-HT$_{1B}$ receptor-mediated inhibition of forskolin-stimulated cyclicAMP formation

	[^{35}S]-GTPγS, pA$_2$ (95% CL)	*CyclicAMP, pK$_B$ (95% CL)*
Compound		
Methiothepin	9.74 (1.00)	8.55 (0.23)
SB224289	8.73 (0.14)	9.24 (0.37)
GR127935	9.44 (0.50)	>9.62
Ketanserin	5.87[a]	ND

Antagonism of 5-HT-stimulated [^{35}S]-GTPγS binding to membrane preparations of stably transfected C6-glial cells was performed as described in the legend to Figure 6. pA$_2$ values were calculated as described in Methods. pK_B values for antagonism of zolmitriptan (pEC$_{50}$: 7.95, 95% CL: 0.38)-mediated inhibition of forskolin (100 μM)-stimulated cAMP formation was performed as described in Methods. [a]pK_B instead of pA$_2$ value. ND: not determined.

sites measured in guinea-pig cortical membranes correlate with the binding profile of both recombinant gp 5-HT$_{1B}$ and gp 5-HT$_{1D}$ receptors (Bruinvels *et al*., 1992; Zgombick *et al*., 1996).

Functional analysis of the recombinant gp 5-HT$_{1B}$ receptor with the agonist-stimulated [^{35}S]-GTPγS binding assay, suggests that C6-glial/gp 5-HT$_{1B}$ membrane preparation constitutes a tool to differentiate between slight variations in agonist efficacy. The agonists that stimulated [^{35}S]-GTPγS binding showed a maximal response between 0.32 and 0.85 compared to that of 5-HT (1.00). This was under experimental conditions where the B$_{max}$ of 5-HT-stimulated [^{35}S]-GTPγS binding was four times higher than the B$_{max}$ of [^{3}H]-5-CT binding. This ratio is similar to that for h 5-HT$_{1B}$ receptors in C6-glial cells (3.1, unpublished results) and suggests that both 5-HT$_{1B}$ receptors show the same ability to activate G-proteins in C6-glial cells. On the basis of this assumption the responses of the 5-HT ligands can be compared between both 5-HT$_{1B}$ receptors. The experimental data demonstrate a positive correlation between the potency of the agonists at the gp 5-HT$_{1B}$ receptor and their potency at the h 5-HT$_{1B}$ receptor. Some variation was observed in the maximal responses for the following ligands: zolmitriptan and sumatriptan appeared to be as efficacious as 5-HT in the C6-glial/h 5-HT$_{1B}$ cell line; otherwise, the maximal response to dihydroergotamine was greater at the gp -HT$_{1B}$ receptor than the h 5-HT$_{1B}$ receptor; and intrinsic activity was more important with the putative antagonists GR127935 and 1-naphthylpiperazine in the C6-glial/gp 5-HT$_{1B}$ cell line. Although there is some variation in the maximal response of certain agonists, it can be concluded that the responses of these agonists at the gp 5-HT$_{1B}$ and h 5-HT$_{1B}$ receptor are quite similar. Whereas the maximal responses of the partial agonists are likely to vary between different model systems, the C6-glial cell lines are useful to differentiate between highly efficacious agonists, partial agonists with various degrees of intrinsic activity, and neutral antagonists at 5-HT$_{1B}$ receptors.

The gp 5-HT$_{1B}$ receptor-mediated [^{35}5S]-GTPγS response was partially inhibited by GR 127935, thus extending previous observations (see Pauwels, 1997a) that GR 127935 produces partial intrinsic activity at 5-HT$_{1B}$ receptors. This characterization can also account for some *in vivo* data; Yu *et al*. (1997) showed that GR 127935 acts as a partial antagonist of sumatriptan-mediated inhibition of neurogenic plasma extravasion within guinea-pig dura mater. Otherwise, methiothepin and SB224289 potently antagonized the 5-HT response in a competitive manner. They also yielded negative intrinsic activity, like ritanserin. This was evident at gp 5-HT$_{1B}$ as well as h 5-HT$_{1B}$ receptors at concentrations relevant to their binding affinities. Similar results were obtained with methiothepin in a CHO-K1/h 5-HT$_{1B}$ cell line (Thomas *et al*., 1995). *In vivo* evidence for 5-HT$_{1B}$ receptor inverse agonists is scarce. Methiothepin has been postulated as an inverse agonist at the terminal autoreceptor in the rat hypothalamus (Moret & Briley, 1993). However, neither methiothepin nor the selective 5-HT$_{1B}$ inverse agonist SB224289 have been found to increase extracellular 5-HT in brain terminal regions upon systemic administration (Moret & Briley, 1995; Roberts *et al*., 1997a,b). This may be a result of the attenuation of the endogenous 5-HT tone at cell body autoreceptors in the raphe. Such an increase in local 5-HT levels could then stimulate 5-HT$_{1A}$ receptors to inhibit cell firing and hence decrease 5-HT levels in the terminal regions. Despite the lack of *in vivo* supportive data at the present time, it may be more appropriate to develop inverse agonists to enhance 5-HT release than neutral antagonists because the latter would only operate in the presence of a sufficient 5-HT tone (Pauwels, 1997b). Inverse agonists may also be expected to block various organs under basal neurochemical control (Kenakin *et al*., 1995) or attenuate the stimulation of receptors, constitutively active, in pathological conditions (see Lanier *et al*., 1996). Hence, the search for and development of inverse agonists may be an important target for drug discovery.

In conclusion, the recombinant gp 5-HT$_{1B}$ receptor has been investigated in a C6-glial cell line and shows besides its sequence homology important pharmacological similarities with the h 5-HT$_{1B}$ receptor. The finding of inverse agonist activity with some of the ligands further emphasizes the need to differentiate between neutral antagonists and inverse agonists at 5-HT$_{1B}$ receptors.

Note added in proof

During the review of this manuscript another publication has appeared on the cloning of the guinea-pig 5-HT$_{1B}$ receptor (J.M. Zgombick *et al*., 1997, *Neuropharmacology*, **36,** 513-524).

We sincerely thank J-C. Blanchet, C. Cathala and F. Lestienne for excellent technical assistance, and S. Cecco for fruitful secretarial assistance.

References

ADHAM, N., ROMANEINKO, P., HARTIG, P., WEINSHANK, R.L. & BRANCHEK, T. (1992). The rat 5-hydroxytryptamine1B receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol. Pharmacol*., **41,** 1–7.

ADHAM, N., TAMM, J.A., SALON, J.A., VAYSSE, P.J.J., WEINSHANK, R.L. & BRANCHEK, T.A. (1994). A single point mutation increases the affinity of serotonin 5-HT$_{1D\alpha}$, 5-HT$_{1D\beta}$, 5-ht$_{1E}$ and 5-ht$_{1F}$ receptors for β-adrenergic antagonists. *Neuropharmacology*., **33,** 387–391.

BACH, A.W.J., UNGER, L., SPRENGEL, R., MENGOD, G., PALACIOS, J., SEEBURG, P.H. & VOIGT, M.M. (1993). Structure, functional expression and spatial distribution of a cloned cDNA encoding a rat 5-HT$_{1D\text{-like}}$ receptor. *J. Recept. Res*., **13,** 479–502.

BRADFORD, M.M. (1976). 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.

BRANCHEK, T.A., BARD, J.A., KUCHAREWICZ, S.A., ZGOMBICK, J.M., WEINSHANK, R.L. & COHEN, M. L. (1995). Migraine relationship to cloned canine and human 5-HT$_{1D}$ receptors. In *Experimental Headache Models*, ed. Olsen, J. & Moskowitz, M.A. pp. 125-129, New York: Raven press.

BRUINVELS, A.T., LERY, H., NOZULAK, J., PALACIOS, J.M. & HOYER, D. (1992). 5-HT$_{1D}$ binding sites in various species: similar pharmacological profile in dog, monkey, calf, guinea-pig and human brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 479 – 485.

CHIU, T.T., YUNG, L.Y. & WONG, Y.H. (1996) Inverse agonist effect of ICI-174,864 on the cloned δ-opioid receptor: role of G protein and adenylyl cyclase activation. *Mol. Pharmacol.*, **50,** 1651 – 1657.

FINK, K., ZENTER, J. & GOTHERT, M. (1995). Subclassification of presynaptic 5-HT autoreceptors in the human cerebral cortex as 5-HT$_{1D\beta}$ receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352,** 451 – 454.

GUAN, X.M., PEROUTKA, S.J. & KOBILKA, B.K. (1992). Identification of a single amino acid residue responsible for the binding of a class of β-adrenergic receptors antagonists to 5-hydroxytryptamine$_{1A}$ receptors. *Mol. Pharmacol.*, **41,** 695 – 698.

HAMBLIN, M.W. & METCALF, M.A. (1991). Primary structure and functional characterization of a human 5-HT$_{1D}$-type serotonin receptor. *Mol. Pharmacol.*, **40,** 143 – 148.

HAMBLIN, M.W., McGUFFIN, R.W., METCALF, M.A., DORSA, D.M. & MERCHANT, K. M. (1992). Distinct 5-HT$_{1B}$ and 5-HT$_{1D}$ serotonin receptors in rat: structural and pharmacological comparison of the two cloned receptors. *Mol. Cell. Neurosci.*, **3,** 578 – 587.

HARWOOD, G., LOCKYER, M., GILES, H. & FAIRWEATHER, N. (1995). Cloning and characterisation of the rabbit 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors. *FEBS Lett.*, **377,** 73 – 76.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPRHEY, P.P.A. (1994). VII International union of pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 158 – 203.

HJORTH, S., SUCHOWSKI, S.S. & GALLOWAY, M.P. (1995). Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. *Synapse*, **19,** 170 – 176.

JACOBS, B.L. & AZMITIA, E.C. (1992). Structure and function of the brain serotonin system. *Physiol. Rev.*, **72,** 165 – 229.

JOHNSON, S.W., MERCURI, N.B. & NORTH, R.A. (1992). 5-Hydroxytryptamine$_{1B}$ receptors block the GABA$_B$ synaptic potential in rat dopamine neurons. *J. Neurosci.*, **12,** 2000 – 2006.

KENAKIN, T., MORGANE, P. & LUTZ, M. (1995). On the importance of the 'antagonist assumption' to how receptors express themselves. *Biochem. Pharmacol.*, **50,**, 17 – 26.

LANIER, S. M., LAFONTAN, M., LIMBIRD, L. E. & PARIS, H. (1996). Summary of the ASPECT-sponsored Colloquium: *alpha*-2 adrenergic receptors: structure, function, and therapeutic implications. *J. Pharmacol. Exp. Ther.*, **277,** 10 – 16.

LIBERT, F., PARMENTIER, M., LEFORT, A., DINSART, C., VAN SANDE, J., MAENHAUT, C., SIMONS, M-J., DUMONT, J. & VASSART, G. (1989). Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science*, **244,** 569 – 572.

LUYTEN, W.H.M.L., VAN DE WEYER, I., NOBELS, G., PARKER, A., VAN GOMPEL, P., ERCKEN, M., LESAGE, A. & LEYSEN, J.E. (1996). Genomic cloning and heterologous expression of a recombinant guinea pig serotonin 5-HT$_{1D\beta}$-receptor. *Soc. Neurosci.*, **22,** 1329.

METCALF, M.A., McGUFFIN, R.W. & HAMBLIN, M.W. (1992). Conversion of the human 5-HT$_{1D\beta}$ serotonin receptor to the rat 5-HT$_{1B}$ ligand-binding phenotype by Thr^{355} Asn site directed mutagenesis. *Biochem. Pharmacol.*, **44,** 1917 – 1920.

MORET, C. & BRILEY, M. (1993). The unique effect of methiothepin on the terminal serotonin autoreceptor in the rat hypothalamus could be an example of inverse agonism. *J. Psychopharmacol.*, **7,** 331 – 337.

MORET, C. & BRILEY, M. (1995). The effect of terminal 5-HT autoreceptor antagonists on 5-HT release in the guinea-pig brain. *Br. J. Pharmacol. Proc. Suppl.*, **115,** 31P.

OKSENBERG, D., MASTERS, S.A., O'DOWD, B.F., JIN, H., HAVLIK, S., PEROUTKA, S.J. & ASHKENAZI, A. (1992). A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT$_{1B}$ receptors. *Nature*, **360,** 161 – 163.

PARKER, E.M., GRISEL, D.A., IBEN, L.G. & SHAPIRO, R.A. (1993). A single amino acid difference accounts for the pharmacological distinction between the rat and human 5-hydroxytryptamine$_{1B}$ receptor. *J. Neurochem.*, **60,** 380 – 383.

PAUWELS, P.J. (1997a). Pharmacological properties of a putative 5-HT$_{1B/D}$ receptor antagonist GR 127,935. *CNS Drug Rev.*, **2,** 415 – 428.

PAUWELS, P.J. (1997b). 5-HT$_{1B/D}$ receptor antagonists. *Gen. Pharmacol.*, **29,** 293 – 304.

PAUWELS, P.J. & COLPAERT, F.C. (1996) Stereoselectivity of 8-OH-DPAT enantiomers at cloned human 5-HT$_{1D}$ receptor sites. *Eur. J. Pharmacol.*, **300,** 137 – 139.

PAUWELS, P.J., PALMIER, C., WURCH, T., & COLPAERT, F.C. (1996). 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-Schmiedeberg's Arch. Pharmacol.*, **353,** 144-156.

PAUWELS, P.J., TARDIF, S., PALMIER, C., WURCH, T. & COLPAERT, F.C. (1997). 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.

PRICE, G.W., BURTON, M.J., ROBERTS, C., WATSON, J., DUCKWORTH, M., GASTER, L., MIDDLEMISS, D.N. & JONES, B.J. (1996). SB 216641 and BRL 15572 pharmacologically discriminate between h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. *Br. J. Pharmacol. Proc. Suppl.*, **119,** 301P.

ROBERTS, C., PRICE, G.W. & JONES, B.J. (1997a). The role of 5-HT$_{1B/1D}$ receptors in the modulation of 5-hydroxytryptamine levels in the frontal cortex of the conscious guinea-pig. *Eur. J. Pharmacol.*, **326,** 23 – 30.

ROBERTS, C., PRICE, G.W., JONES, B.J., MIDDLEMISS, D.N., GASTER, L. & ROUTLEDGE, C. (1997b). Importance of 5-HT$_{1B}$ selectivity for 5-HT terminal autoreceptor activity: an *in vivo* microdialysis study in the freely-moving guinea-pig. *Br. J. Pharmacol. Proc. Suppl.*, **120,** 140P.

SAUDOU, F., AMARA, D.A., DIERICH, A., LeMEUR, M., RAMBOZ, S., SEGU, L., BUHOT, M-C. & HEN, R. (1994). Enhanced aggressive behavior in mice lacking 5-HT$_{1B}$ receptor. *Science*, **265,** 1875 – 1878.

THOMAS, D.R., FARUQ, S.A., BALCAREK, J.M. & BROWN, A.M. (1995). Pharmacological characterisation of [^{35}S]-GTPγS binding to Chinese hamster ovary cell membranes stably expressing cloned human 5-HT$_{1D}$ receptor subtypes. *J. Recept. Signal Transduct. Res.*, **15,** 199 – 211.

VAN WIJNGAARDEN, I., TULP, M.T.M. & SOUDIJN, W. (1990). The concept of selectivity in 5-HT receptor research. *Eur. J. Pharmacol. Mol. Pharmacol. Section*, **188,** 301 – 312.

VOIGT, M.M., LAURIE, D.J., SEEBURG, P.H. & BACH, A. (1991). Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine receptor. *EMBO J.*, **10,** 4017 – 4023.

VOIGT, M. (1997). Sibling rivarly: the 5-HT$_{1B}$/5-HT$_{1D}$ receptors. *Serotonin ID Research Alert*, *2,* 47 – 51.

WEINSHANK, R.J., ZGOMBICK, J.M., MACCHI, M.J., BRANCHEK, T.A. & HARTIG, P.R. (1992). Human serotonin$_{1D}$ receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 3630 – 3634.

WURCH, T., CATHALA, C., PALMIER, C., VALENTIN, J-P., JOHN, G.W., COLPAERT, F.C. & PAUWELS, P.J. (1996). Molecular cloning and identification of a rabbit saphenous vein 5-HT$_{1D\beta}$ receptor gene. *Neurosci. Res. Commun.*, **18,** 155-162.

WURCH, T., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1997a). Recombinant saphenous vein 5-HT$_{1B}$ receptors of the rabbit: comparative pharmacology with human 5-HT$_{1B}$ receptors. *Br. J. Pharmacol.*, **120,** 153-159.

WURCH, T., PALMIER, C., COLPAERT, F.C. & PAUWELS, P.J. (1997b). 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.

YU, X-J., CUTRER, F.M., MOSKOWITZ, M.A. & WAEBER, C. (1997). The 5-HT$_{1D}$ receptor antagonist GR 127,935 prevents inhibitory effects of sumatriptan but not CP-122,288 and 5-CT on neurogenic plasma extravasion within guinea-pig dura mater. *Neuropharmacology*, **36,** 83-91.

ZIFA, E. & FILLION, G. (1992). 5-Hydroxytryptamine receptors. *Pharmacol. Rev.*, **44,** 402 – 458.

ZGOMBICK, J.M., BARD, J.A., KUCHAREWICZ, S.A., URQUHART, D.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1996). Cloning and characterization of recombinant guinea-pig 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors. *Soc. Neurosci.*, **22,** 1330.

ZGOMBICK, J.M., SCHECHTER , L.E., KUCHAREWICS, S.A., WEINSHANK, R.L. & BRANCHEK, T.A. (1995). Ketanserin and ritanserin discriminate between recombinant human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes. *Eur. J. Pharmacol. Mol. Pharmacol. Section*, **291** 9–15.

(Received July 21, 1997
Revised September 30, 1997
Accepted September 30, 1997)