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Running title : Pharmacological profile of the human 5-HT$_{4(e)}$ receptor

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Abstract

1 RT-PCR technique was used to clone the human 5-HT$_{4(e)}$ receptor (h5-HT$_{4(e)}$) from heart atrium. We showed that this h5-HT$_{4(e)}$ receptor splice variant is restricted to brain and heart atrium.

2 Recombinant h5-HT$_{4(e)}$ receptor was stably expressed in CHO and C6-glial cell lines at 347 and 88 fmol mg^{-1} protein, respectively. Expression of h5-HT$_{4(e)}$ receptors at the cell membrane was confirmed by immunoblotting.

3 The receptor binding profile, determined by competition with [^{3}H]GR113808 of a number of 5-HT$_4$ ligands, was consistent with that previously reported for other 5-HT$_4$ receptor isoforms. Surprisingly, we found that the rank order of potencies (EC_{50}) of 5-HT$_4$ agonists obtained from adenylyl cyclase functional assays was inversely correlated to their rank order of affinities (K_i) obtained from binding assays. Furthermore, EC_{50} values for 5-HT, renzapride and cisapride were two fold lower in C6-glial cells than in CHO cells.

4 ML10302 and renzapride behaved like partial agonists on the h5-HT$_{4(e)}$ receptor. These results are in agreement with the reported low efficacy of the these two compounds on L-type Ca^{2+} currents and myocyte contractility in human atrium.

5 A constitutive activity of the h5-HT$_{4(e)}$ receptor was observed in CHO cells in the absence of any 5-HT$_4$ ligand and two 5-HT$_4$ antagonists, GR113808 and ML10375, behaved as inverse agonists.

6 These data show that the h5-HT$_{4(e)}$ receptor has a pharmacological profile which is close to the native h5-HT$_4$ receptor in human atrium with a functional potency which is dependent on the cellular context in which the receptor is expressed.

Keywords: Human, atrial arrhythmia, serotoninergic receptors, 5-HT$_4$ ligands, inverse agonism, G-protein coupled receptors, adenylyl cyclase, benzamides.

Abbreviations:

- 5-HT, 5-hydroxytryptamine
- 5-MeOT, 5-methoxytryptamine
- BIMU1, endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide
- C6 cells, rat glioma cells
- CHO cells, chinese hamster ovary cells
- cisapride, *cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide
- GR113808, [1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate)
- GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'- methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide
- h5-HT$_4$, human 5-HT$_4$ receptor
- I_{Ca}, L-type Ca^{2+} channel current
- I_f, pacemaker current
- ML10302, 2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride
- ML10375, 2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate
- renzapride (BRL 24924), (±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride
- RS23597, 3-(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride
- RS39604, 1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone
- RS67333, 1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone
- SB204070, 8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride

Introduction

Several receptors mediate the action of serotonin [5-hydroxytryptamine (5-HT)] both in the central nervous system and the periphery (Saxena, 1995). The 5-HT$_4$ receptor is a member of the seven transmembrane-spanning G protein-coupled family of receptors which is positively coupled to adenylyl cyclase (Bockaert & Pin, 1999). Functional responses of the 5-HT$_4$ receptor have been described in a wide variety of vertebrate tissues including brain, heart, gastrointestinal tract, bladder and adrenal gland where they produce many physiological effects (Eglen *et al.*, 1995 ; Hedge & Eglen, 1996). In addition, 5-HT$_4$ receptors are thought to be involved in a variety of central and peripheral human disorders including gastroparesis (Hedge & Eglen, 1996) and neurodegenerative disorders such as Alzheimer's disease (Reynolds *et al.*, 1995; Wong *et al.*, 1996).

With respect to the heart, activation of 5-HT$_4$ receptors can induce strong positive chronotropic, inotropic and lusitropic effects in human and pig atrium (Kaumann, 1991; Kaumann *et al.*, 1991). These cardiac effects of 5-HT$_4$ receptors are associated with an increase in intracellular cAMP leading to an activation of cAMP-dependent protein kinase which phosphorylates several key proteins involved in the excitation-contraction coupling such as the L-type Ca^{2+} channel (Ouadid *et al.*, 1991; Hove-Madsen *et al.*, 1996; Blondel *et al.*, 1997). Furthermore, Pino and colleagues (1998) have recently reported that 5-HT$_4$ receptor stimulation can increase the pacemaker current I_f in human isolated atrial myocytes. Therefore, given the ability of 5-HT$_4$ receptors to activate

two potential arrhythmogenic currents, the L-type Ca^{2+} channel current I_{Ca} and I_f, it has been suggested that these receptors may be involved in the genesis of atrial arrhythmias (Kaumann, 1994; Workman & Rankin, 1998). In support of this hypothesis, serotonin-induced arrhythmic contractions have been demonstrated in isolated human atrial strips (Kaumann & Sanders, 1994) and these effects were abolished by specific 5-HT$_4$ antagonists indicating mediation through 5-HT$_4$ receptors. Thus, 5-HT$_4$ receptors may represent a new potential therapeutic target for the treatment of cardiac arrhythmias as well as other 5-HT$_4$ receptor associated disorders. In order to design novel potent and selective 5-HT$_4$ ligands an expeditious pharmacological characterisation of 5-HT$_4$ receptors is necessary.

A large number of pharmacological studies have been performed in a wide variety of tissues and species including rat oesophagus, rat and human brain, human and porcine heart (Ford & Clarke, 1993; Eglen *et al.*, 1995). 5-HT$_4$ receptors have a unique pharmacology which is clearly different from that of the other members of the 5-HT receptor family (Ford & Clarke, 1993). Interestingly, despite the existence of a typical 5-HT$_4$ pharmacological profile, heterogeneity has been reported on the basis of differences in the potency and intrinsic activity of 5-HT$_4$ ligands in different biological models. For instance, the benzoate ML10302 is a highly potent, selective, and partial 5-HT$_4$ agonist in guinea pig and rat oesophagus (Langlois *et al.*, 1994). In contrast, it displays a poor agonistic effect on I_{Ca} in human atrial myocytes (Blondel *et al.*, 1997) and behaves as an antagonist in mouse colliculi neurones (Ansanay *et al.*, 1996). Yet, one of the main pharmacological differences between 5-HT$_4$ receptors in different tissues and species, comes from the observation that benzamides behave either as full agonists in mouse colliculi neurones (Dumuis *et al.*, 1989) or as partial agonists in human heart, rat distal colon and oesophagus (Ouadid *et al.*, 1991; Wardle & Sanger, 1993; Bockaert *et al.*, 1998). Taken together, these findings suggest the existence of several 5-HT$_4$

receptors which may account for the apparent pharmacological discrepancies regarding the efficiency of 5-HT$_4$ ligands tested in different tissues.

Recently, remarkable progress in understanding the pharmacological behaviour of 5-HT$_4$ receptors has been made with the molecular identification of four splice variants of the human 5-HT$_4$ receptor (h5-HT$_4$) (Blondel *et al.*, 1997; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998a). These splice variants have been named h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$, and h5-HT$_{4(d)}$ and are generated by splicing events that occur in the C-terminus of the h5-HT$_4$ receptor. Tissue distribution studies revealed some degree of specificity in the pattern of expression of the different human isoforms with h5-HT$_{4(a)}$, h5-HT$_{4(b)}$ and h5-HT$_{4(c)}$ being expressed in cardiac atria and brain (Blondel *et al.*, 1998a). This distinct spatial distribution may explain the difference in efficacy of 5-HT$_4$ ligands on 5-HT$_4$ receptor-mediated responses in various tissues. Alternatively, the coupling efficiency of each 5-HT$_4$ receptor isoform may be influenced by the cellular environment. In support of this hypothesis, alternative splicing in the C-terminal sequence of other G-protein coupled receptors was shown to contribute to the coupling specificity between receptor and G proteins and hence to determine the specificity of the signalling pathway of a given receptor (Namba *et al.*, 1993; Spengler *et al.*, 1993).

To further understand the pharmacology of the h5-HT$_4$ receptor and its pathological implication in atrial arrhythmias, we searched for other splice variants in human heart. In this study, we report the molecular identification and the tissue expression of an additional h5-HT$_4$ splice variant cloned from human atrium. Sequence analysis revealed that this isoform corresponds to the recently identified h5-HT$_{4(e)}$ receptor isolated from human brain (Claeysen *et al.*, 1999). Tissue distribution studies showed that this h5-HT$_{4(e)}$ receptor splice variant is restricted to brain and heart atrium. In order to provide a pharmacological profile of this newly described h5-HT$_{4(e)}$ receptor and to analyse the influence of the cellular environment

on its signalling pathway, we stably introduced h5-HT$_{4(e)}$ receptor expression vectors into chinese hamster ovary cells (CHO) and rat glioma cells (C6). Development of specific antibodies against the second extracellular loop of the h5-HT$_4$ receptor allowed us to demonstrate the presence of the h5-HT$_{4(e)}$ receptor in these cellular clones. Finally, binding studies and the functional profile of the h5-HT$_{4(e)}$ were compared in these two cellular systems using a number of standard 5-HT$_4$ agonists and antagonists.

Materials and Methods

Surgery

All protocols for obtaining human tissue were approved by the ethics committee of our institution (GREBB, Hôpital de Bicêtre, Université de Paris-Sud). Specimens of right atrial appendages were obtained from patients undergoing heart surgery for coronary artery diseases or valve replacement at the Institut Hospitalier Jacques Cartier, Massy, France.

PCR cloning of the human 5-HT$_{4(e)}$ cDNA

Primers HHT45 and HHT43C (Blondel *et al.*, 1998a; accession number Y08756) corresponding to the beginning of the receptor sequence and the 3' untranslated part of the splice variant h5-HT$_{4(a)}$ respectively, were used to amplify 2 μg of total RNA from human atrium. This RNA was then reverse transcribed with oligo(dt) primers and Superscript reverse transcriptase (Life Technologies Inc., Cergy-Pontoise, France). Products of this first reaction were used as templates for a nested PCR amplification using primers HTS5 and HHT43D, a 3' primer specific for h5-HT$_{4(a)}$ isoform (Blondel *et al.*, 1998a; accession number Y08756). Both PCR reactions were performed using the following cycle conditions : denaturation for 1 min at 94°C, annealing for 45 s at 55°C, and extension for 1 min 30 at 72°C with the final extension for 8 min. The PCR products were electrophoresed on a 2% agarose gel containing 0.01% ethidium bromide and photographed under U.V. irradiation at 320 nm. Photograph analysis of the gel revealed two DNA fragments of about 760 and 850 bp, which were cloned into the pGEMT-easy vector (Promega,

Charbonnieres, France) and sequenced. We found that the 760 bp PCR product corresponds to the 3′ end of the h5-HT$_{4(a)}$ variant and the 850 bp DNA fragment is the 3′ end of the h5-HT$_{4(e)}$ recently isolated by Claeysen *et al.* (1999) (accession number AJ011371) from human brain. To obtain the full cDNA of the h5-HT$_{4(e)}$ receptor, a PCR reaction was performed using primers HHT45 and G2S (5'-TGCTCTGTACACCGTTCTGC-3', accession number AJ011371). PCR was performed with Taq DNA polymerase (Boehringer Mannheim, Meylan, France), 94°C for 3 min, followed by 35 cycles of denaturation (30 sec at 94°C), annealing (40sec at 52°C) and elongation (1min at 72°C). These conditions yielded a band of approximately 1180 bp that was subcloned into the pGEMT-easy vector (Promega, Charbonnieres, France) and sequenced with a T7 DNA Polymerase sequencing kit (Amersham Pharmacia Biotech, Orsay, France) according to the manufacturer instructions.

Tissue localisation studies

Total RNA was prepared from human brain and peripheral tissues using the Trizol RNA purification system (Life Technologies Inc., Cergy-Pontoise, France). cDNA was prepared using the same procedure as described above and cDNA specific for h5-HT$_{4(e)}$ receptor was detected using a nested PCR amplification. A first reaction was performed using 100 ng of cDNA together with specific primers HHT45 and G1rev (5'-GCAGAAGAGCAGGAGGAAGC-3' ; accession number AJ011371) designed to the 5'-

and 3'-end of the cloned h5-HT$_{4(e)}$ receptor, respectively. Products of this first reaction were used as templates for a nested PCR amplification with specific primers HTS5 (Blondel *et al.*, 1998a; accession number Y08756) and G2rev (5'- GAGACAGGGGAACAGCCACT -3' ; accession number AJ011371). The PCR products were run on a 1.5% agarose gel. To assess relative quantities of cDNA from different tissue sources, a single-PCR amplification was performed using reverse and forward primers specific for the rat/human β-actin (Blondel *et al.*, 1997). All PCR reactions in tissue localisation studies were performed as follows: 28 cycles (30 sec at 94°C, 45 sec at 55°C and 1.5 min at 72°C) and a final elongation (8 min at 72°C).

Cell culture

Cell culture materials and reagents were obtained from Life Technologies (Cergy Pontoise, France). CHO, a chinese hamster ovary cell line and C6, a rat glioma cell line were purchased from ATCC (Rockville, USA). Stock cultures of CHO and C6-glial cells were grown at 37°C and 5% CO_2 in HamsF12 medium and DMEM medium respectively, supplemented with 10% foetal calf serum, 10 mM HEPES (pH 7.4) and antibiotics.

Stable expression of the h5-HT$_{4(e)}$ receptor in CHO and C6-glial cell lines

The full coding region of the h5-HT$_{4(e)}$ receptor was subcloned into the expression vector pRC/CMV containing the neomycin selection gene (Invitrogen, Carlsbad, CA, USA). Briefly, confluent cells were transfected with 10 μg of the expression vector by electroporation using a gene pulser transfection apparatus (Biorad, Ivry sur Seine, France; setting 960 μF, 250 V). Forty eight hours after the transfection period, neomycin (1.25 mg ml^{-1}) was added to the dishes for selection. The antibiotic-containing medium was replaced every 2-3 days over 2

weeks. Isolated colonies were selectively trypsinized for further selection, subcloning and propagation of cell clones. h5-HT$_{4(e)}$ expressing clones were detected both by their ability to stimulate cAMP production after treatment with 5-HT and to bind a specific 5-HT$_4$ antagonist, [^{3}H]GR 113808.

Cyclic AMP radioimmunoassay

For measurement of intracellular cAMP accumulation, stably transfected cells were grown to confluence and were incubated with serum-free medium 4 hours before the beginning of the assay. Then, the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline, 10 μM pargyline and 1 μM GR127935 in CHO cells to block the activity of endogenous 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. 5-HT or other serotoninergic ligands were then added for an additional 15 min. The reaction was stopped by aspiration of the medium and addition of 500 μl of ice-cold ethanol. After 30 min incubation at room temperature, the ethanol fraction was collected and evaporated under vacuum. The pellet was reconstituted and cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Immunotech, Marseille, France). Student's *t*-tests were performed using the QuickTTest software.

Membrane preparation and radioligand binding assays

Membrane preparation and radioligand binding assays were performed as previously described (Blondel *et al.*, 1998a). Briefly, cells grown at confluence were washed twice with Phosphate-Buffered Saline (PBS) and centrifuged at 300 g for 5 min. The resulting pellet was resuspended in 1 ml of ice-cold HEPES buffer (50 mM, pH 7.4), centrifuged at 40,000 g for 15 min at 4°C. The final pellet containing intracellular membrane component as well as

plasma membranes was resuspended in 1 ml HEPES buffer and protein concentrations were determined by the method of Bradford (1976).

Radioligand binding assays were performed in 500 µl buffer (50 mM HEPES, pH 7.4) containing 20 µl of [^{3}H]GR113808, 50 µg of membrane preparation and 20 µl of displacing drug. Saturation experiments were performed using [^{3}H]GR113808 at nine concentrations ranging from 0.01 to 4 nM. Non specific binding was measured in the presence of 10 µM ML10375 and subtracted from total binding to determine the affinity of [^{3}H]GR113808 for its receptor (K_d, nM) and the total number of receptors (B_{max}, fmol mg^{-1} protein). At a concentration of [^{3}H]GR113808 corresponding to K_d, the total radioactivity was >500 dpm and non specific binding <30% of total binding for the saturation experiment to be considered as valid. Competition assays were performed in the presence of nine concentrations of the displacing ligands (10^{-12} to 10^{-4} M) and a concentration of [^{3}H]GR113808 corresponding to its K_d for the receptor. Incubations were performed at 25°C for 30 min and the reaction was terminated by rapid filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data were analysed by computer-assisted nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA).

Antibody production and immunoblotting

A peptide (sequence: G I I D L I E K R K F N Q N S N S T Y C V) corresponding to the second extracellular loop of the 5HT$_4$ receptor (G21V) was synthesised in an automated synthesiser using the Fmoc technique (Neimark & Briand, 1993). The peptide was then purified by HPLC and checked by mass spectrophotometry.

Rabbits were immunised subcutaneously with a mix of 0.25 mg peptide and 3 mg methylated bovine serum albumin emulsified in complete Freund's adjuvant. Three booster injections were given at the same concentration but in incomplete Freud's adjuvant. Sera were collected one week after the last booster injection. Rabbit sera were precipitated at 40% $(NH_4)_2SO_4$ saturation and dissolved in PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) at 1:1 dilution.

The G21V peptide was coupled to activated EAH-Sepharose (Pharmacia diagnostics AB, Uppsala, Sweden) according to the standard procedure. The rabbit immunoglobulin fractions were diluted 10 times with PBS and centrifuged at 200g for 5 min. They were then passed through the column with a flow of 6 $ml.h^{-1}$ for 3 h at 4°C. Finally the adsorbed antibodies were eluted with 3 ml of 3 M KSCN and dialysed directly against 6 l PBS overnight at 4°C. Specificity of antibodies was checked by enzyme immunoassay as described previously (Lebesgue *et al.*, 1998).

Western blotting was performed as previously described (Lezoualc'h *et al.*, 1998). Briefly, membrane proteins (30 μg) from CHO and C6-glial cells, prepared as described above, were mixed with SDS sample buffer containing 4% β-mercaptoethanol and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a membrane (Hybond-P, Amersham Pharmacia Biotech, Orsay, France) and incubated with the anti-5-HT$_4$ receptor antibody (see below) overnight at 4°C. The primary antibody was detected by counterstaining with a horseradish peroxidase-linked antibody and visualized by the ECL detection kit (Amersham Pharmacia Biotech, Orsay, France). A part of the SDS gel was stained with Coomassie blue to verify whether equal amounts of proteins had been used.

Drugs

GR113808 ([1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate) and GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'- methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide) were gifts from Glaxo Research Group (Ware, Hertfordshire, UK). [^{3}H]GR113808 was purchased from Amersham (Orsay, France). ML10302 (2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate hydrochloride) and ML10375 (2-[*cis*-3,5-dimethylpiperidino]ethyl 4-amino-5-chloro-2-methoxybenzoate) were synthesised as previously described (Langlois *et al.*, 1994; Yang *et al.*, 1997). 5-HT (5-hydroxytryptamine) and 5-MeOT (5-methoxytryptamine) were from Aldrich (L'Isle d'Abeau Chesnes, France). BIMU1 (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide) and cisapride (*cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide) were synthesised in our laboratory. Renzapride (BRL 24924) ((±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride) and SB204070 (8-amino-7-chloro-(N-butyl-4-piperidyl)-methylbenzo-1,4-dioxan-5-carboxylate hydrochloride) were generously given by SmithKline Beecham (U.K.). RS23597 (3-(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxybenzoate hydrochloride), RS39604 (1-(5-chloro-2(3,5-dimethoxy)benzyloxy-4-aminophenyl)-3-(N-(methylsulfamido)ethyl-4-piperidyl)propanone) and RS67333 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-n-butyl-4-piperidinyl)-1-propanone) were from Tocris Interchim (Montluçon, France).

Results

Primary structure of the h5-HT$_{4(e)}$ receptor

Two DNA fragments of approximately 760 and 850 bp, were isolated when human heart cDNA was used as a template in a nested RACE-PCR amplification using oligonucleotide primers derived from the central region and the 3'-end of the h5-HT$_{4(a)}$ receptor subtype (see Materials and Methods). The nucleotide sequences of the two amplified fragments were found to correspond to the h5-HT$_{4(a)}$ isoform and to the novel h5-HT$_4$ receptor named h5-HT$_{4(e)}$ receptor. It is suggested that this new h5-HT$_{4(e)}$ isoform is the ortholog of the corresponding mouse 5-HT$_{4(e)}$ [m5-HT$_{4(e)}$] and rat 5-HT$_{4(e)}$ [r5-HT$_{4(e)}$] variants (Claeysen *et al.*, 1999). The h5-HT$_{4(e)}$ receptor is identical to the other h5-HT$_4$ splice variants up to the codon coding for Leu$_{358}$ (Blondel *et al.*, 1998a; Claeysen *et al.*, 1999). A schematic representation of the h5-HT$_{4(e)}$ receptor and its deduced amino acid sequence starting from the splicing site at Leu$_{358}$ are shown in Fig. 1. Interestingly, we found that the 3' untranslated part of h5-HT$_{4(e)}$ receptor cDNA corresponds to the coding region of h5-HT$_{4(a)}$ cDNA (Fig. 1).

(Figure 1 near here)

Tissue-specific expression of the 5-HT$_{4(e)}$ receptor

The expression of h5-HT$_{4(e)}$ transcripts was analysed by amplification of cDNA derived from RNA isolated from various human tissues using a nested RT-PCR technique with specific primers for the h5-HT$_{4(e)}$ isoform (see Materials and Methods). PCR resulted in a fragment of around 664 bp as predicted from the nucleotide sequence of the splice

variant. Gel electrophoresis of these PCR products revealed that h5-HT$_{4(e)}$ transcripts are expressed both in human atrium and brain (Fig. 2). In contrast, we found no detectable level of h5-HT$_{(e)}$ mRNA in other 5-HT$_4$ receptor target tissues such as the kidney and the colon (Fig. 2). Interestingly, the h5-HT$_{(e)}$ in the human heart was expressed at high levels in the atrium, but not in the ventricle (Fig. 2). We also demonstrated the presence in all tissues of cDNA corresponding to the constitutively expressed β-actin gene (Fig. 2), as well as the absence of actin PCR product in a minus reverse transcriptase control (Fig. 2). Therefore, signals obtained in our study were not due to any contaminating genomic DNA since no bands were observed when RNA was directly amplified.

(Figure 2 and Table 1 near here)

Pharmacological characterisation of the h5-HT$_{4(e)}$ receptor

The coding region of the h5-HT$_{4(e)}$ receptor was cloned in an expression vector containing the neomycin selection gene and was stably expressed in CHO and C6-glial cells at 347±7 fmol mg^{-1} protein and 88±7 fmol mg^{-1} protein respectively (Fig. 3, Table 1). The expression of the receptor at the plasma membrane was monitored by Western blotting with an affinity rabbit purified polyclonal antibody directed against the second extracellular loop of the h5-HT$_4$ receptor (Fig. 4). Figure 4 shows that CHO and C6-glial cells transfected with the h5-HT$_{4(e)}$

receptor displayed a single specific band migrating around 60 kDa. No specific signal was detected in the control cell clones transfected with the control vector.

(Figure 3 and Figure 4 near here)

Saturation analysis revealed a single saturable binding site of high affinity for [^{3}H]GR113808 in each cellular clone (Fig. 3). K_d values were 0.22±0.02 and 0.08±0.03 nM in CHO and C6 clones expressing h5-HT$_{4(e)}$ receptor, respectively (Fig. 3, Table 1). K_d values are similar to the other h5-HT$_4$ isoforms previously cloned (Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997 ; Blondel *et al.*, 1998a). For each cell line, non-specific binding increased linearly with increasing ligand concentration (Fig. 3). In addition, no detectable binding was found in non transfected CHO and C6-glial cells (data not shown).

In order to determine and to compare the pharmacological properties of h5-HT$_{4(e)}$ receptor in both cell lines, various selective 5-HT$_4$ agonists and antagonists were tested for the inhibition of [^{3}H]GR113808 binding in CHO and C6 membranes. All the displacement curves were monophasic, giving a Hill coefficient of 0.9 to 1.1**.** Inhibition curves of radioligand binding in membranes from transfected CHO and C6-glial cells are shown in Fig. 5. Comparison analysis of the binding affinities (K_i) between CHO and C6-glial cells expressing the h5-HT$_{4(e)}$ receptor revealed a similar rank order of potency for all the ligands tested in each cell line (Table 1). The data summarised in Table 1 demonstrate that the pharmacological profile of the h5-HT$_{4(e)}$ receptor in terms of rank order of potencies of the different ligands tested, is similar to those found for native 5-HT$_4$ receptors as studied *in vivo* in human atria (Kaumann *et al.*, 1996), rat striatum (Langlois *et al.*, 1994; Yang *et al.*, 1997), mouse colliculi (Ansanay *et al.*, 1996), or after expression of cloned human, mouse or rat 5-HT$_4$ receptor isoforms in cell lines (Gerald *et al.*, 1995; Adham *et al.*, 1996; Claeysen *et al.*,

1996; Blondel *et al.*, 1997; 1998a). The rank order of apparent antagonist and agonists affinities were, respectively, SB204070 > ML10375 > RS39604 > RS23597 and ML10302 > RS67333 > BIMU1 > renzapride ≥ cisapride > 5-HT > 5-MeOT (Table 1). However, the benzamide derivative renzapride had a lower affinity for the h5-HT$_{4(e)}$ receptor than 5-HT in C6-glial cells (Table 1). Furthermore, we found a slightly better affinity (about two fold) for all the ligands tested in CHO cells compared to C6-glial cells, to the exception of the gastroprokinetic agent, cisapride (Table 1).

(Figure 5 near here)

Constitutive activity of the h5-HT$_{4(e)}$ receptor

The ability of the h5-HT$_{4(e)}$ receptor to stimulate adenylyl cyclase activity was analysed by measuring cAMP production. A constitutive activity of the h5-HT$_{4(e)}$ receptor was observed in the absence of any 5-HT$_4$ ligand (Fig. 6). Indeed, we found that basal cAMP levels were increased about 3-fold in CHO cells expressing the h5-HT$_{4(e)}$ receptor as compared to non transfected cells (Fig. 6). These data indicate that expression of h5-HT$_{4(e)}$ splice variant induced a spontaneously active receptor state. In addition, two selective 5-HT$_4$ antagonists, GR113808 (1 μM) and ML10375(1 μM), significantly decreased basal cAMP production to values which were only 64±21% and 65±24% above the level of cAMP production in non transfected cells, respectively (Fig. 6). These results show that these two antagonists behaved as inverse agonists.

(Figure 6 near here)

Functional effects of 5-HT$_4$ agonists

5-HT (1 μM) had no effect on cAMP production in non transfected cells (data not shown). In vector transfected cells, 5-HT significantly induced stimulation of basal cAMP up to 8 fold in both cell lines (Fig. 6). Dose response curves using the most representative 5-HT$_4$ agonists are shown in Fig. 7A and 7B (see Ford & Clarke, 1993 ; Eglen *et al.*, 1995 ; for details and bibliography on the compounds used). All 5-HT$_4$ agonists used in these experiments stimulated cAMP production in a dose dependent manner, both in CHO (Fig. 7A) and C6-glial cells (Fig. 7B). However, depending on the cell line we found some differences in their rank order of potency in stimulating cAMP formation (Table 2). Rank orders based on mean EC_{50} values were as follows: 5-MeOT>5-HT>ML10302=BIMU1>renzapride>cisapride for h5-HT$_{4(e)}$ expressed in CHO cells and 5-HT>renzapride>5-MeOT>BIMU1>cisapride for h5-HT$_{4(e)}$ expressed in C6-glial cells (Table 2). Most importantly EC_{50} values for 5-HT, renzapride and cisapride were twice lower in C6-glial cells than in CHO cells, while the EC_{50} for the indoleamine 5-MeOT was 10-fold higher in C6 glial than in CHO cells indicating that the cellular environment can influence the coupling efficiency of the h5-HT$_{4(e)}$ receptor to its effector. With regard to agonist activities, 5-HT and 5-MeOT displayed full agonist properties, whereas BIMU1, ML10302, renzapride and cisapride acted as partial agonists (Fig. 7A and 7B, Table 2). In addition, the rank order of affinities obtained with the agonists from binding assays was inversely correlated to their rank order of potencies obtained from functional studies in both cell lines (compare Table 1 and 2). For instance, 5-MeOT had the lowest affinity for competing with [^{3}H]GR113808 binding (Table 1) whereas, in functional studies, 5-MeOT exhibited the highest coupling efficiency at the cloned h5-HT$_{4(e)}$ (Table 2). BIMU1 behaved as a highly potent 5-HT$_4$ agonist and displayed only 47±5% and 63±5% of the 5-HT stimulatory effect in CHO and C6-glial cells, respectively (Table 2).

(Figure 7 near here)

Functional effects of 5-HT$_4$ antagonists

Antagonists were tested at a concentration of 10 times K_i and caused a parallel concentration-dependent rightward displacement of the 5-HT curve without depression of the maximum response (Fig. 7C and 7D). As previously observed for EC_{50} values obtained from functional studies with 5-HT$_4$ agonists, estimated K_b values were twice lower in C6-glial cells compared to CHO cells (Table 2). Among the different 5-HT$_4$ antagonists tested in our system, SB204070 was the most potent antagonist in inhibiting 5-HT -induced cAMP production (Table 2). In addition, rank orders of K_b values obtained for the different antagonists tested from functional studies in our cellular systems are comparable with those obtained with K_i values derived from binding experiments (Table 1 and 2).

(Table 2 near here)

Discussion

In this paper, we report the cloning of a novel h5-HT$_4$ receptor from human heart atrium. Sequence analysis revealed that this isoform corresponds to the recently identified h5-HT$_{4(e)}$ receptor isolated from human brain which is considered to be the human counterpart of rat and mouse isoforms (Claeysen *et al.*, 1999). This splice variant together with previously cloned h5-HT$_{4(a)}$, h5-HT$_{4(b)}$, h5-HT$_{4(c)}$ and h5-HT$_{4(d)}$ isoforms is generated by splicing events occurring in the C terminus of the h5-HT$_4$ receptor, just after the amino acid Leu$_{358}$ (Blondel *et al.*, 1997; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998a). Interestingly, we found that the 3' untranslated part of the h5-HT$_{4(e)}$ cDNA is represented by the h5-HT$_{4(a)}$ as a non coding sequence (Fig. 1). This molecular event is not only restricted to the h5-HT$_4$ receptor since m5-HT$_{4(a)}$ is also found in the 3' untranslated parts of m5-HT$_{4(e)}$ and m5-HT$_{4(f)}$ (Claeysen *et al.*, 1999). Although an analysis of the exon-intron organisation of this receptor gene has still to be performed, these observations suggest that the organisation of the 5-HT$_4$ receptor gene is well conserved among animal species.

The h5-HT$_{4(e)}$ receptor isoform is not restricted to the brain as reported for the m5-HT$_{4(e)}$ and m5-HT$_{4(f)}$ variants (Claeysen *et al.*, 1999) but we found in this study that this receptor is also expressed in human heart atrium. However, as reported for the h5-HT$_{4(a)}$, h5-HT$_{4(b)}$ and h5-HT$_{4(c)}$ isoforms (Blondel *et al.*, 1998a), h5-HT$_{4(e)}$ mRNA is expressed in human atrium but not in ventricle. This is in agreement with a number of functional studies showing positive inotropic and chronotropic effects of 5-HT which are located exclusively in the human atrial tissue (Kaumann, 1991; Schoemaker *et al.*, 1993). Concerning the distribution pattern of 5-HT$_4$ receptor messengers, some differences exist

between species. For instance, m5-HT$_{4(b)}$ mRNA is present in the bladder and kidney whereas h5-HT$_{4(b)}$ messenger is not detectable in these organs (Blondel *et al.*, 1998a; Claeysen *et al.*, 1999). Only the development of specific antibodies against 5-HT$_4$ subtypes will enable us to assess 5-HT$_4$ receptor expression at the protein level and will confirm 5-HT$_4$ isoform differential distributions between species.

A major issue is to understand the physiological relevance of the restricted expression of 5-HT$_4$ receptors. Four 5-HT$_4$ receptor isoforms have been shown to be expressed in human atria. Detailed analysis of 5-HT$_4$ receptor pharmacology is therefore crucial for the elucidation of their pathological implication in atrial arrhythmias. We have characterised in this study the radioligand binding and functional properties of recombinant h5-HT$_{4(e)}$ receptor stably expressed in two different cellular systems, CHO and C6-glial cells. To assess the expression of the h5-HT$_{4(e)}$ receptor in the transfected cell lines, polyclonal antibodies were produced against a peptide corresponding to the second extracellular loop of the h5-HT$_4$ receptor. The anti-5-HT$_4$ receptor antibody recognised the transfected h5-HT$_{4(e)}$ receptor as shown by western blotting experiments (Fig. 4). We anticipate that this antibody which is the first anti-5-HT$_4$ receptor antibody available will be a useful tool for studying the anatomic distribution, regulation and function of h5-HT$_4$ receptors.

Using the radiolabelled specific antagonist [^{3}H]GR113808, we found that densities of specific binding sites in CHO and C6 cells were 347±7 fmol mg^{-1} protein and 88±7 fmol mg^{-1} protein, respectively (Fig. 3). These values are comparable to those found in rat and human brains where levels of native 5-HT$_4$ receptor expression have been reported to vary between 20 and 400 fmol mg^{-1} protein depending on the considered brain region (Waeber *et al.*, 1993; 1994). On the contrary, the density of human atrial binding sites with 5-HT$_4$ receptor characteristics have been found to be around 4 fmol mg^{-1} protein which is much lower than in our cellular systems and in the central nervous system (Kaumann *et al.*, 1996).

Binding experiments with [^{3}H]GR113808 showed a typical 5-HT$_4$ profile for the h5-HT$_{4(e)}$ receptor expressed in CHO and C6-glial cells (Fig. 3). K_d values (Fig. 3, Table 1) measured in our study are in good agreement with those reported in human atria (Kaumann *et al.*, 1996), in human brain membranes (Waeber *et al.*, 1993), and in COS-7 cells transiently transfected with other h5-HT$_4$ receptor splice variants (Blondel *et al.*, 1997; Claeysen *et al.*, 1997; Van den Wyngaert *et al.*, 1997; Blondel *et al.*, 1998a). The rank order of affinity of compounds used for the competition of [^{3}H]GR113808 binding on CHO and C6 membranes matched the expected pharmacological profile. The ligand found to have the lowest K_i values was SB204070 (Table 1). This benzodioxane derivative is the most potent and selective 5-HT$_4$ antagonist described so far (Gaster & Sanger, 1994). On the other hand, 5-MeOT had the lowest affinity for competing with [^{3}H]GR113808 binding in both cell lines (Table 1). This latter observation correlates well with that obtained in binding studies using preparations such as human brain membranes (Waeber *et al.*, 1993). Furthermore, we found a good correlation between the affinity constants of 5-HT and 5-MeOT for the cloned h5-HT$_{4(e)}$ receptor and those obtained for the same agonists when binding to the native cardiac 5-HT$_4$ receptors in human and pig atria (Kaumann *et al.*, 1995; 1996).

Interestingly, we found that the rank order of affinities obtained with the agonists from binding assays was inversely correlated to their rank order of potencies obtained from functional studies in both cell lines (Tables 1 and 2). A similar observation has already been reported for r5-HT$_{4(a)}$ and r5-HT$_{4(b)}$ receptors when expressed in recombinant systems and it was suggested that some ligands might differentially activate a receptor reserve (Gerald *et al.*, 1995). Clearly, these pharmacological differences between the affinity of a given ligand and

its functional effects will have to be taken into consideration in the future when designing novel 5-HT$_4$ drugs.

A constitutive activation was found with the h5-HT$_{4(e)}$ receptor expressed in CHO cells. Such constitutive coupling has already been described for other 5-HT$_4$ receptor splice variants such as h5-HT$_{4(c)}$, m5-HT$_{4(e)}$ and m5-HT$_{4(f)}$ receptors (Blondel *et al.*, 1998b, Claeysen *et al.*, 1999). In addition we report that two 5-HT$_4$ antagonists, GR113808 and ML10375, significantly decreased basal cAMP values indicating that these antagonists behave as inverse agonists. This was not due to the presence of 5-HT in the culture medium since cells were deprived of serum (see Materials and Methods). ML10375 also reduced basal adenylyl cyclase activity in COS cells transfected with the h5-HT$_{4(c)}$ receptor (Blondel *et al.*, 1998b). Therefore, these inverse agonistic properties are likely to be of therapeutic relevance in the treatment of disorders involving 5-HT$_4$ receptors, particularly if these disorders are directly related to constitutive h5-HT$_4$ receptor activation.

Since the h5-HT$_{4(e)}$ receptor had similar ligand binding properties in CHO and C6-glial cell lines and a higher expression level (B_{max}) in CHO cells, one would have expected to obtain lower EC_{50} values and higher maximal stimulation (E_{max}) of adenylyl cyclase activity induced by 5-HT$_4$ agonists in C6-glial as compared to CHO cells. However, 5-HT induced a similar 8-fold maximal stimulation of adenylyl cyclase activity in both cell lines. Moreover, with the exception of 5-MeOT and BIMU1, all other 5-HT$_4$ agonists had a higher potency in activating adenylyl cyclase (lower EC_{50} values) in C6-glial cells than in CHO cells (Table 2). This is in contradiction with the previously reported inverse relationship between receptor number and EC_{50} value observed for the β_2-adrenergic receptor-mediated cAMP accumulation in several heterologous expression systems (Bouvier *et al.*, 1988; Whaley *et al.*, 1994). Since functional interactions between G protein-coupled receptors and G proteins are strongly influenced by their relative expression level (Kenakin, 1996), one may speculate that

C6-glial cells have a higher level of G_s protein expression than CHO cells. Alternatively, C6-glial and CHO cells may possess different isoforms of G_s proteins with different coupling efficiency to 5-HT$_4$ receptors and/or adenylyl cyclase. Indeed, two splice variants of G_s have been described, a short ($G_s\alpha_S$) and a long isoform ($G_s\alpha_L$) (Bray *et al.* 1986 ; Robishaw *et al.*, 1986), with $G_s\alpha_S$ being more efficiently coupled to adenylyl cyclase than $G_s\alpha_L$ (Seifert *et al.*, 1998). Expression analysis of $G_s\alpha$ splice variants in CHO and C6-glial cell lines will be necessary to test these hypotheses.

Some interesting features were found with the effects of the 5-HT$_4$ agonists, cisapride and 5-MeOT. Human detrusor muscle showed an unusually low potency for 5-MeOT compared to other tissues (Candura *et al.*, 1996). The fact that 5-MeOT behaved as a full agonist in our cellular models excludes the possibility that the h5-HT$_{4(e)}$ receptor mediates 5-HT$_4$ effects in the bladder. Cisapride was a partial and not highly potent agonist whereas 5-MeOT displayed full agonist properties in stable cell lines expressing the h5-HT$_{4(e)}$ receptor (Table 2). *In vivo* studies have shown that cisapride acts as partial agonist in human heart whereas it behaves as a full and highly potent agonist in brain (reviewed in Eglen *et al.*, 1995). Additional data show that the pharmacological behaviour of the h5-HT$_{4(e)}$ is close to the cardiac native h5-HT$_4$ receptor. Indeed, ML10302 and renzapride behaved like partial agonists on the h5-HT$_{4(e)}$ with stimulatory effects that were 30% and 66% of the maximal 5-HT-induced cAMP synthesis in CHO cells, respectively (Table 2). These results are in agreement with the reported low efficacy of the these compounds in activation of I_{Ca} currents or myocyte contractility in human atrium (Sanders *et al.*, 1995; Blondel *et al.*, 1997). However, previous results from our laboratory have shown that the pharmacological profile of the h5-HT$_{4(a)}$ receptor in recombinant systems is also very similar to the native h5-HT$_4$ receptor in human atrium (Blondel *et al.*, 1997). Thus, it is crucial in the future to determine

the relative contribution of each h5-HT$_4$ receptor isoform to the positive inotropic, chronotropic and lusitropic effects of 5-HT in human atrium and to the genesis of pathological events such as cardiac arrhythmias.

In conclusion, we have isolated the h5-HT$_{4(e)}$ receptor from human atrium and characterised its pharmacological profile in two cellular systems, CHO and C6-glial cell lines. We did not observe any major differences in the binding affinity of the different ligands tested between the h5-HT$_{4(e)}$ receptor, other 5-HT$_4$ receptor splice variants and the two cell lines used in the study. However, the rank order of affinities obtained with the agonists from binding assays was inversely correlated to their rank order of potencies obtained from functional studies. Furthermore, functional potency of h5-HT$_{4(e)}$ receptor ligands was dependent on the cellular context in which the receptor was expressed. Finally, we found that the h5-HT$_{4(e)}$ receptor has a pharmacological profile which is very close to that of the native h5-HT$_4$ receptor in human atrium.

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Table 1. Affinities of various 5-HT$_4$ ligands that compete with the binding of 0.2 nM and 0.08 nM [^{3}H]GR113808 in CHO and C6-glial cells, respectively, stably expressed with h5-HT$_{4(e)}$ receptor

	CHO cells	C6-glial cells
^{3}[H]GR113808		
K_d (nM)	0.22±0.02	0.08±0.03
B_{max} (fmol mg^{-1} prot.)	347±7	88±7
	K_i (nM) (95% confidence intervals)	
AGONISTS		
5-HT	629 (433-914)	1046 (557-1965)
5-MeOT	1132 (780-1640)	6920 (4970-9640)
ML10302	4.2 (3-5.8)	5.6 (3.3-9.6)
Renzapride	238 (130-290)	1723 (1064-2789)
Cisapride	248 (176-348)	173 (129.7-232)
RS67333	6.1 (4.7-7.8)	15.2 (10.3-22.4)
BIMU1	38 (29-51)	76 (53-109)
ANTAGONISTS		
ML10375	0.11 (0.06-0.18)	0.34 (0.24-0.47)
RS23597	29 (20-43)	42 (29-61)
RS39604	2.99 (2.15-4.15)	5.2 (3.4-7.9)
SB204070	0.059 (0.04-0.08)	0.095 (0.06-0.16)

K_d and B_{max} values were obtained from saturation binding experiments with [^{3}H]GR113808 in CHO and C6-glial cells stably transfected with the h5-HT$_{4(e)}$ receptor. Non specific binding was determined with 10 µM ML10375. K_i values were obtained from competition binding assays with 0.2 nM (CHO cells) or 0.08 nM (C6-glial cells) [^{3}H]GR113808. IC_{50} values correspond to a displacement of 50% of specific radioligand binding and were converted to K_i values according to the equation: $K_i = IC_{50}/(1+(\text{concentration of } [^3\text{H}]\text{GR113808}/K_d))$. The data were obtained using nine concentrations of ligand and all experiments were performed in triplicate.

Table 2. Pharmacological profile of cAMP response using the h5-HT$_{4(e)}$ receptor stably transfected in CHO and C6 glial cells in response to 5-HT$_4$ agonists and antagonists

	CHO cells		C6-glial cells	
	EC_{50} (nM)	**E_{max}** (% maximal response to 5-HT)	**EC_{50}** (nM)	**E_{max}** (% maximal response to 5-HT)
AGONISTS				
5-HT	25±6	100	12±2	100
5-MeOT	7.0±0.7	108±2	50±4	106±10
renzapride	107±41	66±6	35±19	45±5
cisapride	180±35	85±4	94±17	66±3
BIMU1	52±23	47±5	72±22	63±5
ML10302	51±23	30±3	ND	ND
	EC_{50} (nM)	**K_b** (nM)	**EC_{50}** (nM)	**K_b** (nM)
ANTAGONISTS (concentration)				
GR113808	79±12 (3 nM)	1.38	81±22 (0.8 nM)	0.53
ML10375	74±17 (1 nM)	1.01	79±9 (3 nM)	0.52
SB204070	443±133 (1 nM)	0.06	285±157 (1 nM)	0.036
RS39604	166±45 (30 nM)	5.3	106±6 (50 nM)	6.25

cAMP production was measured in CHO and C6-glial cells expressing the h5-HT$_{4(e)}$ receptor. EC_{50} values correspond to the concentration of agonist required to obtain half-maximal stimulation of adenylyl cyclase. The maximum response produced by each drug was normalised to the 5-HT induced maximum response. For the antagonists, EC_{50} values correspond to the concentration of 5-HT required to produce half-maximal stimulation of adenylyl cyclase in the presence of the indicated concentration of antagonist. K_b values were calculated with the formula: $K_b=[B]/[(A'/A)-1]$ where [B] is the concentration of antagonist and A' and A the EC_{50} values of 5-HT measured respectively in the presence and absence of antagonist. Values are the means ± S.E.M. of three experiments performed in triplicate. They were analysed by non linear regression analysis. N.D, non determined.

Figure Legends

Figure 1. Schematic representation of the h5-HT$_{4(e)}$ receptor cDNA and its C terminal amino acid sequence.

The numbers indicate the position of base pairs in the cDNA sequence (accession number AJ011371). In all of the cloned h5-HT$_4$ receptors, the sequence diverges after Leu$_{358}$.

Figure 2. Expression analysis of h5-HT$_{4(e)}$ transcripts in human tissues.

Reverse Transcription-PCR analysis was performed with mRNA extracted from various human tissues. The PCR products were analysed on a 1.7% agarose gel and photographs of the ethidium bromide stained gels are shown. The PCR primers used for this analysis and expected length of the PCR products are described in Materials and Methods. A positive control was performed using rat/human actin primers on mRNA samples treated with (+RT) or without (-RT) reverse transcriptase. Positions of three molecular weight markers are indicated in bp. This figure is representative of three separate determinations of h5-HT$_{4(e)}$ mRNA expressions obtained by RT-PCR. $\overline{\text{M}}$, molecular weight markers.

Figure 3. Saturation analysis of [^{3}H]GR113808 binding to the h5-HT$_{4(e)}$ receptor stably expressed in CHO and C6-glial cells.

Membranes harvested from stably transfected CHO (A) and C6-glial cells (B) were incubated with 9 concentrations of [^{3}H]GR113808 for 30 min at 25°C. Non-specific binding was determined with 10 μM ML10375. Results are from single experiments but are representative of three such experiments.

Figure 4. Western blot detection of the h5-HT$_{4(e)}$ receptor in h5-HT$_{4(e)}$ receptor transfected subclones.

CHO and C6-glial cells were transfected either with the h5-HT$_{4(e)}$ receptor neo-vector or the corresponding control (CT) and selected for their resistance to the antibiotic. Membrane protein extracts were separated on a polyacrylamide gel and analysed by immunoblotting with antiserum raised against the second extracellular loop of the h5-HT$_{4(e)}$ receptor (see Materials and Methods). In CHO and C6-glial cells stably transfected with the h5-HT$_{4(e)}$ receptor, a specific band migrating to the level of 60 kD was detected whereas in control cells no labelling was detected at this position. This immunoblot is representative of three independent experiments. Molecular weight marker positions are indicated in kilodaltons.

Figure 5. Inhibition of specific [^{3}H]GR113808 binding to the h5-HT$_{4(e)}$ receptor in CHO and C6-glial cells.

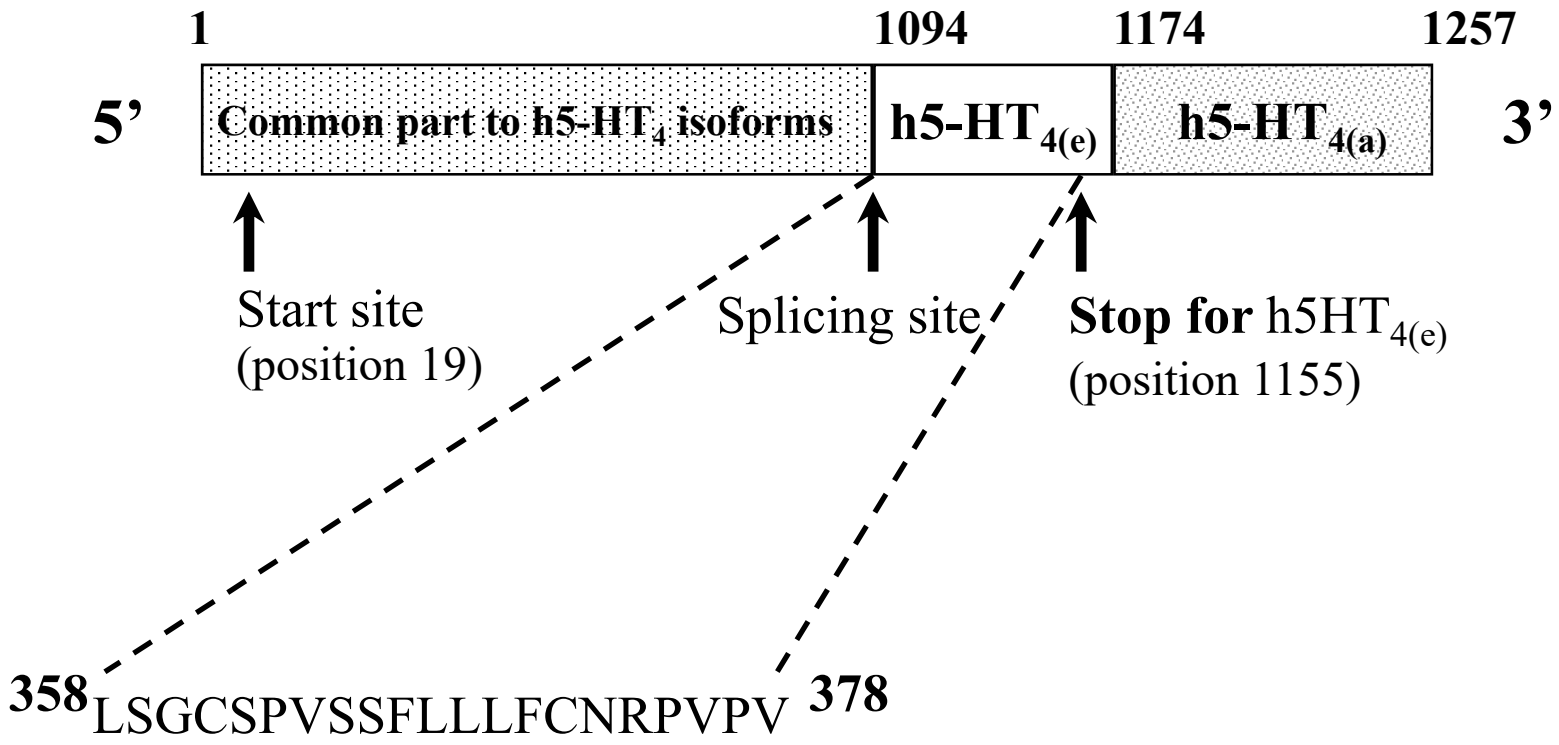
Membranes from stable CHO (A, C) and C6-glial (B, D) cells expressing the h5-HT$_{4(e)}$ receptor were incubated with 0.2 nM and 0.08 nM of [^{3}H]GR113808, respectively, in the presence or absence of increasing concentrations of 5-HT$_4$ agonists (A, B) or antagonists (C, D). Non-specific binding was defined by 10 µM ML10375. Data are presented as a percentage of specific binding in the absence of displacing drug. Results are from single experiments but are representative of three such experiments using a range of nine concentrations of ligands. Data were analysed by computer-assisted non-linear regression analysis (GraphPad, Prism Software). The corresponding *Ki* values are presented in Table 1.

Figure 6. Inverse agonist effects of GR113808 and ML10375 in CHO cells expressing the h5-HT$_{4(e)}$ receptor.

The effects of 5-HT, GR113808 and ML10375 in the absence or in the presence of 5-HT on cAMP production are expressed as percentage of control (untransfected CHO cells). GR113808, ML10375 and 5-HT were used at a concentration of 1 μM. Absolute values for cAMP production in untransfected cells and under basal conditions were 4.3±0.8 pmoles/well and 13.0±1.6 pmoles/well, respectively. Values are mean ± S.E.M. of three independent experiments performed in duplicate. $^{\#}p<0.05$ versus indicated values by *t*-test.

Figure 7. cAMP responses to various 5-HT$_4$ ligands in CHO and C6-glial cells expressing the h5-HT$_{4(e)}$ receptor.

cAMP measurements were performed as described in Materials and Methods. In A and B, the cells were incubated for 15 min with increasing concentrations of agonists and cAMP production was then quantified. In C and D, the cells were preincubated for 15 min with a concentration of antagonist corresponding to 10 fold the K_i value as measured in binding experiments (see Table 1); increasing concentrations of 5-HT were then added for an additional 15 min before cAMP was measured. Values are expressed as the percentage of 5-HT maximal response. Each point is the mean of at least three independent experiments, each performed in triplicate. EC_{50} and E_{max} values are presented in Table 2.


1
1094
1174
1257
5’
Common part to h5-HT4 isoforms
h5-HT4(e)
h5-HT4(a)
3’
Start site
(position 19)
Splicing site
Stop for h5HT4(e)
(position 1155)
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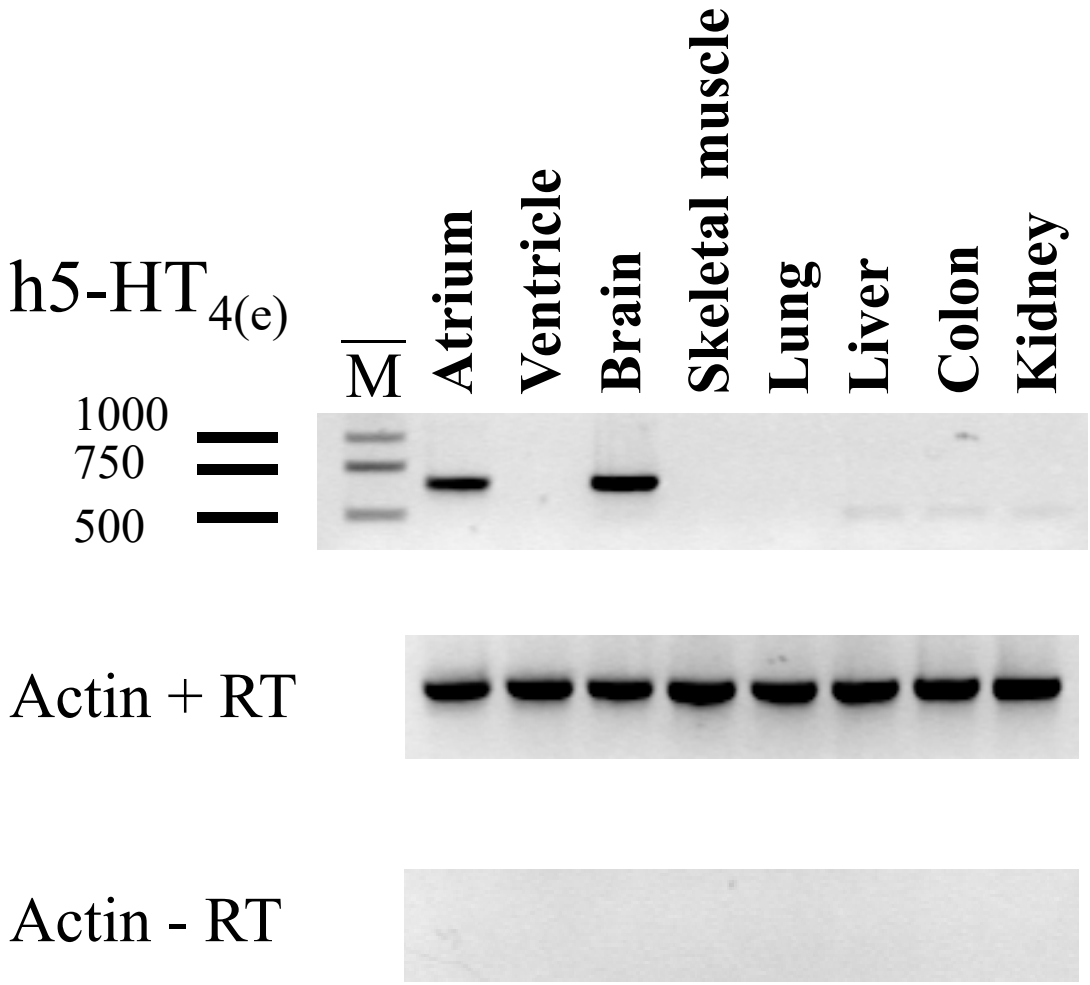
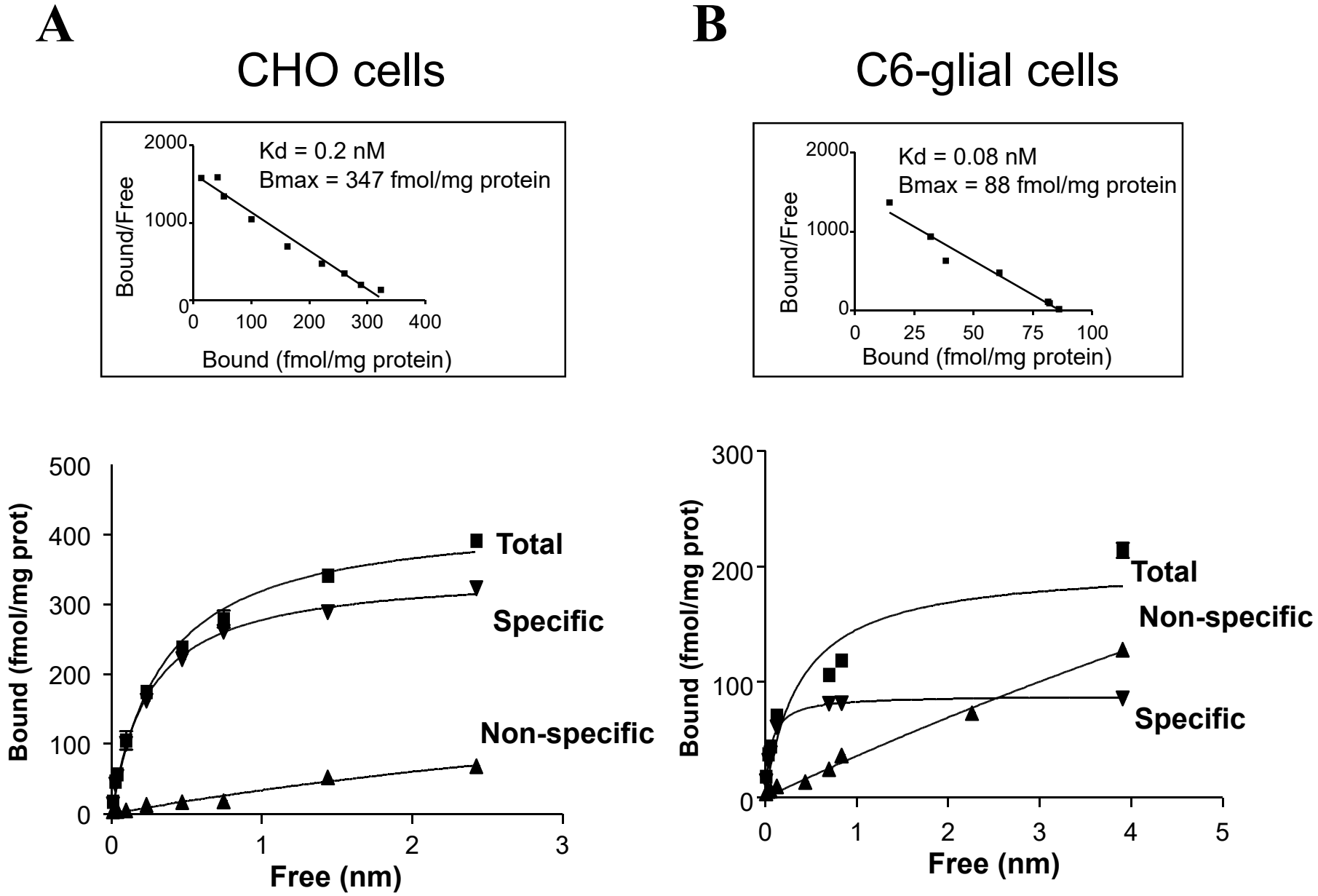
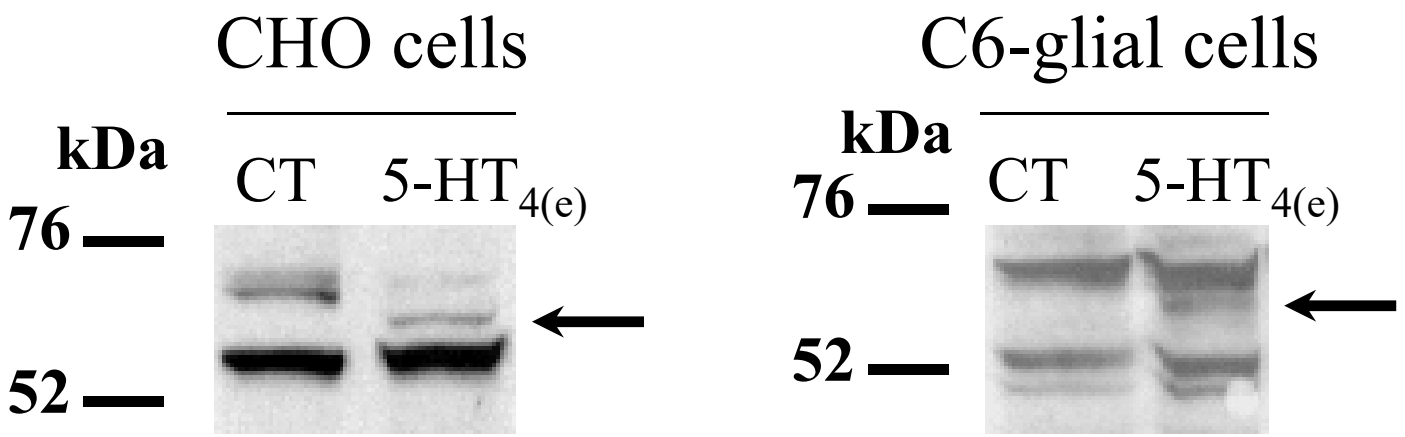
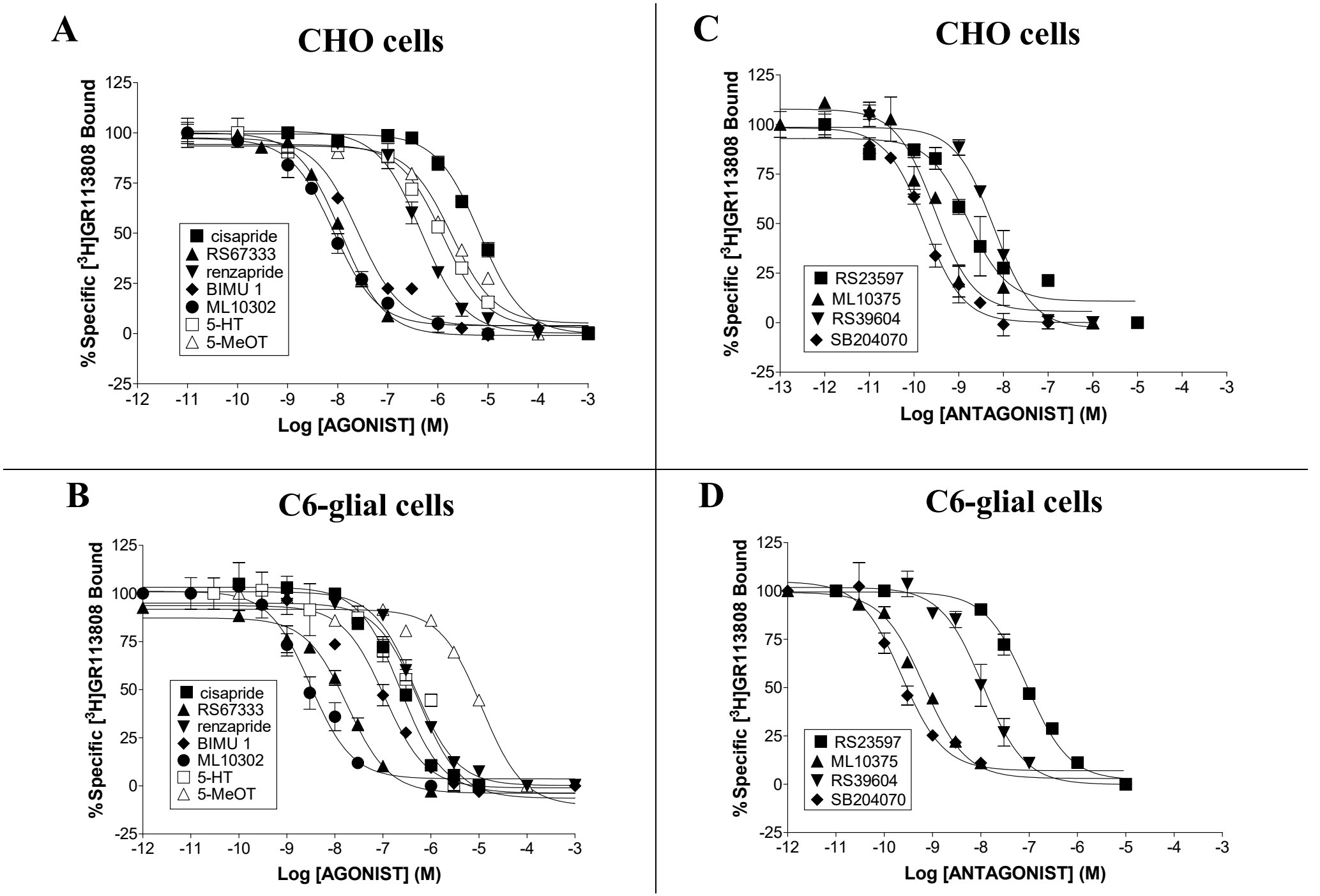
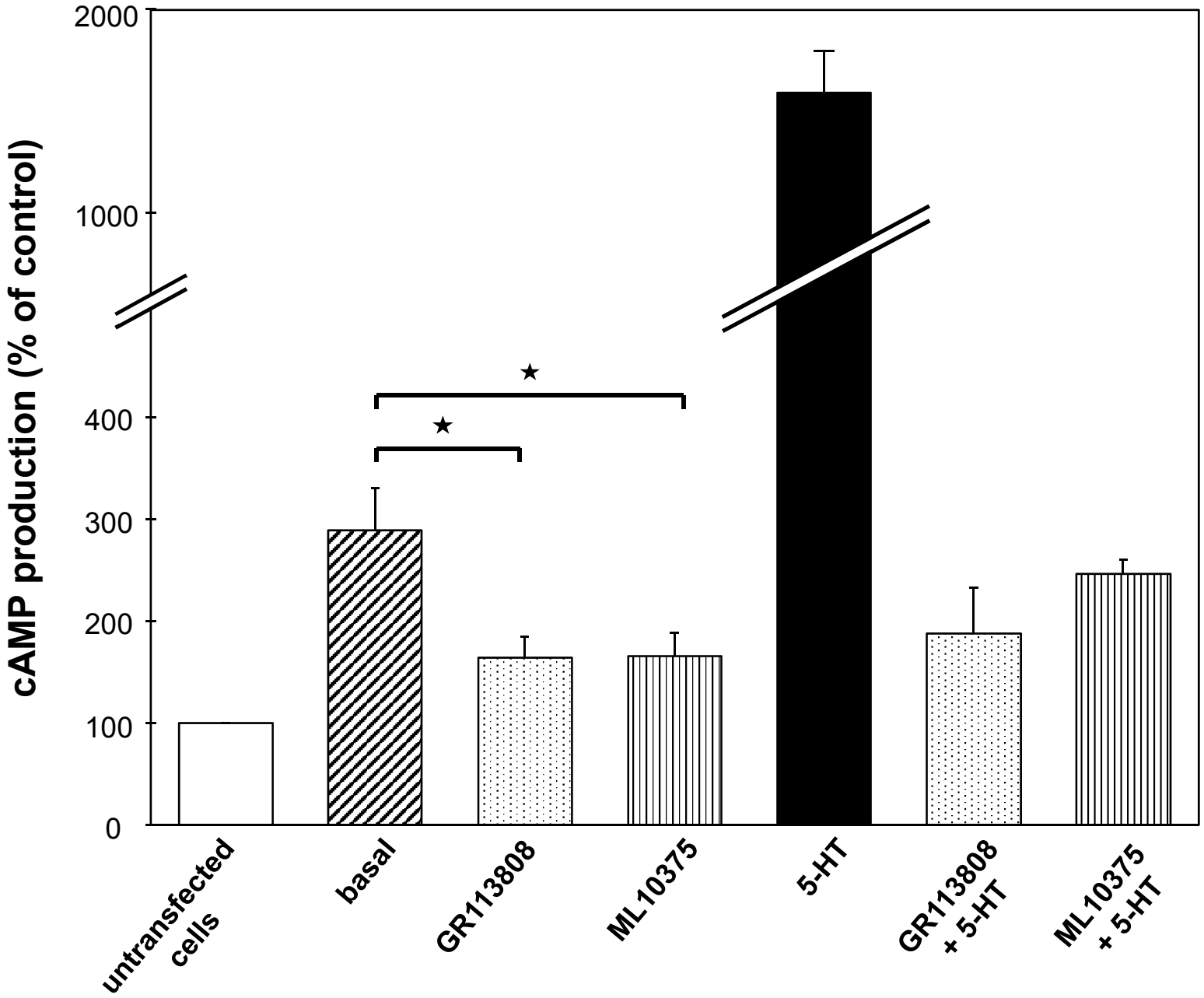
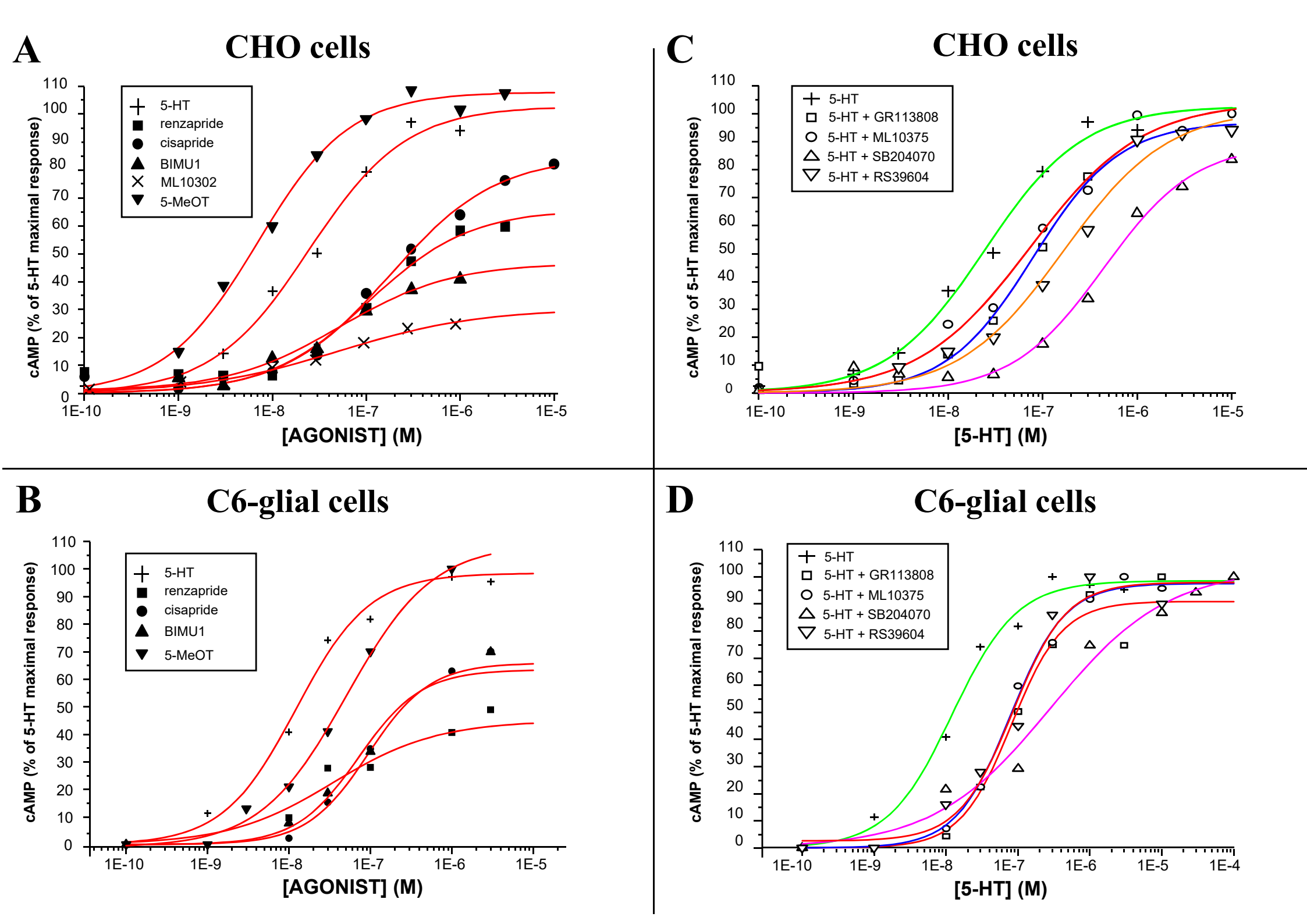

h5-HT4(e)
M
Atrium
Ventricle
Brain
Skeletal muscle
Lung
Liver
Colon
Kidney
1000
750
500
Actin + RT
Actin - RT


A
CHO cells
Kd = 0.2 nM
Bmax = 347 fmol/mg protein
Bound/Free
Bound (fmol/mg protein)
Bound (fmol/mg prot)
Free (nm)
Total
Specific
Non-specific
B
C6-glial cells
Kd = 0.08 nM
Bmax = 88 fmol/mg protein
Bound/Free
Bound (fmol/mg protein)
Bound (fmol/mg prot)
Free (nm)
Total
Non-specific
Specific


CHO cells
kDa
76
52
CT
5-HT4(e)
C6-glial cells
kDa
76
52
CT
5-HT4(e)


A
CHO cells
% Specific [3H]GR113808 Bound
Log [AGONIST] (M)
cisapride
RS67333
renzapride
BIMU 1
ML10302
5-HT
5-MeOT
C
CHO cells
% Specific [3H]GR113808 Bound
Log [ANTAGONIST] (M)
RS23597
ML10375
RS39604
SB204070
B
C6-glial cells
% Specific [3H]GR113808 Bound
Log [AGONIST] (M)
cisapride
RS67333
renzapride
BIMU 1
ML10302
5-HT
5-MeOT
D
C6-glial cells
% Specific [3H]GR113808 Bound
Log [ANTAGONIST] (M)
RS23597
ML10375
RS39604
SB204070


cAMP production (% of control)
2000
1000
400
300
200
100
0
★
★
untransfected cells
basal
GR113808
ML10375
5-HT
GR113808 + 5-HT
ML10375 + 5-HT


A
CHO cells
5-HT
renzapride
cisapride
BIMU1
ML10302
5-MeOT
cAMP (% of 5-HT maximal response)
[AGONIST] (M)
B
C6-glial cells
5-HT
renzapride
cisapride
BIMU1
5-MeOT
cAMP (% of 5-HT maximal response)
[AGONIST] (M)
C
CHO cells
5-HT
5-HT + GR113808
5-HT + ML10375
5-HT + SB204070
5-HT + RS39604
cAMP (% of 5-HT maximal response)
[5-HT] (M)
D
C6-glial cells
5-HT
5-HT + GR113808
5-HT + ML10375
5-HT + SB204070
5-HT + RS39604
cAMP (% of 5-HT maximal response)
[5-HT] (M)