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5-HT$_4$ Receptors: Cloning and Expression of New Splice Variants

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ABSTRACT

On the basis of differences in the potencies and intrinsic activity of 5-HT$_4$ receptor agonists in different biological models it has been suggested that there is heterogeneity among 5-HT$_4$ receptors. Here, we report the molecular cloning of several 5-HT$_4$ receptor splice variants in mouse, rat, and human brain. Our data suggest that the differences in efficacy of 5-HT$_4$ ligands on 5-HT$_4$ receptor-mediated responses in several tissues is due to differences in coupling efficiency rather than to the presence of different 5-HT$_4$ receptor isoforms.

Heterogeneity among 5-HT_4 receptors has been suggested on the basis of differences in the potencies and intrinsic activity of 5-HT_4 receptor agonists in different biological models. It has been proposed that these differences may be due to the existence of distinct 5-HT_4 receptor subtypes.[1,2] Although it has been proposed that the existence of 5-HT_4 receptor splice variants[3,4] might explain some tissular pharmacological differences, no clear data have yet been provided.

In this study, we report the molecular cloning of several other 5-HT_4 receptor splice variants in mouse, rat, and human brain. We present data suggesting that the difference in efficacy of 5-HT_4 ligands on 5-HT_4 receptor-mediated responses in several tissues was not due to the presence of different 5-HT_4 receptor isoforms but was due rather to their coupling efficiency.

CLONING OF TWO NEW SPLICE VARIANTS OF MOUSE 5-HT_4 RECEPTOR: m5-$HT_{4(c)}$ AND $_{(d)}$

We have recently cloned the mouse 5-HT_{4L} (or m5-$HT_{4(b)}$) receptor[5] and found that the two isoforms L (or (b)) and S (or (a)) of the receptor are expressed whatever the structure of the brain studied. This observation, which was confirmed by Vilaro *et al.*,[6] prompted us to clone the short isoform of the m5-HT_4 receptor. From whole brain RNA preparation, we isolated the full-length cDNA of the m5-$HT_{4(a)}$ receptor, corresponding to a deduced sequence of 387 amino acids. The nucleotide and peptide sequences were 94% and 96% identical to those of the r5-$HT_{4(a)}$, respectively.[3] We also cloned two splice variants of the m5-HT_4 receptor that we named m5-$HT_{4(c)}$ and m5-$HT_{4(d)}$ according to the NC-IUPHAR recommendation[7] (Fig. 1a). Figure 1b shows the three cDNA fragments obtained by PCR amplification of the C-terminal part of the m5-$HT_{4(a)}$, $_{(c)}$ and $_{(d)}$ receptors. The amino acid sequences, upstream to Leu_{358}, were identical in all m5-HT_4 receptor splice variants. After codon 358, m5-$HT_{4(c)}$ cDNA contained two cassettes, whereas m5-$HT_{4(d)}$ cDNA contained only the second one (Fig. 1a). The C-terminal nucleotide sequence of m5-$HT_{4(a)}$ cDNA followed those cassettes. An in-frame stop codon appeared in the common cassette of m5-$HT_{4(c)}$ and $_{(d)}$ receptors. This resulted in two receptors of 371 and 363 amino acids, respectively.

A priori, none of the differences in sequences observed between the four m5-HT_4 receptor isoforms seem to be apt to lead to different pharmacological responses. Indeed, comparing the binding affinity constants for a series of agonists or antagonists or the affinity constants for stimulating adenylyl cyclase activity, when expressing in COS-7 cells, a significant correlation ($r > 0.95$) was found between the four m5-HT_4 isoforms.

HYPOTHESIS FOR THE ORGANIZATION OF THE MOUSE 5-HT$_4$ RECEPTOR GENE

For the mouse gene, we can imagine an organization with at least 5 exons, as described in Figure 1c. A first exon may contain the common 5′ part of the four receptors 5-HT$_{4(a)}$ to $_{(d)}$. The first inserted cassette which appears in the m5-HT$_{4(c)}$ receptor and the second one, shared by m5-HT$_{4(c)}$ and $_{(d)}$ receptors which contain the in-frame stop codon, suggest the existence of two additional and separate exonic cassettes (exon 2 and 3, on Fig. 1c). A fourth exon may correspond to the C-terminal part of the m5-HT$_{4(a)}$ receptor, which is also found in the splice variants $_{(c)}$ and $_{(d)}$ as non-coding sequence. The C-terminal part of the m5-HT$_{4(b)}$ receptor could be in a fifth exonic sequence. We cannot exclude that the N-terminal common sequence is composed of more than one exon. Indeed, a splice variant presenting an extended second extracellular loop in the porcine 5-HT$_4$ receptor has been found.[4] To confirm this hypothesis, we have to analyze the exon-intron organization on the 5-HT$_4$ receptor gene as recently done for the 5-HT$_7$ gene[8] which presents four splice variants differing also in the C-termini by alternative splicing.

CLONING OF THE RAT 5-HT$_{4(c)}$ RECEPTOR

We also cloned the r5-HT$_{4(c)}$ receptor from rat brain (Fig. 1a). The nucleotide and peptide sequences of which were also 94% and 96% identical to those of the m5-HT$_{4(c)}$ receptor, respectively. The receptor was 371 residues long, identical in length to m5-HT$_{4(c)}$, and had only one amino acid change in the C-terminal part after the splicing site (Fig. 1d).

CLONING OF TWO SPLICE VARIANTS OF HUMAN 5-HT$_4$ RECEPTOR: h5-HT$_{4(b)}$ AND $_{(e)}$

We isolated the cDNA of three human receptors: the h5-HT$_{4(a)}$ receptor from the brain and the atrium,[9] the h5-HT$_{4(b)}$ receptor and a novel splice variant h5-HT$_4$(e) receptor from the brain. The peptide sequences of the h5-HT$_{4(b)}$ was 92% identical to those of the r5-HT$_{4(b)}$ receptor, but one punctual insertion (an additional cytosine after position 1154) occurring in the last 50 nucleotides of the h5-HT$_4$ cDNA sequence induces a frameshift and the apparition of a stop codon leading to a form shorter by 18 residues (Fig. 1a). Therefore, the h5-HT$_{4(b)}$ receptor was 388 residues long (Fig. 1d). This receptor recently cloned by another group,[10] confirmed the occurrence of the additional cytosine by partial sequencing of human and rat genomic DNA. In contrast to the published sequence, 3 they surprisingly found the same insertion in the rat

genomic DNA; we therefore checked our sequence of the m5-HT$_{4(b)}$ receptor. 5 The additional cytosine was also present, but a compression occurring in this part of the sequence had made us initially find a cytosine doublet instead of a triplet really present in the mouse sequence. Therefore, the m5-HT$_{4(b)}$ receptor was 388 residues long, like the human one and probably like the one of the rat. The second human receptor we cloned, the h5-HT$_4$(e) receptor, presents the insertion of a cassette at the putative splicing site as seen in the m and r5-HT$_{4(c)}$ receptors. This cassette led to a receptor of 378 residues, because of the presence of an in-frame stop codon (Fig. 1a). The species differences are probably due to altered intron-exon organization similar to the one described 5-HT$_7$ receptor gene.[8]

SIGNIFICANT DIFFERENCES OBSERVED IN THE C-TERMINAL AMINO ACID SEQUENCES OF THE CLONED 5-HT$_4$ RECEPTOR SPLICE VARIANTS

The alignment of the C-terminal amino acid sequences of the cloned 5-HT$_4$ receptor isoforms of mouse, rat or human brain showed that the divergence in their sequences started after position L$_{358}$. After these L$_{358}$, all the receptor isoforms had their own specific sequence and length (Fig. 1d). Preliminary experiments indicated that r5-HT$_{4(b)}$ receptor (long form) desensitized more rapidly than the short form 5-HT$_{4(a)}$.[11] Indeed we have reported in a precedent study that a rapid and homologous desensitization of 5-HT$_4$ receptors occur in mouse colliculi neurons.[12] This type of desensitization is classically triggered via phosphorylation of the receptors by specific G-protein coupled receptor kinases called GRKs.[13] The consensus sequences recognized by GRKs in GPCRs, could be S and T residues downstream of an acidic doublet such as DE, ED or EE in the C-terminal end of GPCRs.[14] The r, m, and h5-HT$_4$ splice variants revealed that they shared the DE (331–332) doublet. This was the only acidic doublet present in all the 5-HT$_4$ receptor isoforms after the cysteine 328, putative palmitoylation site (Fig. 1d). It remains to be demonstrated if there are differences in the mechanism and rate of desensitization between the 5-HT$_4$ splice variants. It will also necessary to determine which of the serines and threonines downstream this acidic doublet are indeed phosphorylated during desensitization.

It was interesting to note that the new cloned splice variants m5-HT$_{4(c)}$ and $_{(d)}$, r5-HT$_{4(c)}$ and h5-HT$_{4(e)}$ receptor shared the same four carboxy-terminal residues: PVPV (Fig. 1d). Recently, a variety of proteins with PDZ domains has been identified that interacts with receptors containing a valine at the carboxyl-terminal end.[15] These proteins with PDZ domains seem to act as molecular organizers that cluster receptors in the plasma membrane or mediate

direct protein-protein interaction essential for intracellular signaling. The specificity of the interaction seems to be determined by the adjacent three or four residues before the carboxy-terminal residue. This PVPV sequence present in the four 5-HT_4 receptor splice variants has also been shown at the carboxy-terminal end of receptor tyrosine kinase.[16] The structural feature of the sequence PVPV forming several angles, owing to the presence of two prolines, could determine the specificity of the protein-protein interaction.

CHROMOSOMAL LOCALIZATION

We used the recently cloned h5-$HT_{4(a)}$ receptor cDNA to map the chromosomal localization.[9,17] By using fluorescence *in situ* hybridization, we showed that this gene mapped at the locus 5q31-q33, which was syntenic to the region carrying three other receptors, positively coupled to adenylyl cyclase, the dopamine D_1 receptor, and the β_1-and the β_2-adrenergic receptors.[18,19] The general sequence homologies between these receptors was relatively low (28.4, 29 and 27.8 %, respectively) but high if one considers the important domains interacting with Gs such as the end of the third intracellular loop. Both β-adrenergic (β_1- and the β_2) and 5-HT_4 receptor stimulation increased atrial cAMP levels, activated cAMP-dependent protein kinase A (PKA) and, ultimately, increased heart rate and contractile force (only in atrial cells for 5-HT_4 receptor). However, these receptors differently contributed to the cAMP-dependent positive inotropic response of human atrial cells, which can also vary during pathology. It has been reported that both β_2-adrenergic receptor and 5-HT_4 receptor responsiveness vary during pathology in human atrial tissues from heart failure patients treated chronically with β_1-adrenoreceptor antagonists.[2] The same chromosomal localization might explain the cross-talk expression observed between β_2-adrenoreceptors and 5-HT_4 receptors, which has been previously interpreted as a consequence of increased receptor density. Cells from patients with various forms of 5q syndrome[20] should provide useful material for exploring the effects of such genetic abnormalities on 5-HT_4 receptor expression.

COMPARISON BETWEEN THE PHARMACOLOGICAL PROPERTIES OF 5-HT_4 ANTAGONISTS ON 5-HT_4 RECEPTORS IN COLLICULI NEURONS AND TRANSFECTED COS-7 CELLS: EVIDENCE FOR EXISTENCE OF INVERSE AGONISTS

In COS-7 cells, we observed that all 5-HT_4 receptor isoforms were able to activate adenylyl cyclase in the absence of agonists, even when expressed at physiological density (25–

200 fmol/mg protein). This spontaneous activity was termed constitutive activity in the pioneer work of Costa and Hertz and Cotecchia *et al.*[21,22] It is now accepted that receptors exist in equilibrium between two conformations, an inactive state R and an active state R*. Spontaneous isomerization occurs between these two forms and only the R* conformation can interact efficiently with G proteins. The constitutive activity of the native 5-HT_4 receptor transfected in COS-7 cells (98 ± 16 fmol/mg protein) was confirmed by the discovery of three inverse agonists of 5-HT_4 receptors-GR 113808, GR 125487, and SB 207266-all of which inhibited the basal cAMP formation. SB 207266 had a higher efficacy than the others (Fig. 2c, d). In contrast, they were not able to inhibit basal cAMP formation in colliculi neurons expressing similar amounts of 5-HT_4 receptors (60 ± 23 fmol/mg protein). A comparison in the ability of each isoform to induce constitutive activity at the same receptor density has to be analyzed. It seems that less R* was present in neurons than in COS-7 cells for the same total R +R* concentration.

During the last ten years, the 5-HT_4 receptor has been defined essentially on the basis of transductional and pharmacological criteria in several models including mouse colliculi neurons, guinea pig ileum and colon, rat esophagus, and human heart. 1 Generally, similar pharmacological properties were found in the different models used, despite a few differences in potency and activity (partial or total agonist or antagonist) of some compounds. However, in a recent report Leung *et al.*[23] observed that several novel selective 5-HT_4 compounds showed an opposing pattern of activity when tested in guinea-pig colon or rat esophagus, which is difficult to explain by a difference in coupling efficiency. Here, we show that highly potent and selective 5-HT_4 ligands, described as potent neutral antagonists in colliculi neurons (Fig. 2a, c), were partial agonists, neutral agonists or inverse agonists when tested in COS-7 cells expressing one of the 5-HT_4 receptor isoforms (Fig. 2b, d). GR 113808, GR 125487 and SB 207266 inhibited the constitutive activity of the receptor and behaved as inverse agonists (Fig. 2b). In contrast, RS 100235, described as the most potent 5-HT_4 antagonist in colliculi neurons and in other models,[24] was a neutral antagonist in COS-7 cells; it inhibited only 5-HT-induced cAMP production (Fig. 2a, b). It was also able to competitively inhibit the inverse agonist effect of GR 125487 on the 5-HT_4 receptor transiently expressed in COS-7 or LLC-PK1 cells (not shown). Furthermore, other potent 5-HT_4 antagonists on 5-HT_4 receptor in colliculi neurons: RS 39604, SB 204070, ML 10302, RS 23597 and DAU 6285 behaved as partial agonists (Fig. 2c, d). None of the benzoate compounds tested, which are potent 5-HT_4 receptor antagonists in colliculi neurons, [1,25] displayed any inverse agonistic activity.

These results are a further illustration of differences in pharmacology that could be found when the same receptors are expressed in different cellular contexts. These differences in pharmacological property requests caution when designing the nature of novel 5-HT_4 drugs. Their agonistic or antagonistic effects may depend on the coupling efficiency between the 5-HT_4 receptor and G protein in the biological model selected.

CONCLUSION

The number of splice variants of the 5-HT_4 receptor is on the increase. Although, Gerald *et al.* described only two splice variants (r5-$HT_{4(a)}$ and $_{(b)}$),[3] Ullmer *et al.* have also described the possible existence of 5-HT_4 receptor splice variants in the second extracellular loop.[4] Here, we found two novel splice variants in mouse (m5-$HT_{4(c)}$ and $_{(d)}$) and human (h5-$HT_{4(b)}$ and $_{(e)}$) and one in rat (r5-$HT_{4(c)}$). The new splice variants, like the first two, varied in their C-terminal domain, after L_{358}. Alignment of the C-terminal of all these splice variants indicated that they probably derived from a gene having a complex organization.

We found that 5-HT_4 receptors transfected in COS-7 cells at low density gave a constitutive activity which was inhibited by compounds thought to be neutral antagonists, behaving as inverse agonists. In colliculi neurons, we observed no evidence for a constitutive activity of 5-HT_4 receptors.

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FIGURE LEGENDS

Figure 1

Differences in the C-termini of the mouse, rat and human 5-HT_4 splice variants. **(a)** Schematic representation of the nucleotide cassettes composing the receptors. *, position of the stop codon. **(b)** PCR amplification of cDNA fragments corresponding to the C-terminal part of the mouse splice variants (a), (c) and (d). The PCR was performed on retro-transcribed poly(a)+ RNA from whole mouse brain using the upper primer: 5′ CCAAAGCAGCCAAGACT 3′ (International Patent WO94/14957, Sequence 1, position 864–880) and the lower primer: 5′ TAGTAACCTGTTCATGCAGACACA 3′ (Patent, Sequence 1, position 1302–1325). The fragments are separated by electrophoresis on a 2.5% agarose gel. **(c)** Putative organization of the intron-exon in the mouse 5-HT_4 receptor gene. Schematic representation of the splicing events producing mRNA for the four different isoforms. *, position of the different in-frame stop codons. **(d)** Carboxy-terminal amino acid sequences of the cloned 5-HT_4 receptor splice variants indicated in a.

Figure 2

Comparison between the pharmacological properties of 5-HT_4 antagonists on 5-HT_4 receptors in colliculi neurons and transfected COS-7 cells. Evidence for existence of inverse agonists. Panels **(a)** and **(c)** represent the activity of well-known 5-HT_4 antagonists on 5-HT-stimulated cAMP formation in colliculi neurons. Neuronal cells were exposed to increasing concentrations of each antagonist in the presence of 1μM 5-HT. The results are expressed as a percentage of residual stimulation relative to the stimulatory action of 1μM of 5-HT taken as 100%. Panels **(b) and(d)** represent the activity of the same drugs on the h5-$HT_{4(a)}$ receptor transiently transfected in COS-7 cells expressing 98 ± 16 fmol/mg protein. cAMP production was measured during 30 min and expressed as a percent of control (mock transfected Cos-7 cells).

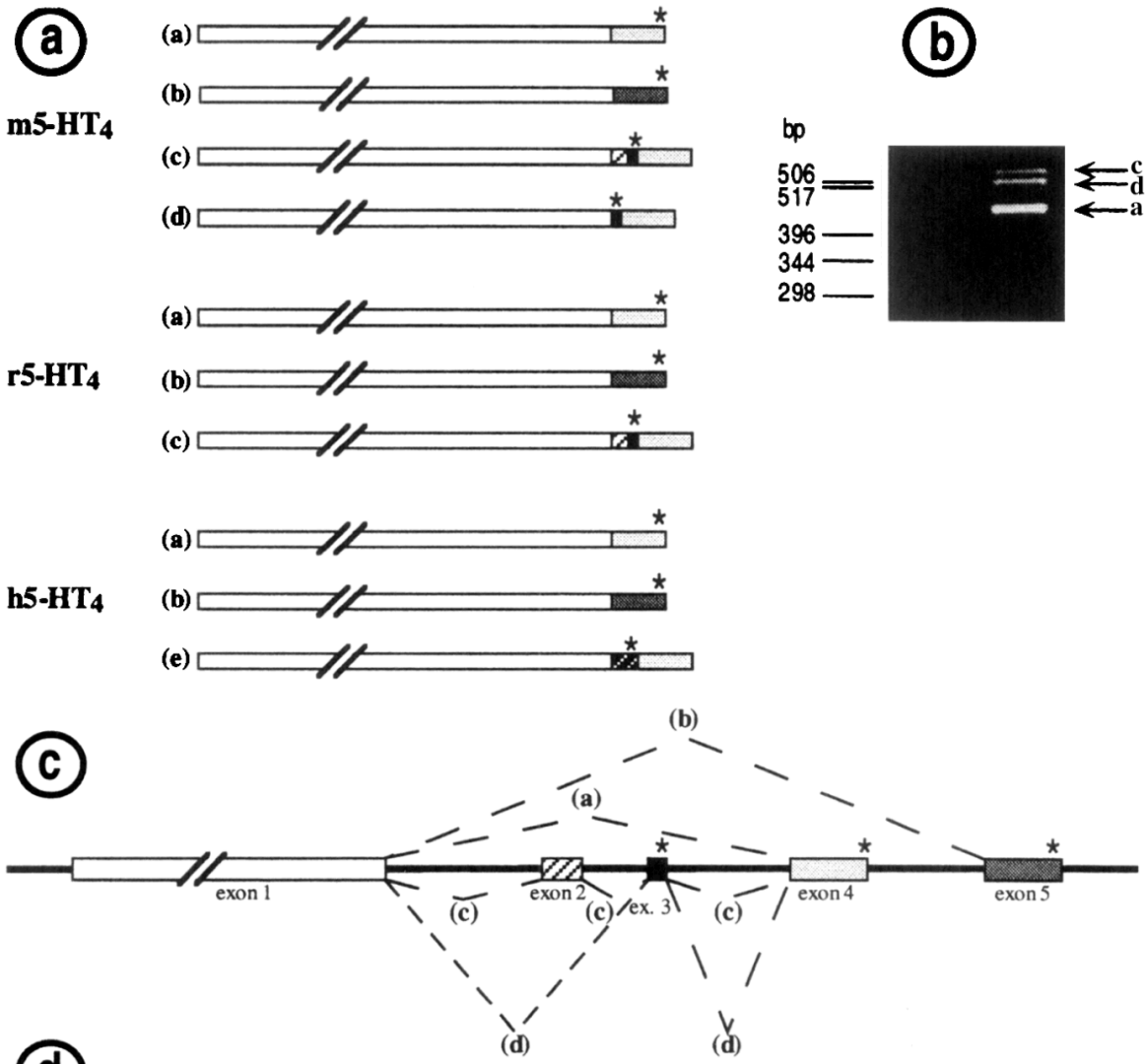

a
m5-HT4
(a)
(b)
(c)
(d)
r5-HT4
(a)
(b)
(c)
h5-HT4
(a)
(b)
(e)
b
bp
506
517
396
344
298
c
d
a
c
(b)
(a)
exon 1
exon 2
ex. 3
exon 4
exon 5
(c)
(c)
(c)
(d)
(d)
d
358
(a) LRYTVLHSGHHQELEKLPIHNDPESLESCF 387
(b) LRDAVECGGQWESRCHLTATSPLVAAQPSDT 388
m5-HT4
(c) LSFPLLFRNRPVPV 371
(d) LRPVPV 363
358
(a) LRYTVLHSGQHQELEKLPIHNDPESLESCF 387
r5-HT4
(b) LRDTVECGGQWESRCHLTATSPLVAAQPSDT 388
(c) LSFPLLFCNRPVPV 371
358
(a) LRYTVLHRGHHQELEKLPIHNDPESLESCF 387
h5-HT4
(b) LRDAVECGGQWESQCHPPATSPLVAAQPSDT 388
(e) LSGCSPVSSFLLLFCNRPVPV 378

Figure 1

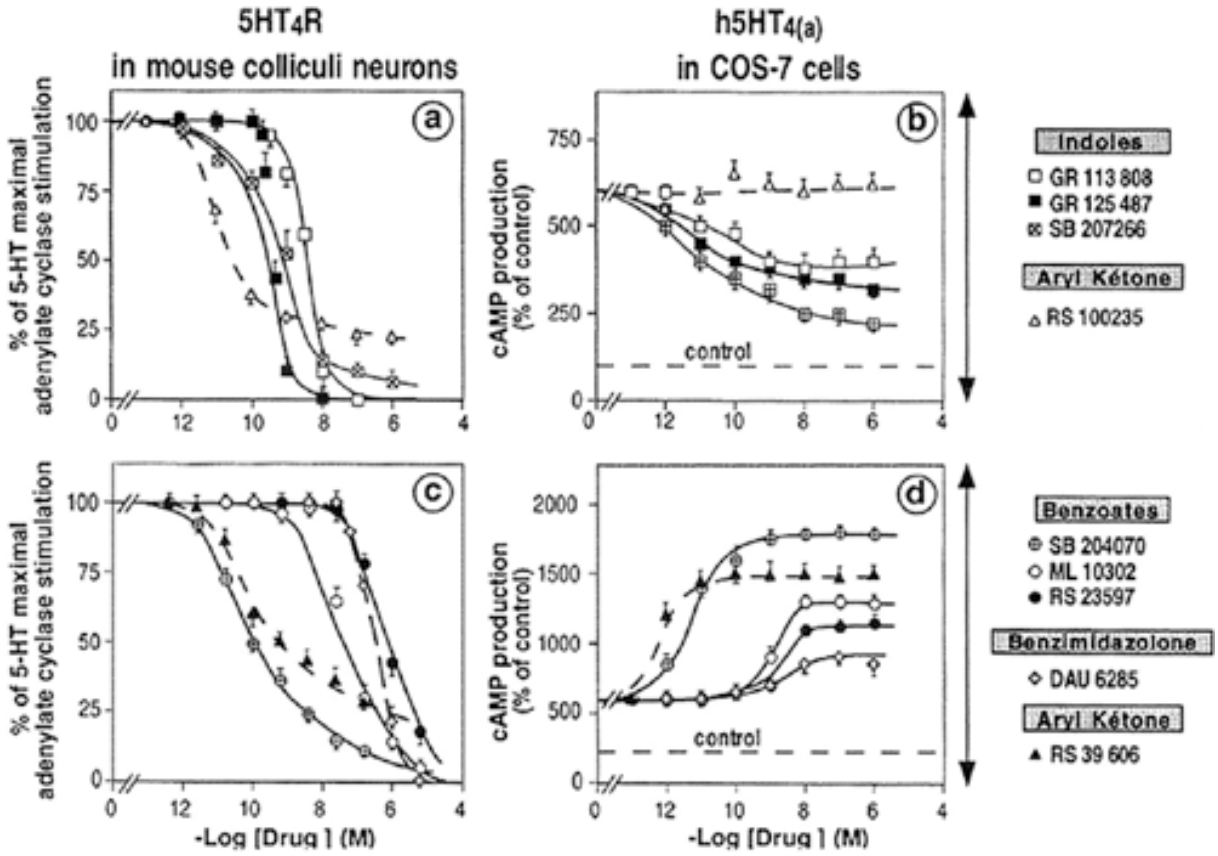

5HT4R
in mouse colliculi neurons
h5HT4(a)
in COS-7 cells
a
b
c
d
% of 5-HT maximal adenylate cyclase stimulation
cAMP production (% of control)
control
-Log [Drug] (M)
Indoles
GR 113 808
GR 125 487
SB 207266
Aryl Kétone
RS 100235
Benzoates
SB 204070
ML 10302
RS 23597
Benzimidazolone
DAU 6285
Aryl Kétone
RS 39 606

Figure 2