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# **Novel brain-specific 5-HT<sub>4</sub> receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain**

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**Running title:** 5-HT<sub>4</sub> receptor splice variants and constitutive activity

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**The abbreviations used are:** GPCRs, G-protein coupled receptors; 5-HT<sub>4</sub>R, 5-Hydroxytryptamine 4 receptor; RT-PCR, reverse transcriptase polymerase chain reaction; dFBS, dialyzed fetal bovine serum;  $\beta_2$ -AR,  $\beta_2$ -adrenergic receptor; CAM, Constitutively active mutant; R, inactive receptor conformation; R\*, active receptor conformation; ON, oligonucleotide; ISO, isoproterenol; TM, transmembrane domain. TSHR, thyrotropin receptor; TRHR, thyrotropin releasing hormone receptor.

## SUMMARY

We have cloned new 5-HT<sub>4</sub> receptor (5-HT<sub>4</sub>R) splice variants from mouse (m5-HT<sub>4(e)</sub>R and m5-HT<sub>4(f)</sub>R), rat and human brain (r and h5-HT<sub>4(e)</sub>R) which differ, as the previously described 5-HT<sub>4</sub>R variants, in the length and composition of their intracellular C-termini after the common splicing site (L358). These new variants have a unique C-terminal sequence made of two PV repeats and are only expressed in brain. All the 5-HT<sub>4</sub>R splice variants have a high constitutive activity when expressed at low and physiological densities (<500 fmols/mg protein). At similar density, they showed a much higher constitutive activity than the native and the mutated  $\beta_2$ -adrenergic receptors ( $\beta_2$ -AR, CAM  $\beta_2$ -AR). The constitutive activity of the new splice variants with short C-terminal sequences (m5-HT<sub>4(e)</sub>R and <sub>(f)</sub>R) was higher than that of the long C-terminal sequence variants (m5-HT<sub>4(a)</sub>R and <sub>(b)</sub>R). This may indicate that the short variants have a higher capacity of isomerize from the inactive (R) to the active conformation (R\*). Moreover, we further identified a sequence, within the C-terminal tail upstream of L358, rich in serine and threonine residues, which played a crucial role in maintaining 5-HT<sub>4</sub>R under its inactive conformation.

## INTRODUCTION

The physiological functions of 5-HT both in the central nervous system and the periphery of vertebrates are mediated by a large number of GPCRs. Among the 13 known genes coding for 5-HT GPCR only three (5-HT<sub>4</sub>R, 5-HT<sub>6</sub>R and 5-HT<sub>7</sub>R) are positively coupled to Gs (Hoyer and Martin, 1997). 5-HT<sub>4</sub> receptors are expressed in a wide variety of tissues of vertebrates including brain, oesophagus, ileum, colon, adreno-cortical cells, urinary bladder, human and porcine heart (Bockaert *et al.*, 1997; Eglen *et al.*, 1995). Benzamide 5-HT<sub>4</sub>R agonists, such as metoclopramide and cisapride, are therapeutically used as prokinetic drugs. A role for 5-HT<sub>4</sub> receptors in learning and memory has been demonstrated in brain (Eglen *et al.*, 1995; Letty *et al.*, 1997; Marchetti-Gauthier *et al.*, 1997). Although the pharmacology of 5-HT<sub>4</sub> receptors is similar in the different tissues studied, some still unexplained differences remain. For example, benzamides are super agonists (efficacy greater than that of 5-HT) in colliculi neurons, the preparation on which 5-HT<sub>4</sub> receptors were first described, whereas in many systems they are partial agonists or even antagonists (Bockaert *et al.*, 1997; Dumuis *et al.*, 1988). A difference in structure may explain some of the observed differences and in particular the super agonism of benzamides in colliculi neurons. This is the reason why we have persevered in our cloning effort of 5-HT<sub>4</sub> receptors especially in brain tissues and further analyzed the coupling efficiency of variants cloned in different species. Gerald *et al.* (Gerald *et al.*, 1995) first reported the cloning of two rat variants, (r5-HT<sub>4S</sub>R and r5-HT<sub>4L</sub>R now called r5-HT<sub>4(a)</sub>R and r5-HT<sub>4(b)</sub>R) which differ in their C-terminal tail after the splicing site at L358. We and others have cloned the human and mouse m5-HT<sub>4(a)</sub> and m5-HT<sub>4(b)</sub> receptor homologues (Blondel *et al.*, 1997; Claeysen *et al.*, 1997a; Claeysen *et al.*, 1996). Blondel *et al.*, however, (Blondel *et al.*, 1998) reported the existence of two different human variants (h5-HT<sub>4(c)</sub>R and (d)R) also displaying divergent sequences after L358. In this study, we have cloned two additional splice variants in mouse (m5-HT<sub>4(e)</sub>R and m5-HT<sub>4(f)</sub>R). The (e) form has also been cloned in rat and human brain (r and h5-HT<sub>4(e)</sub>R). All these variants share the same sequence up to L358, followed by specific C-termini. Splice variants of GPCRs generally differ in their intracellular domains in particular, within the third intracellular loop and the C-terminal tail. These two regions are involved in the fine tuning of the coupling of GPCRs to G proteins (Journot *et al.*, 1994). The role of C-terminus in maintaining the receptor in an inactive conformation (R) in

the absence of agonist has been highlighted by recent studies [Mary, 1998 #1556; Hasegawa, 1996 #1654; (Weiss *et al.*, 1994) Matus-Leibovitch, 1995 #1651; Parker, 1991 #1652]. These data support the hypothesis of a GPCR structure likely selected during evolution to constrain allosteric transition from the inactive (R) to active (R\*) forms in the absence of agonist (Cotecchia *et al.*, 1990; Cotecchia *et al.*, 1992; Kenakin, 1995; Kjelsberg *et al.*, 1992). This transition is governed by the allosteric transition constant J, which is an index of the magnitude of receptor constraint (Samama *et al.*, 1993). The lower the constant, the higher the signal to noise ratio.

The aptitude for a given receptor to convert from R to R\*, in the absence of agonist, is also an important physio-pathological parameter to take into account because of: (a) the increasing number of pathologies associated with constitutively active receptors (under the R\* form in the absence of agonist) (Spiegel *et al.*, 1993) and (b) the potential therapeutic use of inverse agonists. Not all receptors have the same propensity to isomerize from R to R\* in the absence of ligand. TSHR is one of the GPCRs which has the highest propensity to isomerize. This is probably the reason why mutations of residues localized throughout the TSHR structure, lead to the constitutively active states found in thyroid hyperplasia (Van Sande *et al.*, 1995). Here, we report that 5-HT<sub>4</sub> receptors like TSHRs show marked constitutive activity when expressed at low physiological densities. At a similar receptor density, the newly cloned 5-HT<sub>4</sub> receptor splice variants (m5-HT<sub>4(e)</sub>R and (f)R) have, in their native conformation, an agonist-independent activity level above the mutated  $\beta_2$ -AR (CAM  $\beta_2$ -AR).

## MATERIALS AND METHODS

### Isolation and sequencing of the mouse 5-HT<sub>4(a)</sub>, (e) and (f) receptors cDNA by RT-PCR:

Total RNA was isolated from whole adult Swiss mouse brain. Poly(A)<sup>+</sup>RNA was extracted with Dynabeads Oligo(dT)<sub>25</sub> (Dynal) and reverse transcribed using Superscript II RNase H Reverse Transcriptase (Life Technologies) and random nonamers. RT-PCR was performed with the following oligonucleotides, ON1 (Claeyssen *et al.*, 1996) (accession number Y09585, position 1-24): 5' TAATGGACAACTTGATGCTAATG 3', and ON2 designed on International Patent WO 94/14957, (Sequence 1, position 1302-1325): 5' TAGTAACCTGTTCATGCAGACACA 3'. ON1 is located in the 5' translated region of m5-HT<sub>4(b)</sub> receptor and ON2 is located in the 3' untranslated part of the r5-HT<sub>4S</sub>R cDNA. PCR was performed with the Taq DNA Polymerase (Eurobio): 94°C/3 min followed by 30 cycles of denaturation (94°C/30 sec), annealing (55°C/30 sec) and elongation (72°C/1 min). These conditions yielded a large band of near 1228 bp which was subcloned into pCR®2.1 vector using the Original TA Cloning® Kit (Invitrogen) and plasmid DNA was prepared for insert sequencing. Nucleotidic sequence was performed using Sanger dideoxy-nucleotide chain-termination method on denatured double-stranded plasmid templates using T7 DNA polymerase (Pharmacia kit). Three different clones were isolated: the m5-HT<sub>4(a)</sub>R cDNA which is the equivalent of the previously cloned r5-HT<sub>4S</sub>R in rat (Gerald *et al.*, 1995) and two other cDNA sequences presenting additional cassettes at position 1079 that we named m5-HT<sub>4(e)</sub>R and m5-HT<sub>4(f)</sub>R, taking into account the other published 5-HT<sub>4</sub> receptors and the order of cloning. To confirm the sequence of the 5' part of the receptor around the ATG codon, we performed a PCR reaction using the following primers: ON3 (Patent, Sequence 1, position 21-41) 5' GGTGGAAGGAGGAGGATGCT 3', in the 5' non coding sequence, and ON4 (Patent, Sequence 1, position 699-721) 5' GAGGAGAAACGGGATGTAGAAGG 3', in the middle of the receptor sequence. The PCR conditions with the Taq DNA Polymerase (Eurobio) were the same as above. Fragments of the expected size (701 bp) were subcloned using the Original TA Cloning® Kit (Invitrogen). To define the exact sequence of all the 5-HT<sub>4</sub> receptor splice variant cDNAs, the sequences were performed for each cDNA, on three clones obtained from three independent PCR reactions.

**Isolation and sequencing of the rat 5-HT<sub>4(e)</sub> receptor cDNA by RT-PCR:**

cDNA encoding rat brain 5-HT<sub>4(e)</sub> receptor, a novel rat variant, has been cloned by RT-PCR on total rat brain poly(A)<sup>+</sup>RNA (Clontech) following the same procedures as described above. Primers used were: ON5 (Gerald *et al.*, 1995) (accession number U20906, position 19-42) and ON2.

**Isolation and sequencing of the human 5-HT<sub>4(e)</sub> receptor cDNA by RT-PCR:**

cDNA encoding human brain 5-HT<sub>4(e)</sub> receptor, a novel human variant, has been cloned by RT-PCR on total human brain poly(A)<sup>+</sup>RNA (Clontech) following the same procedures as described above. Primers used were: ON6 (Claeyssen *et al.*, 1997a) (accession number Y0586, position 1-24) and ON2.

**Tissue localization studies:**

Brain areas and organs were dissected from adult Swiss mice. cDNA was obtained as described above from poly(A)<sup>+</sup> RNA. Primers designed to carry out PCR amplification were: one sense primer in the common part of the variant sequences, ON7 (Patent WO 94/14957, Sequence 1, position 864-880): 5'-CCAAGGCAGCCAAGACT-5' and two antisense primers, ON2 for the amplification of the C-termini of the (a), (e) and (f) forms and ON8 (Patent WO 94/14957, Sequence 3, position 1322-1340) 5' TTGCCTCATGCTCTTGAA 3', for the amplification of the C-terminus of the (b) form. For the amplification of  $\beta$ -actin as control, we used ON9 (J00691, position 2252-2271) 5' GACTCCGGAGACGGGGTCAC 3' and ON10 (J00691, position 2482-2501) 5' CGCATCCTCTTCCTCCCTGG 3'. The PCR conditions were those described above. The template cDNA used per tube was retro-transcribed from 250 or 500 ng of poly(A)<sup>+</sup> RNA. The expected sizes of the PCR fragments were 463 bp for m5-HT<sub>4(a)</sub>R, 503 bp for m5-HT<sub>4(f)</sub>R, 527 bp for m5-HT<sub>4(e)</sub>R (primer couple ON7/ON2), 528 bp for m5-HT<sub>4(b)</sub>R (primer couple ON7/ON8) and 450 bp for  $\beta$ -actin (primer couple ON9/ON10). PCR products were run on 3% MetaPhor® agarose (FMC®)/ethidium bromide gels and photographed. Positive and negative controls (with or without reverse transcriptase) were performed on the  $\beta$ -actin amplification (data not shown). Southern blotting was done after transfer of the DNA to charged nylon membranes (Qiabond, Qiagen). The hybridization probe was an RT-PCR fragment obtained in the same PCR conditions with r5-HT<sub>4(a)</sub>R cDNA as template (a



generous gift from Dr. Christophe Gerald) and the two primers ON7 and ON2. The probe was labeled using the Prime-It II Random Primer Labeling Kit (Stratagene).

### **$\beta_2$ -adrenergic receptor and CAM- $\beta_2$ -AR cDNAs:**

The human  $\beta_2$ -adrenergic receptor cDNA (h $\beta_2$ -AR) and the CAM- $\beta_2$ -AR cDNA were obtained from Susanna Cotecchia.

All the receptors were subcloned in the eucaryotic expression vector pRK5, a CMV based expression vector (Schall *et al.*, 1990).

### **Construction of truncated 5-HT<sub>4(a)</sub> receptor cDNA:**

Constructs were obtained by inserting a stop codon after residues 327, 346 or 359 in the 5-HT<sub>4(a)</sub>R cDNA sequence, using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Sense primers used:

$\Delta 327$ : 5'-CATCCTCTAGAAGTGATGATGAGCG-3'

$\Delta 346$ : 5'-TCCCCTGTTAACCCACAACCATTA-3'

$\Delta 359$ : 5'-CATGTACTAAGGTAGACAGTTTTGCAC -3'

### **Construction of mutated m5-HT<sub>4(e)</sub> receptor cDNA:**

The mutant A258L was obtained by exchanging the endogenous A258 to L in the m5-HT<sub>4(e)</sub>R cDNA sequence, using the QuikChange™ Site-Directed Mutagenesis Kit (STRATAGENE). Sense primer used: 5'-AGACCAAGGCACTCAAGACTTTATGTGT-3'

### **Construction of epitope-tagged m5-HT<sub>4</sub>R splice variants:**

The cDNA of all the 5-HT<sub>4</sub>R variants cloned was subcloned into pRK5, a CMV based expression vector (Schall *et al.*, 1990). We then tagged the m5-HT<sub>4(a)</sub>, (b), (e) and (f) receptors with a stretch of nucleotides coding for a 10-amino acid epitope (EQKLISEEDL) of the human c-Myc protein (Ramsay *et al.*, 1984) in the N-terminal extremity using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Sense primer used:

5'GATTGAATTCTAAT**G**GAACAAAAGCTTATTTCTGAAGAAGACTTGGACAAACTTGATGC-3' (in bold letters: start codon, underlined: cMyc epitope).

### **Cell culture and transfection:**

The cDNAs, subcloned into pRK5, were introduced into LLC-PK1 or in COS-7 cells by electroporation as described by (Claeysen *et al.*, 1996). Briefly, cells were trypsinized, centrifuged, resuspended in EP 1X (50 mM K<sub>2</sub>HPO<sub>4</sub>/20 mM CH<sub>3</sub>CO<sub>2</sub>K/20 mM KOH/26.7 mM MgSO<sub>4</sub>/pH 7.4) with 25 to 2000 ng of receptor cDNA. The total amount of cDNA was kept constant at 15 µg/transfection using wild type pRK5 vector. After 15 min at room temperature, 300 µl of cell suspension (10<sup>7</sup> cells) were transferred to a 0.4 cm electroporation cuvette (Bio-Rad) and pulsed using a Gene pulser apparatus (setting 1000 µfarads, 270V). Cells were diluted in DMEM (10<sup>6</sup> cells/ml) containing 10% dialyzed and de complemented fetal bovine serum (dFBS) and plated on 10 cm-Falcon Petri dishes or into 12-well clusters at the desired density. Using epitope tagged 5-HT<sub>4</sub> receptors, we estimated the percent of cells that expressed the receptors.

### **Immunocytochemical fluorescence labeling of epitope-tagged 5-HT<sub>4</sub> receptors in cells:**

5-HT<sub>4</sub> receptors on intact cells were visualized using the anti c-Myc primary antibody and a rhodamine conjugated secondary anti-mouse antibody. Cells were grown on coverslips and fixed 24 hours after transient transfection in 4% paraformaldehyde diluted in PBS (pH 7.4) for 30 min at room temperature, washed three times in a glycine buffer (0.1M, pH 7.4) and incubated for 2h at 37°C with monoclonal antibody 9E10, ascite fluid was diluted 1:250 in PBS-gelatine 0.2%. The cells were then washed and incubated for 1h with a rhodamine conjugated secondary anti-mouse antibody 1:40 in PBS-gelatine 0.2%. Coverslips were washed and mounted on glass slides using gel mount (Biomedica Corp., Foster City, CA.). Cells were viewed on Zeiss Axioplan 2 microscope.

### **Determination of cAMP production in intact cells:**

Six hours after transfection, cells were incubated overnight in DMEM without dFBS with 2 µCi [<sup>3</sup>H]-adenine/ml to label the ATP pool and cAMP accumulation was measured as described by (Dumuis *et al.*, 1988).

**Membrane preparation and radioligand binding assay:**

Membranes were prepared from transiently transfected cells plated on 15 cm-dishes and grown in DMEM with 10% dFBS for 6 hours and 20 hours in DMEM without dFBS as previously described (Ansanay *et al.*, 1996). The cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS and centrifuged at 4°C (900 x g for 4 min). The pellet was resuspended in buffer containing 10 mM Hepes (pH 7.4), 5 mM EGTA, 1 mM EDTA and 0.32 M sucrose and homogenized 10 times with a glass-Teflon potter at 4°C. The homogenate was centrifuged at 20,000 x g for 20 min, the membrane pellet was resuspended in 50 mM Hepes (pH 7.4) (5 mg of protein in 1 ml solution) and stored at -80°C until used.

To perform radioligand binding studies with [<sup>3</sup>H]GR 113808 (specific activity: 83 Ci/mmol) 100 µl of membrane suspension, prepared as described above, were diluted 5 times with 50 mM HEPES (pH 7.4) (2 to 20 µg of protein) containing 10 mM pargyline and 0.01% ascorbic acid and were incubated at 20°C for 30 min with 100 µl [<sup>3</sup>H]GR 113808 and 50 µl of buffer or competing drugs. For saturation analysis assays, various concentrations of [<sup>3</sup>H]GR 113808 (0.001 to 1 nM) were used. For competition binding experiments, the [<sup>3</sup>H]-GR 113808 concentrations was 0.15 nM. Receptor expression was determined using a saturating concentration of [<sup>3</sup>H]GR 113808 (0.3-0.5 nM). 5-HT (0.5 µM) was used to determine non specific binding.

For determination of β<sub>2</sub>-adrenergic and CAM β<sub>2</sub>-adrenergic receptor expression in COS-7 cells, membranes preparation and binding studies were performed as described previously (Chidiac *et al.*, 1994). The specific radioligand [<sup>125</sup>I]cyanopindolol was used at saturating concentrations (300-500 pM; K<sub>d</sub> = 21 nM) and propranolol at 10 µM was added to determine non-specific binding. For binding experiments, membranes were incubated in a buffer containing 7 mM Tris-HCl, pH 7.4, 12.5 mM MgCl<sub>2</sub>, 2 mM EDTA, and proteins (5 to 20 µg) for 90 min at 20°C.

In both assays, the incubation was terminated by vacuum filtration over Whatman GF/B filters pre-soaked with 0.1% polyethylenimine using a Brendel 48 well cell harvester; the filters were then washed with 3 x 4 ml ice-cold buffer (10 mM HEPES). Filtration was performed in less than 10 seconds. Radioactivity was counted using a complete phase combining system for liquid scintillation counting (Amersham, Les Ullis, France). Protein concentration in the samples was determined using the Bio-Rad protein assay .

## Drugs

GR 113808 ([1-[2(methylsulphonyl-amino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3-carboxylate, maleate) and GR 125487 ([1-[2-(methylsulphonyl-amino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1*H*-indole-3-carboxylate, hydrochloride) were generously donated by Glaxo (U.K.); [<sup>3</sup>H]GR 113808 was purchased from Amersham (U.K.); 5-HT (serotonin) and isoproterenol were purchased from Sigma Chemical Corp., St. Louis, MO, USA. RS 100235 (1-(8-amino-7-chloro-1,4-benzodioxan-5-yl)-3-[[3,4-dimethoxyphenyl]prop-1-yl]piperidin-4-yl]propan-1-one) generously donated by Roche-Syntex Laboratories, Palo Alto, USA.

## RESULTS

### Primary structure of the different 5-HT<sub>4</sub>R splice variants:

We have published the isolation of m5-HT<sub>4(b)</sub>R, previously named long form (Claeysen *et al.*, 1996) which has also been isolated in rat and human (Gerald *et al.*, 1995; Van den Wyngaert *et al.*, 1997). We performed RT-PCR experiments with poly(A)<sup>+</sup>RNA prepared from mouse brain using primers designed to clone the variant (a) (described in the experimental procedure section). We obtained three different full length cDNA fragments. One of them corresponded to variant (a) previously cloned in rat and human (Blondel *et al.*, 1997; Claeysen *et al.*, 1997a; Gerald *et al.*, 1995), whereas two new clones generated from the same RT-PCR corresponded to new 5-HT<sub>4</sub> receptor cDNAs, coding for variants significantly shorter than the (a) and (b) forms. Blondel *et al.* have reported the existence of two unrelated h5-HT<sub>4</sub>R splice variants (c) and (d) (Blondel *et al.*, 1998) (Fig. 1). Taking into account the already published human variants, we named the present new variants m5-HT<sub>4(e)</sub>R and m5-HT<sub>4(f)</sub>R (Fig. 1). We also cloned a r5-HT<sub>4(e)</sub> receptor, identical, to the m5-HT<sub>4(e)</sub>R in length and amino acid sequence after the splicing site by performing RT-PCR experiments with poly(A)<sup>+</sup>RNA prepared from rat brain. In human, we have also isolated, from brain, a new splice variant very close but not identical in sequence to the (e) form. When comparing its nucleotidic and amino acid sequences to those of rat and mouse (e) forms, we can suggest that this new human variant produced by alternative splicing is the human orthologue of the mouse and rat (e) forms. We therefore called it h5-HT<sub>4(e)</sub>R. Whatever the species, mouse, human or rat, the different splice variants have an identical sequence up to L358, whereas the length as well as the composition of the C-terminal end, is specific for each splice variant (Fig. 1). Note the specific characteristics of the last four residues of the C-termini of the new clones described here. The r5-HT<sub>4(e)</sub>, m5-HT<sub>4(e)</sub>, (f) as well as of the h5-HT<sub>4(e)</sub> receptors contain two PV repeats.

### Novel mouse 5-HT<sub>4</sub>R splice variants are brain-specific:

Using pairs of primers, designed as described in the experimental procedure section, the expression of the different m5-HT<sub>4</sub>R transcripts (Fig. 2A) were assayed in brain and in the peripheral tissues. Note that our RT-PCRs performed either with 250 or 500 ng of poly(A<sup>+</sup>) RNA are non-saturating, ie.

the ratio between the different bands amplified remain the same whatever the quantity used (Fig. 2B). The most interesting observation was the total absence of m5-HT<sub>4(e)</sub>R and (f)R mRNA expression in peripheral tissues. In brain, we detected a high level of the long form (a) and (b) mRNAs in all of the structures studied (Fig. 2C). The two new variants (e) and (f) were expressed in colliculi, striatum and hippocampus. In the cerebellum and the cortex the (e) form was not detected. We observed a relatively high level of the m5-HT<sub>4(a)</sub>R and (b)R mRNA in heart and kidney but also in bladder, ileum and colon. Oesophagus and adrenal gland showed very low levels of signal and liver did not express detectable amount of any of the four splice variants. As for m5-HT<sub>4(e)</sub>R, (f)R, the r5-HT<sub>4(e)</sub>R and h5-HT<sub>4(e)</sub>R mRNAs have been detected only in brain (data not shown).

### **5-HT<sub>4(a)</sub> receptors have a high constitutive activity:**

Since we previously observed that native m5-HT<sub>4(a)</sub>R and h5-HT<sub>4(b)</sub>R expressed high basal constitutive activities in the absence of agonist (Claeysen *et al.*, 1997a; Claeysen *et al.*, 1997b), we decided to investigate the capacity of 5-HT<sub>4</sub> receptors to convert from R to R\* by studying their constitutive activity as a function of receptor density. In COS-7 cells, in the absence of 5-HT, a two-fold increase in cAMP production as compared with mock transfected cells, was obtained for a 5-HT<sub>4(a)</sub> receptor density (150/200 fmol/mg protein) comparable to those found in brain tissues (Waeber *et al.*, 1994) (Fig. 3A). Because comparison of receptor density is rather complex we have determined the percent of cells expressing the 5-HT<sub>4</sub> receptors. Cells were transiently transfected with the epitope-tagged receptor cDNA and visualized using the anti c-Myc primary antibody. We found that  $57 \pm 16\%$  (n=6) of the cells expressed the transfected epitope-tagged 5-HT<sub>4</sub> receptor isoforms, whatever the splice variant considered.

The constitutive activity of 5-HT<sub>4(a)</sub>R was almost linearly proportional to the receptor density and independent of the species origin of the receptor (Fig. 3A). Such a constitutive activity, was not specific to COS-7 cells since similar results were obtained in LLC-PK1 cells (Fig. 3B). In this cell lane, a two fold increase in cAMP production was observed for a receptor density of 100 fmol/mg proteins. Fig. 3A shows that native 5-HT<sub>4(a)</sub> receptors had a much higher constitutive activity than the native h $\beta$ <sub>2</sub>-AR when expressed in COS-7 cells.

Although our experiments were performed with dialyzed serum, it was important to check whether some contamination by 5-HT, known to stick to plastic surfaces was responsible for the observed intrinsic activity. We therefore used highly potent selective 5-HT<sub>4</sub>R antagonists, some (like RS 100235) were neutral antagonists and had no effect on basal 5-HT<sub>4(a)</sub> receptor-induced constitutive activity (Fig. 4A). However other antagonists like GR 125487, behaved as inverse agonists because they reduced this basal constitutive activity (Costa and Hertz, 1989). In addition, both antagonists used in Fig. 4A were highly potent in inhibiting the 5-HT-induced cAMP production in transfected COS-7 cells expressing 5-HT<sub>4(a)</sub> receptors (Fig. 4B). These experiments indicated that the observed constitutive activity was not due to 5-HT contamination.

#### **Mouse 5-HT<sub>4</sub> receptor splice variants exhibit different constitutive activities:**

It was of particular interest to check if the difference in the structure of the C-terminus of the different m5-HT<sub>4</sub> receptor splice variants influence their propensity to induce high basal constitutive activity. We actually found that although the (a) and (b) forms gave a similar constitutive activity when studied as the function of receptor density, the shortest forms (e) and (f) presented a two times higher basal constitutive activity (Fig. 5). In comparison, in this experiment, hβ<sub>2</sub>-AR do not modify cAMP production when expressed at this density (Fig. 5)

#### **A C-terminal domain of 5-HT<sub>4</sub> receptors inhibits constitutive activity:**

Since we found that the shortest splice variants had the highest constitutive activity (Fig. 5) we wondered if the C-terminal domain was important for constraining 5-HT<sub>4</sub> receptors in an inactive state. This turned out to be true since progressive deletions of the C-terminal part of the 5-HT<sub>4(a)</sub> receptor (Fig. 6A) resulted in a progressive increase in constitutive activity for a similar density of wild type and truncated receptors (Fig. 6B). The truncated 5-HT<sub>4</sub>Δ359 receptor identical in length and sequence to the 5-HT<sub>4(f)</sub>R variant without its PVPV sequence, represents the common region of the four splice variants. This truncated receptor displayed a similar constitutive activity to that of 5-HT<sub>4(f)</sub>R, whereas a larger deletion mutant (deletion of 13 residues: 5-HT<sub>4</sub>Δ346 receptor) dramatically activated the receptor. A larger deletion of the C-terminal (Δ327) did not further increase the

constitutive activity compared to  $\Delta 346$ . Both deletions ( $\Delta 346$  and  $\Delta 327$ ) induced a 5-fold activation of basal cAMP production for receptor densities below 200 fmol/mg protein.

### **The C-terminal part of the third intracellular loop (i3) constrains the 5-HT<sub>4</sub> receptor in its inactive form:**

A comparison between the sequences of the C-terminal part of i3 of m5-HT<sub>4</sub> receptors on one hand and of the h $\beta$ <sub>2</sub>-AR on the other hand indicated that among the last 7 residues, 4 of them were identical. One of them, alanine in 5-HT<sub>4</sub> receptor, leucine in  $\beta$ <sub>2</sub>-AR at position -3 upstream of TM VI, was interesting because the mutation of leucine to alanine was one of the four mutations in i3 of  $\beta$ <sub>2</sub>-AR that produced the CAM h $\beta$ <sub>2</sub>-AR (Samama *et al.*, 1993) (Fig. 7A). It is also this mutation (leucine to alanine) which is the most potent mutation in inducing the constitutive activity of CAM h $\beta$ <sub>2</sub>-AR (S. Cotecchia, personal communication). Therefore, one possible explanation for the highest constitutive activity of native 5-HT<sub>4</sub> receptors as compared to native  $\beta$ <sub>2</sub>-AR, was the presence, at this homologous position, of an alanine (A258) in the wild type 5-HT<sub>4</sub> receptor (Fig. 7A) instead of a leucine in  $\beta$ <sub>2</sub>-AR. We therefore mutated this A258 to L in the 5-HT<sub>4(e)</sub> receptor. Instead of reducing the constitutive activity of the receptor we obtained a dramatic increase (Fig. 7B). For comparison we note that the constitutive activity of the native 5-HT<sub>4(e)</sub> receptors was as high as the constitutive activity of CAM h $\beta$ <sub>2</sub>-AR .

### **Basal and 5-HT stimulated cAMP production in cells transfected with 5-HT<sub>4(a)</sub>, (e) receptors and 5-HT<sub>4</sub> mutated receptors:**

The short C-terminal sequence 5-HT<sub>4(e)</sub> receptors had a higher basal constitutive activity than the long C-terminal terminal sequence 5-HT<sub>4(a)</sub> receptors as already described in fig.5. However, they had identical activity in the presence of 5-HT (Fig.8A). In this experiment, we expressed about 1000 fmol/mg protein of 5-HT<sub>4(a)</sub> and (e) receptors. The identical 5-HT-stimulated activity observed with these two 5-HT<sub>4</sub> variants was not due to a limiting step in cAMP formation since increasing the number of expressed receptors from 1000 to 4000 fmol/mg protein increased the 5-HT stimulated activity proportionally (data not shown). Fig. 8B illustrates the relative efficacy of 5-HT to stimulate over the basal constitutive activity of the wild types and the mutated receptors. The higher the basal



constitutive activity, the lower the relative efficacy of 5-HT. Note that the 5-HT-maximum response was slightly lower in severely truncated receptors (Fig.8 A). A decrease in the mutated receptor stability during incubation or an increase in desensitization can be proposed as an explanation (Gether *et al.*, 1997; Pei *et al.*, 1994).

**Substitution of A258 by L within the third intracellular loop i3 and C-terminal truncations ( $\Delta 346$  and  $\Delta 327$ ) of m5-HT<sub>4(e)</sub> receptors increase the affinity of 5-HT and its potency in stimulating cAMP:**

The extended ternary complex (Samama *et al.*, 1993) as well as the cubic ternary complex (Kenakin and Weiss, 1996) introduced for the modeling of the G protein receptor isomerization R to R\* and their interaction with G proteins, indicated that the observed affinity of agonist increased as the function of the isomerization constant J. Indeed, we observed that the affinity of 5-HT for both the mutated 5-HT<sub>4(e)</sub>R A258L and C-terminal deleted ( $\Delta 346$  and  $\Delta 327$ ) 5-HT<sub>4</sub> receptors expressed at similar densities, increased by almost two logs<sub>10</sub> when compared with that of the native receptor. 5-HT-binding was measured by competition. The K<sub>d</sub> values for 5-HT were  $47 \pm 12$  nM for the wild type 5-HT<sub>4(e)</sub>R,  $0.53 \pm 0.6$  nM and  $1.34 \pm 0.15$  nM for the truncated 5-HT<sub>4</sub>R  $\Delta 346$  and 5-HT<sub>4</sub>R  $\Delta 327$  respectively and  $0.85 \pm 0.10$  nM for the mutated 5-HT<sub>4(e)</sub>R A258L (Fig. 9A). The difference in affinity observed was certainly not due to a difference in the coupling to G proteins (different proportion of the high affinity state), since a high GppNHp concentration (10  $\mu$ M) did not modify this difference (data not shown). In contrast to these results, we found no difference between the affinity of 5-HT for the different natural splice variants. The EC<sub>50</sub> of 5-HT to stimulate cAMP were about 10 times lower when determined in mutant receptor transfected cells rather than in native receptors transfected cells (Fig. 9B) The EC<sub>50</sub> values were  $8 \pm 2$  nM,  $0.3 \pm 0.10$  nM,  $0.7 \pm 0.10$  nM and  $0.9 \pm 0.15$  nM for 5-HT<sub>4(e)</sub>R, 5-HT<sub>4</sub>R  $\Delta 346$ , 5-HT<sub>4</sub>R  $\Delta 327$  and 5-HT<sub>4(e)</sub>R A258L, respectively.

## DISCUSSION

We report the cloning of new 5-HT<sub>4</sub>R splice variants (m5-HT<sub>4(e)</sub>R and (f)R; r5-HT<sub>4(e)</sub>R and h5-HT<sub>4(e)</sub>R) which possess original C-terminal domains compared to already cloned 5-HT<sub>4</sub>R splice variants (Blondel *et al.*, 1998; Blondel *et al.*, 1997; Claeysen *et al.*, 1997a; Claeysen *et al.*, 1996; Gerald *et al.*, 1995; Van den Wyngaert *et al.*, 1997). They have in common an intriguing and unique PVPV C-terminal sequence that is also found in the C-termini of some EPH/ELK family of receptor tyrosine kinases (Wicks *et al.*, 1992). We can only speculate on the possible roles for this sequence but one could be the interaction of these splice variants with proteins similar to PDZ-domain-containing proteins. Because of the roles of 5-HT<sub>4</sub> receptors in learning and memory (Ansanay *et al.*, 1995; Eglen *et al.*, 1995; Letty *et al.*, 1997; Marchetti-Gauthier *et al.*, 1997) it is interesting to note that the newly cloned PVPV-containing receptors are only expressed in brain tissues.

In view of the large number of 5-HT<sub>4</sub> receptor variants only differing in their C-termini domains it was of particular interest to analyze the role of these domains in G protein coupling. Over the past ten years our view on how GPCRs are activated has been re-assessed based on the pioneering works of Costa and Lefkowitz's laboratories (Costa and Hertz, 1989; Cotecchia *et al.*, 1990). They demonstrated that GPCRs can isomerize from an inactive form R to an active form R\* in the absence of agonist. GPCRs are no longer considered as shifting from an inactive to an active conformation when interacting with the agonist but it is now proposed that the agonist is only stabilizing the R\* form. Thus, GPCRs can be considered as allosteric molecules which already includes ionotropic receptors such as nicotinic receptors (Changeux and Edelstein, 1998; Monod *et al.*, 1965). Among the numerous theoretical consequences of this view is the idea that the structure of the receptor has been selected to constrain the receptor under the R form in the absence of agonist. The higher the constraint (low isomerization J constant), the higher the signal to noise ratio in the presence of agonist. The evaluation of the capacity of a native receptor to spontaneously isomerize from R to R\* is certainly a very important pharmacological characteristic, because this may explain part of its physiological and possible pathological behavior as well as the effect of drugs classified as inverse agonists (able to reverse the isomerization because of their higher affinity for R than for R\*) on this receptor.

Native 5-HT<sub>4(a)</sub> receptors of different species (mouse, rat, human) have a much higher capacity to convert from R to R\* than most of the other GPCRs studied so far. Indeed, when expressed at low density (100 to 200 fmol/mg), 5-HT<sub>4(a)</sub> receptors produced a two-fold increase in the cAMP production compared to mock transfected cells. In comparison, hβ<sub>2</sub>-AR do not modify cAMP production when expressed at this density (Fig. 3 and (Samama *et al.*, 1993)). Indeed in COS-7 cells, less than a two fold increase in cAMP production, was obtained when native β<sub>2</sub>-AR were expressed at a density of 20 pmol/mg protein (Samama *et al.*, 1993). Increasing native 5-HT<sub>4(a)</sub> receptor density to 400 fmol/mg protein was sufficient to increase cAMP production by four fold as compared to mock transfected cells. This high capacity to isomerize was not restricted to expression of the receptor in COS-7 cells, since in LLC-PK1 cells, when the receptor density reached a density as low as 150 fmol/mg protein, the cAMP produced was 3 times that found in mock transfected cells.

The C-terminal domains of the intracellular i3 loop of 5-HT<sub>4</sub>R and hβ<sub>2</sub>-AR are remarkably similar. Upstream of the TM VI, 4 of the last 7 residues are identical. One of the divergent position is an alanine residue in 5-HT<sub>4</sub> receptor which is a leucine in hβ<sub>2</sub>-AR (localized at residue -3 upstream of TM VI). This position is critical for keeping GPCR in an inactive form (Kjelsberg *et al.*, 1992; Samama *et al.*, 1993). Mutation of this position in α<sub>1B</sub>-AR (Kjelsberg *et al.*, 1992) and TSHRs (Parma *et al.*, 1993) increases their constitutive activity. In CAM β<sub>2</sub>-AR, in which four residues of the C-terminal part of i3 were mutated (Samama *et al.*, 1993), this position is crucial for constitutive activation (S. Cotecchia, personal communication). Since native 5-HT<sub>4</sub> receptors have a much higher constitutive activity than native hβ<sub>2</sub>-AR and have, as CAM hβ<sub>2</sub>-AR, an alanine at this position we mutated the alanine of 5-HT<sub>4</sub> receptor to leucine to see if the constitutive activity would drop to the level of native hβ<sub>2</sub>-AR. This was not the case and, in contrast, a further and dramatic increase in 5-HT<sub>4</sub> receptor constitutive activity was observed. However, this further indicates that the C-terminal part of the i3 loop is also crucial to keep 5-HT<sub>4</sub> receptor in the R inactive state and that the native primary sequence is the one which probably gives the conformation having the lowest constitutive activity.

The highest basal constitutive activity of the short C-terminal sequence splice variant (5-HT<sub>4(e)</sub>R, (f)R) compared to the long C-terminal sequence variants (5-HT<sub>4(a)</sub>R, (b)R) can be explained by either one of the following hypotheses. In the first one, the shortest splice variants have a greater ability to

isomerize from R to R\* (and an equal efficacy of their R\* form to stimulate G proteins). In the second one, the efficacy of their R\* forms to stimulate G proteins are different and their ability to isomerize are identical. It is difficult to completely exclude the second hypothesis. However, the observation that the 5-HT induced maximum responses were identical whatever the splice variant considered argue in favor of a higher capacity of short C-terminal sequence splice variants to isomerize from R to R\*.

One important issue is to know whether such a constitutive activity is present or not in neurons or other peripheral tissues (atria, colon, ileum etc..) in which 5-HT<sub>4</sub> receptors are expressed at comparable density (Eglen *et al.*, 1995; Waeber *et al.*, 1994). Comparison of receptor density is rather complex because even though around 57% of the cells expressed the transfected tagged 5-HT<sub>4</sub> receptors (whatever the splice variant considered), the quantity of cells expressing this receptor in heterogeneous tissues such as brain for example, remains unknown. In addition, the distribution of 5-HT<sub>4</sub> receptors in COS-7 cell and neuronal membranes are completely different. In preliminary experiments, we did not find a reduction of basal cAMP production in primary colliculi neurons expressing 5-HT<sub>4</sub> receptors using the inverse agonist GR 125487 (data not shown). Since the isomerization constant depends on environmental conditions, it is also possible that proteins that interact or chemically modify the C-termini (one may think of a phosphorylation of the serine-threonine rich domain upstream of the splicing site) reduce the J constant in neurons. The search for such putative interacting proteins will certainly be of interest.

There are increasing data to indicate that specific sequences of the C-terminal end of GPCRs modulate the isomerization of these receptors from R to R\*. A cluster of basic residues present in the C-terminal tail of the short variants of mGluRs (mGluR1b, c, d), impairs their ability to isomerize from R to R\*. Removing this cluster by mutation reveals their constitutive activity (Mary *et al.*, 1998). In addition, the effect of this inhibitory cluster is suppressed by a long C-terminal domain of mGluR1a which, in fact, has a high constitutive activity (Mary *et al.*, 1998). Truncation of the last residues of TRHR and prostaglandin E receptors causes constitutive activity (Hasegawa *et al.*, 1996; Matus-Leibovitch *et al.*, 1995). The last 12 residues of bovine rhodopsin have also been proposed to be involved as negative regulator of GTP exchange (Weiss *et al.*, 1994). Removal of the extended C-terminal domain of avian  $\beta_2$ -AR increases its activity (Parker and Ross, 1991).

We found that truncation of the C-terminal domain consisting in the removal of the sequences that are divergent in individual 5-HT<sub>4</sub> receptor splice variants ( $\Delta 359$ ) gave a constitutive activity similar to those of the shortest splice variants (5-HT<sub>4(e)</sub>R and <sub>(f)</sub>R). Since the 5-HT<sub>4(a)</sub>  $\Delta 359$  receptor is identical to the 5-HT<sub>4(f)</sub> receptor with its PVPV sequence removed, the PVPV sequence is not responsible for intrinsic activity of the shortest splice variants (Fig. 6). In contrast truncation of the last 13 residues (12 of which are common in C-terminal tails of all of them) ( $\Delta 346$ ), resulted in a dramatic release on the receptor constraint, the basal activity increased 10-fold when as low as 500 fmol/mg protein of receptors are expressed. A further truncation ( $\Delta 327$ ) did not exacerbate this constitutive activity. This indicates that within the 13 residues comprised between  $\Delta 346$  and  $\Delta 359$ , one set of residues is highly potent in maintaining the receptor under the R form. Interestingly, this sequence contains 6 serine and threonine residues susceptible to be phosphorylated, a post translational modification which may modulate the receptor intrinsic activity. Removal of this inhibitory sequence rendered the receptor so active that it became less sensitive to 5-HT.

In conclusion we can propose a model in which (Fig. 10): (1) the 5-HT<sub>4</sub> receptor protein, up to residue 346, is mainly a constitutive active receptor coupled to Gs, (2) the last common sequence in the C-terminal domain (around 13 residues between 346 and 359) is mainly engaged in the constraint of the receptor under its inactive form 3) the specific sequences of 5-HT<sub>4(e)</sub> and <sub>(f)</sub> receptors are neutral in the modulation of the isomerization because their constitutive activity did not differ from that of  $\Delta 359$ , (4) the specific sequences of the 5-HT<sub>4(a)</sub> and <sub>(b)</sub> receptors reduce the isomerization either by reducing the inhibitory effect of the C-terminal common sequence or by a direct negative effect on the isomerization.

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## LEGENDS TO FIGURES

### **Fig. 1: C-terminal amino acid sequences of the 5-HT<sub>4</sub> receptor splice variants.**

All the cloned 5-HT<sub>4</sub> receptors share the same amino acid sequence up to Leu 358 and differ from Leu 358 on (boxed). †: receptors cloned by Gerald *et al.* (Gerald *et al.*, 1995). \*: receptors cloned by our group (Claeysen *et al.*, 1997a; Claeysen *et al.*, 1996). ‡: receptors previously cloned by Blondel *et al.* (Blondel *et al.*, 1998). §: receptors cloned by Van den Wyngaert *et al.* (Van den Wyngaert *et al.*, 1997). Note that the amino acid sequence after Leu 358 of newly cloned m5-HT<sub>4(e)</sub> and (f), r5-HT<sub>4(e)</sub> and h5-HT<sub>4(e)</sub> receptors are shaded.

The EMBL Nucleotide Sequence Database accession numbers for mouse 5-HT<sub>4(a)</sub>, (b), (e) and (f) receptors are Y09587, Y09585, Y09588 and AJ011369 respectively. For rat 5-HT (a), (b) and (e) receptors the numbers are: U20906, U20907 and AJ011370 respectively and for human 5-HT<sub>4(a)</sub>, (b), (c), (d) and (e) receptors Y09586, Y10437, Y12506, Y12507 and AJ011371 respectively.

### **Fig. 2: Regional distribution of the four mouse 5-HT<sub>4</sub> receptor splice variants.**

**A)** Theoretical PCR results. Lane 1: primer couple ON7/ON2 (see Experimental Procedures) amplifying the mouse (a), (e) and (f) forms. Lane 2: primer couple ON7/ON8 (see Experimental Procedures) amplifying the mouse (b) form.

**B)** Distribution of mouse 5-HT<sub>4</sub>R transcripts. RT-PCR analysis performed with 250 and 500 ng of poly(A<sup>+</sup>)RNA from colliculus (collic.) and cortex of adult Swiss mice using primer couple ON7/ON2 (Lane 1) or primer couple ON7/ON8 (Lane 2) (see Experimental Procedures). 35 µl of a 50 µl reaction were loaded and separated on 3% agarose gels. A photograph of the ethidium bromide stained gels is shown.

**C)** Distribution of mouse 5-HT<sub>4</sub>R transcripts. RT-PCR analysis performed with 500 ng of poly(A<sup>+</sup>)RNA from various structures of adult Swiss mice using primer couple ON7/ON2 (Lane 1) or primer couple ON7/ON8 (Lane 2) (see Experimental Procedures). Upper panel: PCR products were separated on 3% agarose gels, 25 µl of a 50 µl reaction were loaded. A photograph of the ethidium bromide stained gels is shown. Lower panel: southern blot of the PCR results, a 2h exposure

of the autoradiogram is shown. Collic., colliculus; Hippoc., hippocampus; Striat., striatum; Cereb., cerebellum; Ventri., ventricle; Oesoph., oesophagus; Adr.gl., adrenal gland.

**Fig. 3: Basal levels of cAMP in COS-7 and LLC-PK1 cells transiently expressing increasing concentrations of human, rat and mouse 5-HT<sub>4(a)</sub> receptor splice variants.**

**A)** COS-7 cells were transfected with increasing concentrations of human, rat or mouse 5-HT<sub>4(a)</sub>R and h $\beta$ <sub>2</sub>-AR receptor cDNA (25 ng to 1000 ng cDNA/10<sup>7</sup> cells). The total amount of DNA was kept constant at 15 $\mu$ g/10<sup>7</sup> cells using wild type pRK5 vector. The percent conversions of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mocked transfected cells was 0.12  $\pm$  0.03 (control). Basal levels of cAMP productions at different receptor concentrations, measured after 30 min-incubation, are expressed as a percent of control.

**B)** LLC-PK1 cells were transfected with increasing concentrations of h5-HT<sub>4(a)</sub> receptor cDNA/10<sup>6</sup> cells (25 ng to 1000 ng cDNA) or with equivalent concentrations of pRK5 plasmid (mock-transfected cells). The percent conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mock-transfected cells was 0.115  $\pm$  0.021 (control). Basal levels of cAMP production, measured at different receptor concentrations after 30 min-incubation, are expressed as a percent of control.

In A and B, 5-HT<sub>4</sub> receptor densities were determined by using a saturating concentration of [<sup>3</sup>H]GR 113808 (0.4-0.5 nM), h $\beta$ <sub>2</sub>-AR densities were determined by using a saturating concentration of [<sup>3</sup>I]CYP (0.5 nM). Each point is the means  $\pm$  SEM of three independent experiments.

**Fig. 4: Effect of two potent 5-HT<sub>4</sub>R antagonists: RS 100235 and GR 125487 on basal and 5-HT-stimulated 5-HT<sub>4(a)</sub> receptor.**

The effects of RS 100235 and GR 125487 on cAMP production, in the absence (A) and presence (B) of 5-HT (10<sup>-7</sup>M) were evaluated on h5-HT<sub>4(a)</sub> receptor splice variant transiently transfected in COS-7 cells expressing 320  $\pm$  45 fmol/mg protein. Levels of cAMP accumulation were measured after 15 min-incubation and expressed as a percent of control. The percentage conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mock-transfected cells was 0.14  $\pm$  0.018 (control). Each value is the means  $\pm$  SEM of three independent experiments each performed in triplicate.



**Fig. 5: Mouse 5-HT<sub>4</sub> receptor splice variants exhibit different basal activities.**

The activity was measured by increase in cAMP production in COS-7 cells, expressed m5-HT<sub>4(a)</sub>, (b), (e), (f) receptor or hβ<sub>2</sub>-A receptor as a function of receptor density.

COS-7 cells were transfected with increasing concentrations of m5-HT<sub>4(a)</sub>, (b), (e), (f) and hβ<sub>2</sub>-A receptors cDNA (10 ng to 1000 ng cDNA/10<sup>7</sup> cells). Basal levels of cAMP accumulation were measured after 30 min-incubation. The percent conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mocked transfected cells was 0.12 ± 0.03 (control). Basal cAMP production measured at different concentrations of receptor is expressed as a percent of control. 5-HT<sub>4</sub> and hβ<sub>2</sub>-A receptors densities were determined as indicated in Fig. 3. Data for each of the splice variant represent the means ± SEM for at least three independent experiments each performed in triplicate.

**Fig. 6: A common sequence in the C-terminal domain of 5-HT<sub>4</sub> receptor temporizes its basal activity.**

**A)** Schematic representation of the C-terminal tail of the m5-HT<sub>4(a)</sub> and (f) receptor splice variants. The arrows indicate the sites substituted by a stop codon to produce the truncated receptors (5-HT<sub>4</sub>R Δ359, 5-HT<sub>4</sub>R Δ346 and 5-HT<sub>4</sub>R Δ327). Serine-threonine clusters are shaded.

**B)** COS-7 cells were transfected with increasing concentrations of m5-HT<sub>4(a)</sub>R, m5-HT<sub>4(f)</sub>R, m5-HT<sub>4</sub>R Δ359, m5-HT<sub>4</sub>R Δ346 or m5-HT<sub>4</sub>R Δ327 receptor cDNA/10<sup>7</sup> cells (10 ng to 2000 ng cDNA). The percent conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mocked transfected cells was 0.15 ± 0.03 (control). cAMP productions measured at different concentrations of receptor are expressed as a percent of control. Each point is the means ± SEM of three independent experiments each performed in triplicate.

**Fig. 7: Mutations in the C-terminal part of the i3 loop of 5-HT<sub>4(e)</sub> and β<sub>2</sub>-A receptors increase constitutive activity.**

Relationship between receptor density and basal levels of cAMP production measured in COS-7 cells transfected with m5-HT<sub>4(e)</sub> or m5-HT<sub>4(e)</sub> A258L or hβ<sub>2</sub> or CAM hβ<sub>2</sub> receptors.

**A)** Amino acid sequences of the C-termini of the intracellular loop i3 in m5-HT<sub>4</sub>R and hβ<sub>2</sub>-AR, mutated residues in m5-HT<sub>4(e)</sub>R A258L and CAM hβ<sub>2</sub>-AR. The mutated residues are shaded.

**B)** Different receptor densities were obtained by transfecting COS-7 cells by electroporation with varying amounts of cDNA (10 ng to 2000 ng/10<sup>7</sup> cells). The total amount of DNA was kept constant at 15 µg/transfection using wild type pRK5 vector. 5-HT<sub>4</sub> receptor densities were determined by using a saturating concentration of [<sup>3</sup>H]GR 113808 (0.4-0.5 nM), hβ<sub>2</sub>-AR and CAM hβ<sub>2</sub>-AR density was determined by using a saturating concentration of [<sup>3</sup>I]CYP (0.5 nM). The percent conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mocked transfected cells was 0.14 ± 0.03 (control).

**Fig. 8: Mutations in 5-HT<sub>4</sub> receptor decrease responsiveness to an agonist proportionally to increasing constitutive activity.**

**A)** COS-7 cells were incubated for 15 min at 37°C with vehicle (basal) or with 10<sup>-6</sup>M 5-HT for cells expressing wild types or mutated 5-HT<sub>4</sub>R and with vehicle (basal) or with 10<sup>-6</sup>M isoproterenol for those expressing wild type or mutated hβ<sub>2</sub>-AR. The expression levels of 5-HT<sub>4(a)</sub>, 5-HT<sub>4(e)</sub>, 5-HT<sub>4(e)</sub>A258L, 5-HT<sub>4</sub>Δ346, 5-HT<sub>4</sub>Δ327, hβ<sub>2</sub>-A and CAM hβ<sub>2</sub>-A receptors were 940 ± 56, 1060 ± 126, 1180 ± 170, 1090 ± 165, 827 ± 89, 860 ± 35 and 980 ± 105, fmol/ mg protein, respectively. Percent conversion of [<sup>3</sup>H]-ATP to [<sup>3</sup>H]-cAMP in mocked transfected cells was 0.12 ± 0.03 (control). Results are expressed as the percent of control. Data are means ± SEM of four experiments. **B)** Levels of agonist-stimulated cAMP production in the experiment reported in (A) are expressed as the percent over basal cAMP formation.

**Fig. 9: Competition binding and 5-HT-induced cAMP formation in COS-7 cells expressing wild-type or mutated 5-HT<sub>4</sub> receptors.**

**A)** Competition of 5-HT for [<sup>3</sup>H]GR 113808 binding on membranes derived from COS-7 cells expressing comparable receptor densities (between 827 and 1180 fmol/mg protein as in experiments reported in Fig. 8) of m5-HT<sub>4(e)</sub>R, m5-HT<sub>4</sub>R Δ346, m5-HT<sub>4</sub>R Δ327 or m5-HT<sub>4(e)</sub>R A258L. The results expressed as a percent of the specific binding in the absence of a competing agent are from a representative experiment of three to six independent experiments.

**B)** Intracellular cAMP levels were measured at increasing concentrations of 5-HT in the same transfected COS-7 cells as for binding experiments and in experiments reported Fig. 8. The 100%

maximal increase above basal differs between receptors. The basal and 5-HT maximal adenylyl cyclase stimulation values expressed in percent of control, are provided in Fig. 8. The results are expressed as a percent of stimulation and are representative of four independent experiments performed in triplicate.

**Fig. 10: Proposed mechanism for modulation of the constitutive activation of 5-HT<sub>4</sub> receptor splice variants by common and specific carboxyl-terminal domains.**

The 5-HT<sub>4</sub> receptor up to residue 346 which is common to all the four 5-HT<sub>4</sub>R splice variants is mainly a constitutive receptor coupled to Gs. The common sequence in the C-terminal domain between residues 346 and 359 imposes conformational constraints maintaining the receptor under its inactive form. The specific sequences of the (a) and (b) forms reduce its constitutive activation to an even greater extent, either by increasing the inhibitory effect of the C-terminal common sequence or by a direct negative effect on the isomerization. The specific sequences of (e) and (f) splice variants which share the same PVPV C-terminal sequence do not add supplementary constraint to that imposed by the amino acid sequence 346 to 359.

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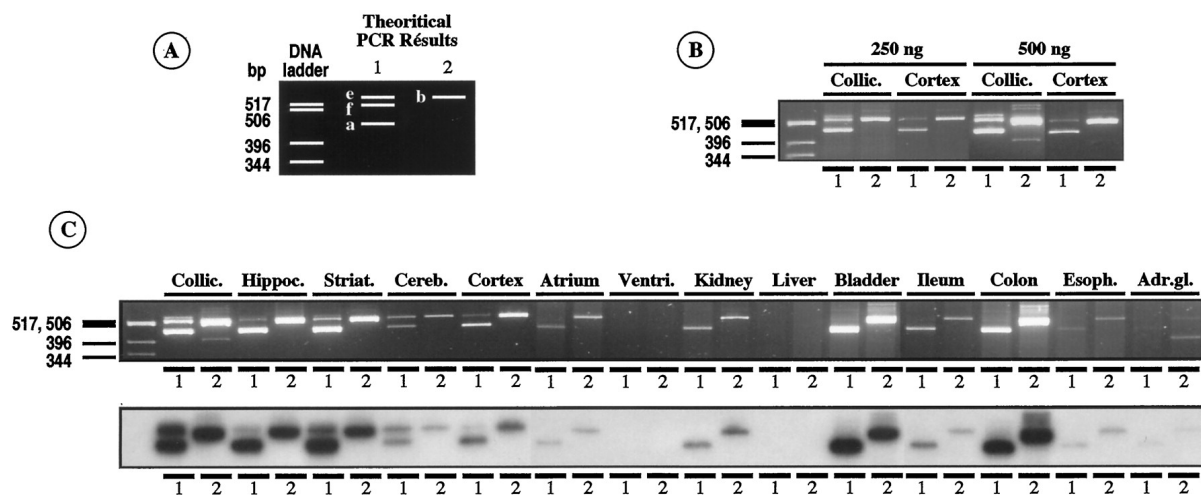
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# C-termini of 5-HT<sub>4</sub>R splice variants

		<b>358</b>		
<b>m5-HT<sub>4</sub>R</b>	(a)	LR YTVLHSGHHQELEKLPIHNDPESLESCF	<b>387</b>	<b>*</b>
	(b)	LRDAVECGGQWESRCHLTATSPLVAAQPSDT	<b>388</b>	<b>*</b>
	(e)	LSFPLLFRNRVPVPV	<b>371</b>	<b>*</b>
	(f)	LRPVPV	<b>363</b>	<b>*</b>
<b>r5-HT<sub>4</sub>R</b>	(a)	LR YTVLHSGQHQELEKLPIHNDPESLESCF	<b>387</b>	<b>†</b>
	(b)	LRDTVECGGQWESRCHLTATSPLVAAQPSDT	<b>388</b>	<b>†</b>
	(e)	LSFPLLFCNRVPVPV	<b>371</b>	<b>*</b>
<b>h5-HT<sub>4</sub>R</b>	(a)	LR YTVLHRGHHQELEKLPIHNDPESLESCF	<b>387</b>	<b>*</b>
	(b)	LRDAVECGGQWESQCHPPATSPLVAAQPSDT	<b>388</b>	<b>§</b>
	(c)	LSSGTETDRRNFGIRKRRLTKPS	<b>380</b>	<b>‡</b>
	(d)	LRF	<b>360</b>	<b>‡</b>
	(e)	LSGCSPVSSFLLFCNRVPVPV	<b>378</b>	<b>*</b>

Figure 1





**Figure 2**

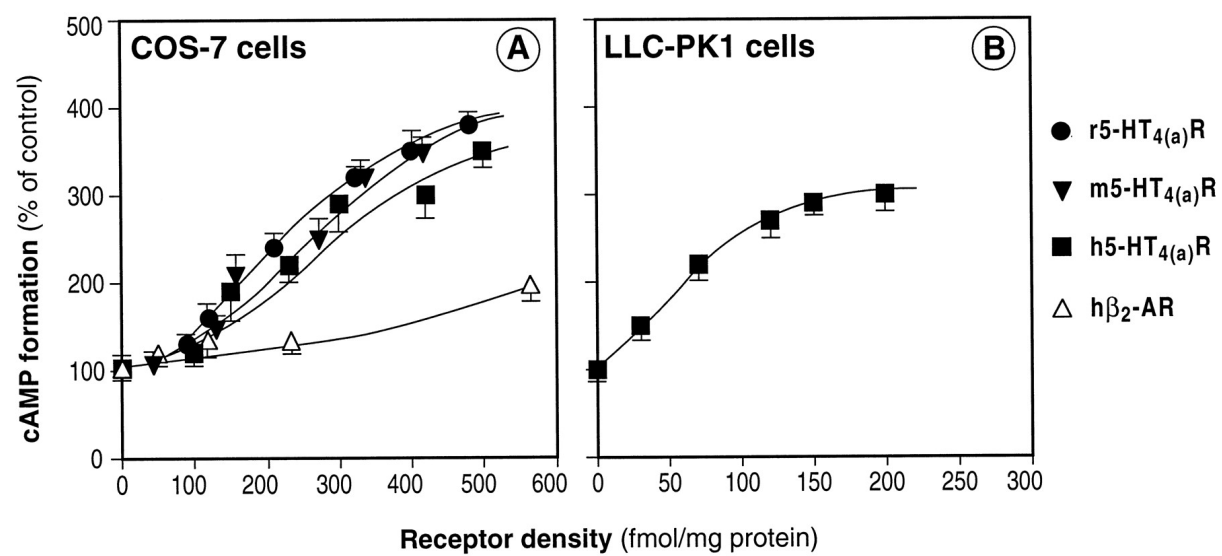


Figure 3

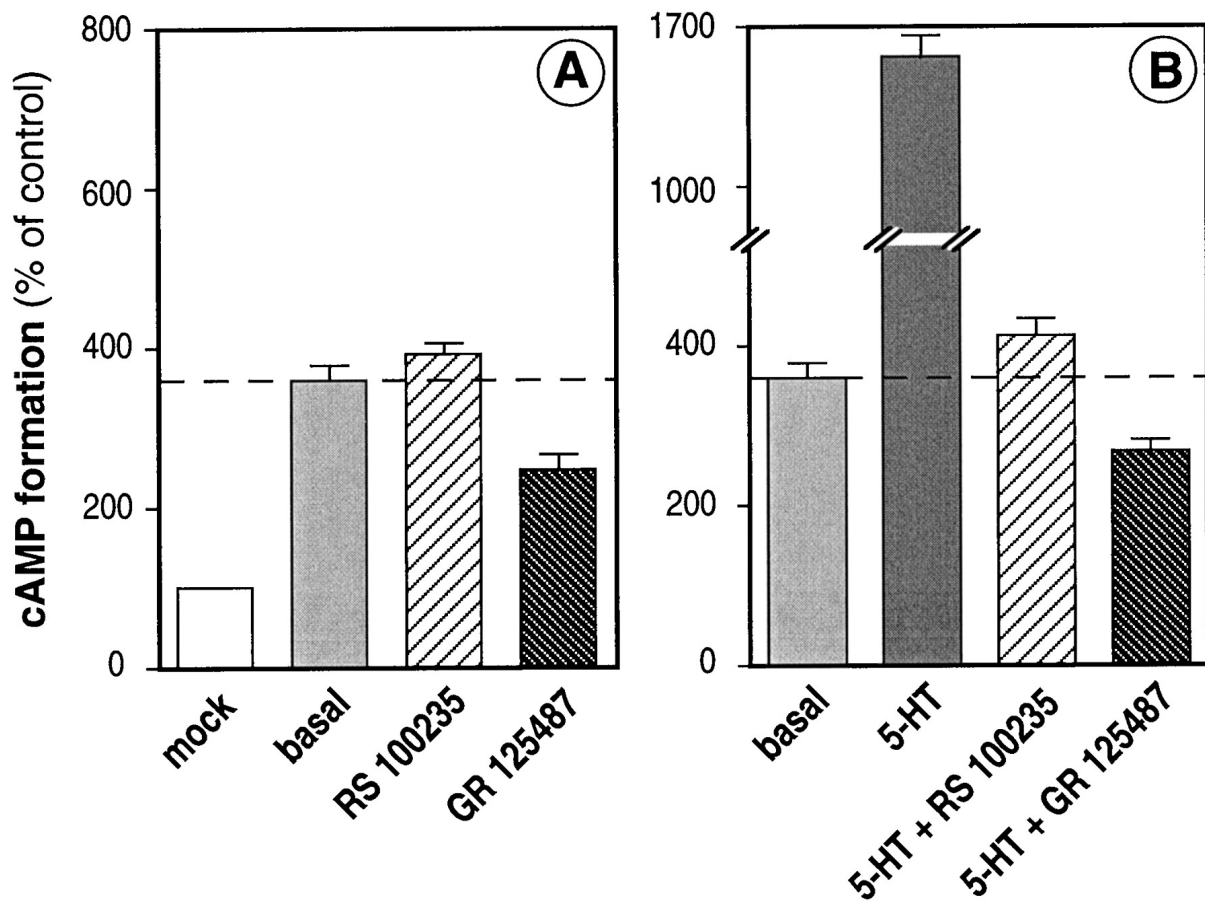
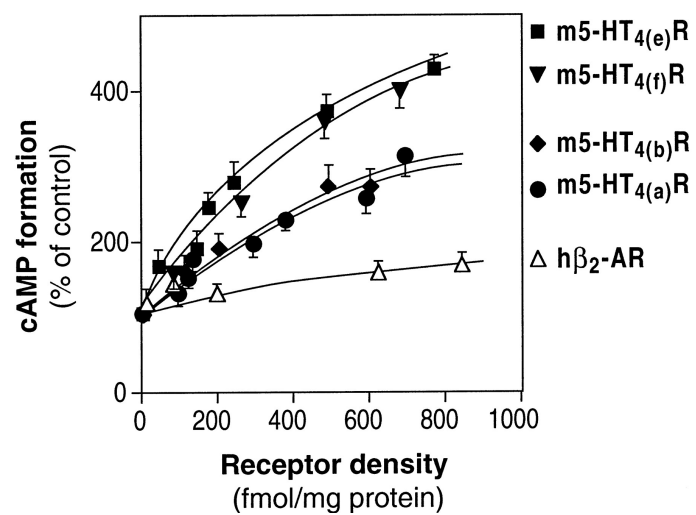
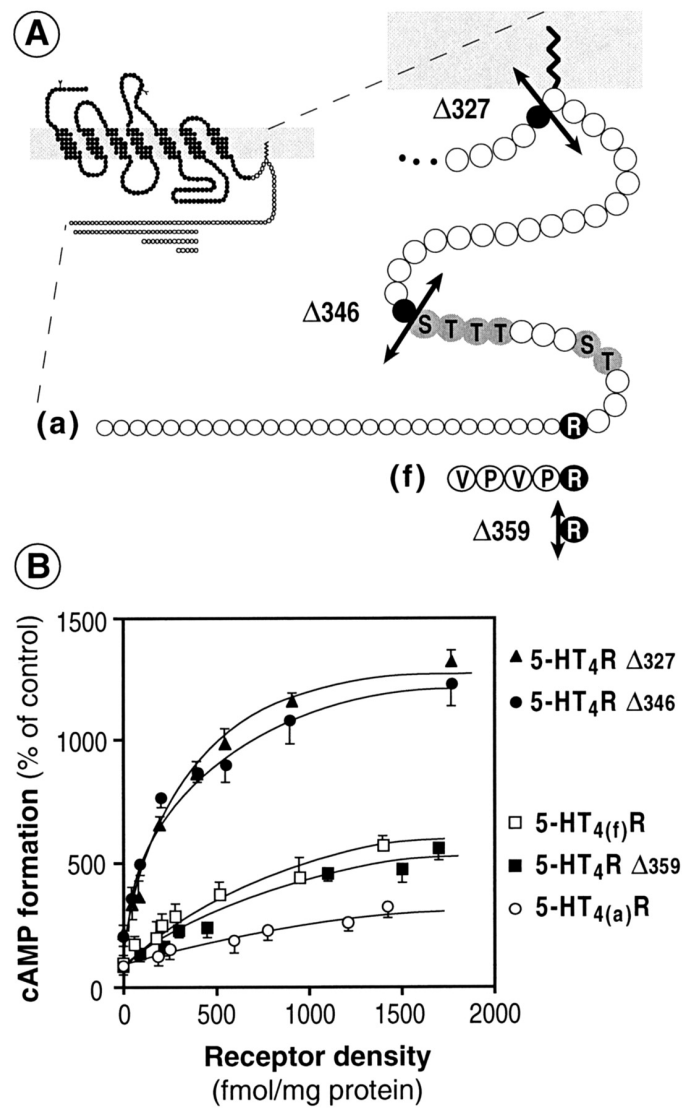


Figure 4



**Figure 5**

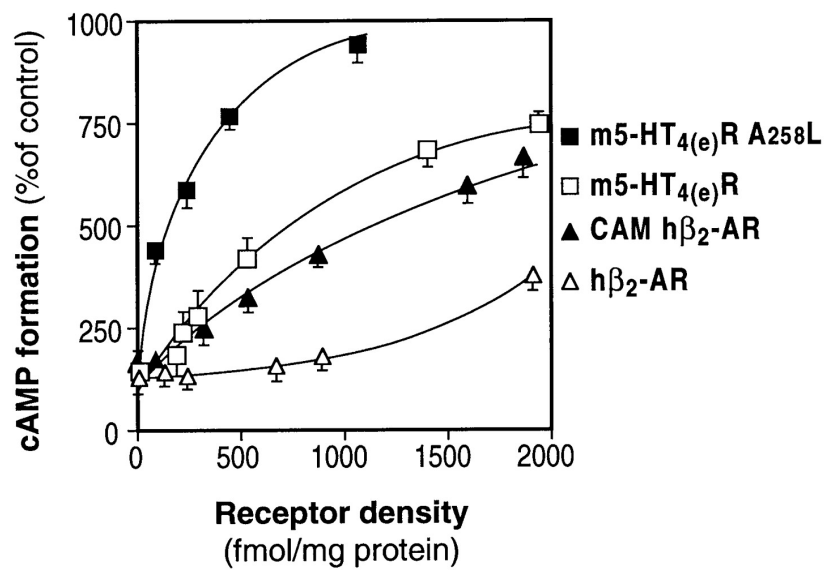


**Figure 6**

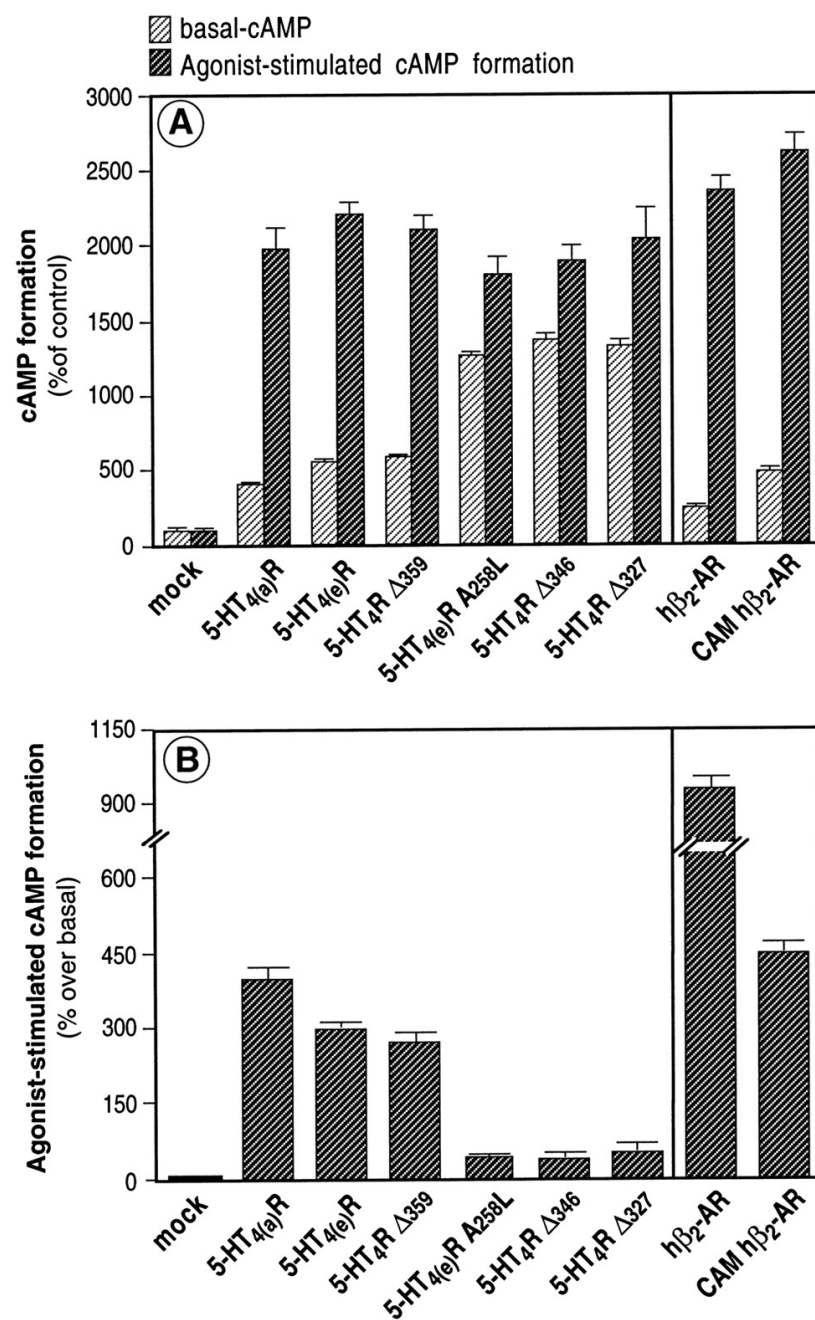
**A**

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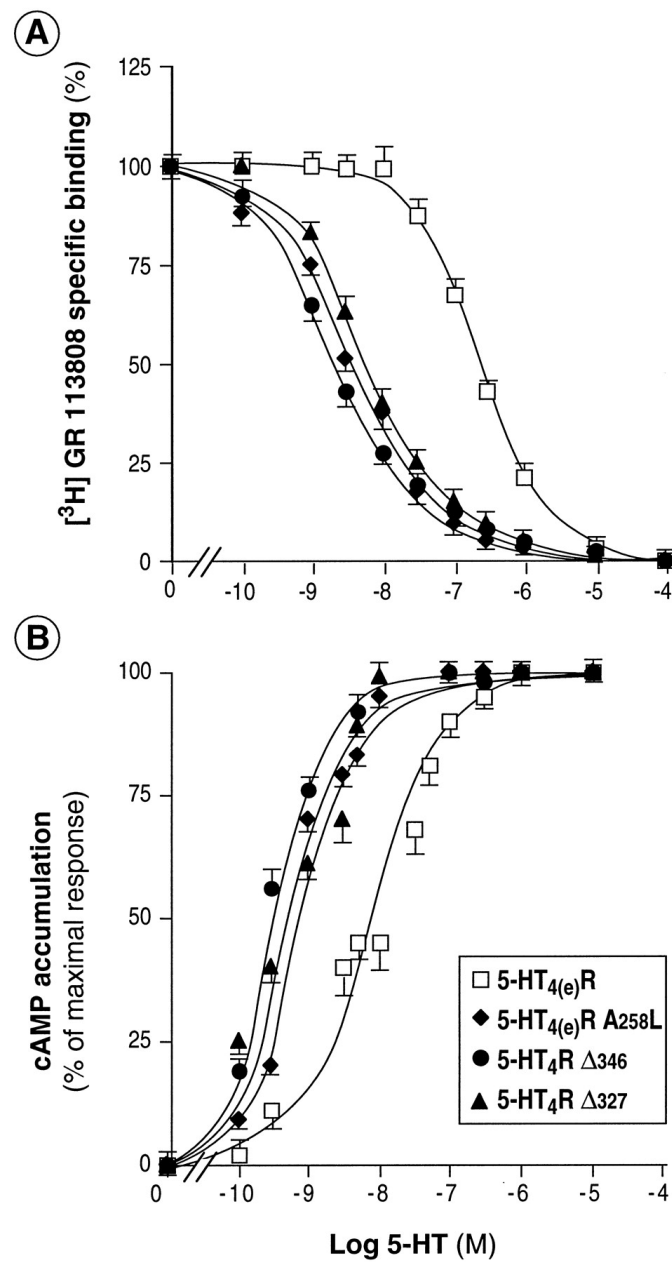
**B**



### Figure 7



**Figure 8**



**Figure 9**



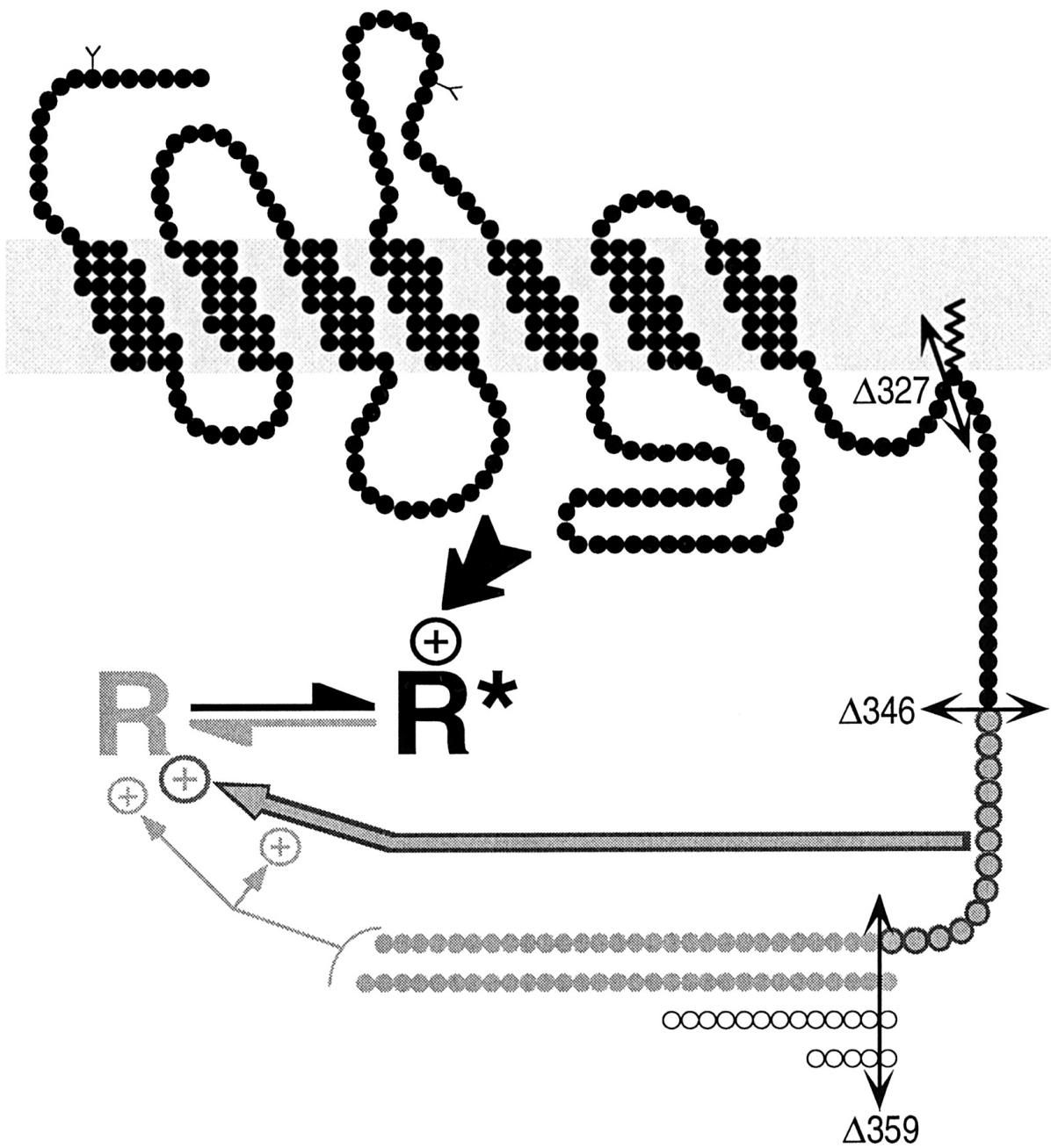


Figure 10