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Pharmacological profile of engineered 5-HT₄ receptors and identification of 5-HT₄ receptor-biased ligands¹

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ABSTRACT

G protein-coupled receptors (GPCRs) can activate simultaneously multiple signaling pathways upon agonist binding. The combined use of engineered GPCRs, such as the Receptors Activated Solely by Synthetic Ligands (RASSLs), and of biased ligands that activate only one pathway at a time might help deciphering the physiological role of each G protein signaling. In order to find serotonin type 4 receptor (5-HT₄R) biased ligands, we analyzed the ability of several compounds to activate the G_s and G_{q/11} pathways in COS-7 cells that transiently express wild type 5-HT₄R, the 5-HT₄R-D¹⁰⁰A mutant (known also as 5-HT₄-RASSL, or Rs1) or the 5-HT₄R-T¹⁰⁴A mutant, which modifies agonist-induced 5-HT₄R activation. This analysis allowed completing the pharmacological profile of the two mutant 5-HT₄Rs, but we did not find any biased ligand for the mutant receptors. Conversely, we identified the first biased agonists for wild type 5-HT₄R. Indeed, RS 67333 and prucalopride acted as partial agonists to induce cAMP accumulation, but as antagonists on inositol phosphate production. Moreover, they showed very different antagonist potencies that could be exploited to study the activation of the G_s pathway, with or without concomitant block of G_{q/11} signaling.

Keywords: 5-HT₄ receptor; G protein-coupled receptor; biased ligand; cyclic AMP, inositol phosphate.

¹Abbreviations: RASSL, Receptor Activated Solely by Synthetic Ligand; DREADD, Designer Receptors Exclusively Activated by Designer Drugs; 5-HT₄R, serotonin type 4 receptor.

HIGHLIGHTS

- We tested the major 5-HT₁R ligands with WT, D¹⁰⁰A and T¹⁰⁰A 5-HT₁Rs.
- We examined 5-HT₁R-induced G_s and G_{q/11} activation.
- We measured cAMP and inositol phosphate accumulation upon 5-HT₁R activation.
- We identified molecules that activate the G_s but not the G_{q/11} pathway.
- We identified the first biased 5-HT₁R ligands: RS 67333 and prucalopride.

1. Introduction

G protein-coupled receptors (GPCRs) convert extracellular messages into intracellular signals to trigger the appropriate cellular responses. As GPCRs can induce multiple pathways at the same time (Rajagopal et al., 2010), it is very difficult to decipher the consequences of the activation of a precise G protein in a physiological context. To overcome this problem, different GPCRs have been engineered over the last decade. First, Receptors Activated Solely by Synthetic Ligands (RASSLs) were generated by site-directed mutagenesis and G_q -, G_s - and G_i -coupled RASSLs are now available (Conklin et al., 2008; Coward et al., 1998; Scearce-Levie et al., 2001). These GPCRs are insensitive to their endogenous ligand and can be activated only by specific synthetic ligands. Then, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) were produced (Dong et al., 2010) by artificially evolving the M_3 and M_4 muscarinic receptors to respond only to a silent drug (clozapine-N-oxide, CNO), and not to their endogenous ligand acetylcholine (Armbruster et al., 2007). A panel of DREADDs was thus created to control all the G-protein signaling pathways (Conklin et al., 2008). Moreover, as GPCRs bind not only to G proteins but also to arrestin family members, the group of J. Wess developed recently an arrestin-biased DREADD (Nakajima and Wess, 2012).

We engineered the first G_s -coupled RASSL, derived from the serotonergic type 4 receptor (5-HT₄R), about ten years ago (Claeysen et al., 2003). This RASSL was obtained by mutating a single aspartate residue (D¹⁰⁰) in the transmembrane domain 3 of 5-HT₄R, a residue that is well conserved in GPCRs and particularly in biogenic amine receptors. The 5-HT₄R-D¹⁰⁰A mutant has lost the ability to bind to serotonin (5-hydroxytryptamine; 5-HT); however, it can be fully activated by a wide variety of highly specific 5-HT₄R agonists (Claeysen et al., 2003). Later, we identified a secondary coupling of 5-HT₄R to the $G_{q/11}$ pathway, leading to inositol 3-phosphate (IP₃) production (Barthet et al., 2007). Therefore, in collaboration with B. Conklin's group, we optimized the 5-HT₄R-D¹⁰⁰A mutant by inserting the additional point mutation D[∞]N or D[∞]A that suppresses 5-HT₄R-induced IP₃ production, in order to obtain a pure G_s -coupled receptor (Chang et al., 2007).

Biased agonism has been described for many GPCRs. Indeed, a wide range of ligands can promote different signaling events downstream of a particular GPCR (Gesty-Palmer et al., 2006; Rajagopal et al., 2010; Thomsen et al., 2012; Wei et al., 2003). A ligand that acts as an agonist for one pathway can behave as an antagonist, an inverse agonist or be without effect on the secondary pathway engaged by the same GPCR. We hypothesized that 5-HT₄R ligands that could differentially activate G_s and $G_{q/11}$ could help deciphering the roles of these different signaling pathways and could also represent new ligands for RASSLs. Thus, from a wide panel of potential 5-HT₄R ligands we selected 22 compounds that belong to different chemical classes in order to cover almost all the different 5-HT₄R ligand families. We then assessed whether these compounds could induce cAMP production upon activation by wild type 5-HT₄R (WT), the 5-HT₄R-D[∞]A mutant (5-HT₄R-RASSL or Rs1) (Claeysen et al., 2003)

or the 5-HT₄R-T¹⁰⁴A mutant (the T¹⁰⁴A mutation modifies agonist-induced 5-HT₄R activation) (Pellissier et al., 2009)). We then measured the ability of 10 of these compounds to induce IP₃ production by quantifying IP₁ accumulation. Finally, we thoroughly characterized the two more interesting 5-HT₄R-biased ligands.

2. Results

2.1. Pharmacological screening of the functional coupling between 5-HT₄R ligands and WT 5-HT₄R, 5-HT₄R-D¹⁰⁰A or 5-HT₄R-T¹⁰⁴A

To identify 5-HT₄R ligands that could differentially activate G_s and G_{q/11}, COS cells were transiently transfected with the WT 5-HT_{4(a)}R, 5-HT₄R-D¹⁰⁰A or 5-HT₄R-T¹⁰⁴A cDNA plasmids (100 ng cDNA / 10⁷ cells, corresponding to 9.5 ± 0.9, 9.2 ± 0.7 and 8.7 ± 0.6 pmol/mg proteins, respectively). Cells were then incubated with each ligand and cAMP and IP₁ (a breakdown product of IP₃) accumulation were quantified using the specific CisBio HTRF kits (see Experimental Procedures). The maximal responses above basal (expressed as percentage of BIMU8 maximal response) are summarized in Tables 1-3. Supplementary Figure 1 shows the raw data in which the basal cAMP or IP₁ accumulation was not subtracted.

2.1.1. cAMP accumulation

As previously described, cAMP accumulation was more strongly induced following binding of 5-HT₄R agonists to 5-HT₄R-D¹⁰⁰A than to WT 5-HT₄R, except for 5-HT and related molecules that belong to the indole class, such as HTF919 (Claeysen et al., 2003) (Table 1 and Supplemental Figure 1B). Conversely, signaling transduction through 5-HT₄R-T¹⁰⁴A resulted in reduced activation of cAMP production, except with the 5-HT₄R agonists of the indole class (5-HT, HTF919) and the benzimidazolones (BIMU1 and BIMU8) (Table 1 and Supplemental Figure 1B).

Compounds that are classically described as 5-HT₄R antagonists or weak, partial agonists acted as partial agonists (GR 113808, GR 125487, for example) or even as full agonists (ML10375, SB 204070) when coupled to 5-HT₄R-D¹⁰⁰A (Table 2 and Supplemental Figure 1A). Inverse agonists, such as RO 116 2617 or RO 116 1148, behaved as neutral antagonists when interacting with this RASSL. Antagonists and inverse agonists did not activate cAMP production via 5-HT₄R-T¹⁰⁴A. Tropisetron, which acts as a 5-HT₄R antagonist or weak partial agonist, was the only drug that retained some activity with the T¹⁰⁴A mutant (Table 2 and Supplemental Figure 1A).

2.1.2. IP₁ accumulation

Receptor-induced IP₁ accumulation (Table 3 and Supplemental Figure 1C) was assessed using nine compounds that were classified as 5-HT₄R agonists based on their ability to induce cAMP accumulation and ML 10375 that acts as a 5-HT₄R antagonist. Dose-response curves were generated to analyze precisely the G_{q/11} activation profiles (Figure 1). For WT 5-HT₄R, the EC₅₀ values of all the tested ligands were shifted to the right compared to G_s activation (for instance, 5-HT EC₅₀ = 4.2 ± 0.8 × 10⁻⁸M for cAMP production and 1.9 ± 0.3 × 10⁻⁷M for IP₁ accumulation) (Tables 1, 3). Molecules of the indole class (5-HT, HTF919) and benzimidazolones (BIMU8) were the most potent and effective activators of the G_{q/11} pathway *via* WT 5-HT₄R (Figure 1A, B). The G_{q/11} activation pattern following coupling of 5-HT₄R-D¹⁰⁰A with the different ligands was comparable to the G_s activation profile obtained for the same molecules (Figure 1C, D and Supplementary Figure 1B, C). Indeed, the majority of the tested compounds could induce IP₁ accumulation upon 5-HT₄R-D¹⁰⁰A stimulation, except molecules belonging to the indole class (5-HT, HTF919). Similarly, RS 67333 was much less efficient in stimulating G_{q/11} signaling than in activating G_s *via* 5-HT₄R-D¹⁰⁰A (Table 1). Finally, 5-HT₄R-T¹⁰⁴A could stimulate IP₁ production only very weakly (Figure 1E, F and Supplemental Figure 1C) and ML 10302 acted as an inverse agonist with this receptor mutant (Table 1, Figure 1F and Supplemental Figure 1C).

2.2. Characterization of biased ligands for WT 5-HT₄R

The results of the functional screening indicated that, among the tested compounds, RS 67333, prucalopride and ML 10302 acted as partial agonists for the G_s pathway and induced only weakly IP₁ accumulation following interaction with WT 5-HT₄R (Figure 2A, B and C). Specifically, dose-response curves showed that the EC₅₀ values for RS 67333-mediated G_{q/11} activation were strongly shifted to the right in comparison to those concerning G_s activation (EC₅₀ = 8.4 ± 1.1 × 10⁻¹⁰M for cAMP and 2.0 ± 0.7 × 10⁻⁷M for IP₁ accumulation) with a significant loss of efficacy (from 49 ± 2% for cAMP to 19 ± 1% for IP₁ accumulation at 10⁻⁵M) (Figure 2C and Tables 1 and 3). Prucalopride and ML 10302 could not induce IP₁ accumulation (Figure 2B, C and Table 3).

In competition experiments, RS 67333 could efficiently inhibit BIMU8-induced IP₁ accumulation with an IC₅₀ of 3.4 ± 0.8 × 10⁻⁹M, similar to the IC₅₀ of GR 113808 (3.8 ± 1.1 × 10⁻⁹M) (Figure 2D). However, RS 67333 could not completely reverse IP₁ production, as this drug possesses a weak partial agonist activity towards the G_{q/11} pathway (Figure 2B, D). Prucalopride was much less potent than RS 67333 in competing with BIMU8-induced IP₁ accumulation (IC₅₀ = 6.2 ± 1.7 × 10⁻⁶M) (Figure 2D). Finally, the efficacy bias factor (eBF) was calculated as the ratio between the maximal response (R_{max}) of the two signaling pathways (cAMP/IP₁ ratio), as described by H. Bräuner-Osborne's group (Thomsen et al., 2012). Taking BIMU8 as the reference, the eBF for 5-HT and HTF919 were 1.1 ± 0.2 and 0.9 ± 0.1, respectively, whereas the eBF value for RS 67333 was 2.6 ± 0.3.

3. Discussion

In order to find pharmacological tools to decipher the role of the different G protein-linked signaling pathways engaged by 5-HT₄R, we tested the ability of several specific compounds to induce cAMP and IP₁ accumulation following coupling with wild type 5-HT₄R and with two previously described receptor mutants (5-HT₄R-D¹⁰⁰A and -T¹⁰⁴A). Our results confirm that a wide range of ligands stimulate more efficiently 5-HT₄-D¹⁰⁰A- than WT 5-HT₄R-mediated signaling; however, we could not identify a molecule that selectively activates only G_s (and not G_{q/11}) upon stimulation of 5-HT₄-D¹⁰⁰A or of 5-HT₄R-T¹⁰⁴A. ML 10302 was the only drug that acted as a weak partial agonist for the WT receptor, a full agonist for the D¹⁰⁰A mutant and an inverse agonist for 5-HT₄R-T¹⁰⁴A concerning IP₁ production. Nevertheless, as recently highlighted by Tschammer et al (Tschammer et al., 2011) who found that a specific residue in the Dopamine D_{2L} receptor is a major determinant of ligand-biased signaling, our analysis revealed that the D¹⁰⁰ residue and to a lesser extent the T¹⁰⁴ residue are crucial elements that have to be taken into account when designing new 5-HT₄R-biased ligands.

The most interesting finding of this study is the identification of two biased ligands (RS 67333 and prucalopride) for wild type 5-HT₄R. Indeed, these two ligands acted as partial agonists for the G_s pathway and as antagonists for the G_{q/11} pathway. Moreover, RS 67333 potentially reversed BIMU8-induced IP₁ production. This drug has already been used *in vivo* to promote learning and memory in rodents, passes well through the blood brain barrier and has a good bioavailability (Freret et al., 2012; Marchetti et al., 2000; Marchetti et al., 2004). Therefore, RS 67333 could be an interesting tool for studying the effect of G_s activation *in vivo*, while selectively blocking coupling of 5-HT₄R to G_{q/11}.

Multiple signaling pathways are linked to 5-HT₄R activation. This receptor is also coupled to G₁₃ and RhoA activation and it has been involved in RhoA-dependent neurite retraction (Ponimaskin et al., 2002). Moreover, 5-HT₄R stimulates the extracellular signal-regulated kinase (ERK) pathway via Src activation and independently of G proteins or β-arrestins (Barthet et al., 2007). It would be interesting to study biased agonism of these additional pathways.

From a therapeutic point of view, 5-HT₄ agonists have been developed and commercialized for gastrointestinal disorders (cisapride, tegaserod, prucalopride, mosapride...). However, some were later withdrawn, as their administration was associated with cardiovascular or other adverse events. These side-effects could be ascribed to the lack of selectivity of the first commercialized drugs for 5-HT₄R and to their affinity also for other molecules, such as the hERG K⁺ channel and the 5-HT₁ receptor (De Maeyer et al., 2008; Tack et al., 2012). Thus, highly specific synthetic agonists are urgently needed. Biased agonism and development of ligand-directed signaling can also help circumventing these adverse effects and thus it could be useful to have ligands that activate the G_s pathway, without triggering G_{q/11} signaling. Indeed, cAMP production has been linked to neuroprotective effects

(Michel and Agid, 1996; Silveira and Linden, 2006), whereas sustained IP₃ production has been associated with tumor formation via PKC activation.

In conclusion, RS 67333 and prucalopride, which belong respectively to the aryl-ketone and benzofuran families, are two 5-HT₄R biased ligands that activate G_s signaling and cAMP production, while blocking the G_{q/11} pathway and constitute promising tools for analyzing the role of G_s activation *in vivo*. Moreover, this study provides important insights for designing new drugs that could be used to treat various disorders related to 5-HT₄R signaling (Bockaert et al., 2011).

4. Experimental procedures

4.1. Plasmid Constructs

Wild type 5-HT_{4(a)}R, 5-HT₄R-D¹⁰⁰A (D¹⁰⁰A) and 5-HT₄R-T¹⁰⁴A (T¹⁰⁴A) cDNA plasmids in pRK5 were previously described in (Barthet et al., 2005; Claeysen et al., 2003; Pellissier et al., 2009).

4.2. Drugs

The following compounds were used: BIMU8 (*endo-N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2-oxo-3-isopropyl-2,3-dihydro-1H-benzimidazole-1-carboxamide), HTF- 919 (5-methoxy-indole-3-carboxaldehyde 4-pentyl-iminosemicarbazone), (*S*)-zacopride ((*S*)-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-4-amino-5-chloro-2-methoxy-benzamide monohydrochloride), SC 53116 (4-Amino-5-chloro-*N*-[[1-(1*S*,7*aS*)-hexahydro-1H-pyrrolizin-1-yl]methyl]-2-methoxy-benzamide), cisapride (*cis-N*-[1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-4-amino-5-chloro-2-methoxy-benzamide), ML 10302 (2-(1-piperidinyl)ethyl-4-amino-5-chloro-2-methoxybenzoate), SL 65.0155 (5-(8-Amino-7-chloro-2,3-dihydro-1,4-benzodioxin-5-yl)-3-[1-(2-phenylethyl)piperidin-4-yl]-1,3,4-oxadiazol-2(3H)-one hydrochloride), Prucalopride (4-Amino-5-chloro-*N*-[1-(3-methoxypropyl)piperidin-4-yl]-2,3-dihydrobenzofuran-7-carboxamide), RS 67333 (1-(4-amino-5-chloro-2-methoxy-phenyl)-3-(1-butyl-4-piperidinyl)-1-propanone), RS 56532 (4-amino-4-chloro-*N*-(quinuclidin-3-yl)-1,8-naphthalimide), BIMU1 (3-ethyl-2,3-dihydro-*N*-[endo-8-methyl-8-azabicyclo(3.2.1)oct-3-yl]-2-oxo-1H-benzimidazole-1-carboxamide, hydrochloride), GR 113308 (1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl 1-methyl-indole-3-carboxylate), Tropisetron (ICS 205 930; [(3*a* tropanyl)-1*H*-indole-3-carboxylic acid ester]), GR 125487 ([1-[2(methylsulphonyl-amino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1-*H*-indole-3-carboxylate, hydrochloride), SDZ 205557 (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino) ethyl ester, hydrochloride), ML 10375 (2-(*cis*-3,5-dimethyl-1-piperidinyl)ethyl-4-amino-5-chloro-2-methoxybenzoate), SB 204070 ((1-butyl-4-piperidinyl)methyl 8-amino-7-chloro-1,4-benzodioxane-5-carboxylate), RS 39604 (1-[4-amino-5-chloro-2-(3,5-dimethoxybenzyl-oxy)phenyl]-3-[1-[2-[(methylsulfonyl)amino] ethyl]-4-piperidinyl]-1-propanone

hydrochloride), Pimozide (2H-Benzimidazol-2-one, 1-(1-(4,4-bis(4-fluorophenyl)butyl)-4-piperidinyl)-1,3-dihydro-), RO116-1148 2,3-dihydrobenzo-(1,4)-dioxine-5-carboxylic acid 1-butylpiperidin-4-ylmethylamide hydrochloride.

4.3. Cell Cultures and Transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal calf serum (dFCS) and antibiotics. Cells were transfected at 60–70% confluence by electroporation as described previously (Claeysen et al., 1999) and then processed for subsequent experiments.

4.4. Determination of cAMP or Inositol Phosphate (IP₁) production in transfected cells

COS-7 cells were transfected with the appropriate plasmids and then seeded in 24-well plates (100 000 cells/well for cAMP and 500 000 cells/well for IP₁ measurement). Twenty-four hours after transfection, cells were incubated with the appropriate drug concentration for 10 min (cAMP) or 30 min (IP₁), as previously described (Barthet et al., 2005). cAMP or IP₁ accumulation was quantified by HTRF[®] using the cAMP Dynamic kit or the IP-One kit (Cisbio International, Bagnols-sur-Cèze, France), according to the manufacturer's instructions.

4.5. Membrane preparation and radioligand binding assay

Membranes were prepared from transiently transfected COS-7 cells plated in 15-cm dishes and grown in DMEM with 10% dFCS as described in Claeysen *et al.* (Claeysen et al., 2003). Membranes were homogenized in 50 mM HEPES (pH 7.4; 5 mg of proteins in 1 ml of solution) and stored at -80°C until use. Membrane suspensions (about 10 µg), diluted in 100 µl of 50 mM HEPES containing 10 mM pargyline and 0.01% ascorbic acid, were incubated with 100 µl [³H]-GR 113808 (specific activity: 82 Ci/mmol) and 50 µl of buffer or competing drugs at 20°C for 30 min. For saturation analysis assays, various concentrations of [³H]-GR 113808 (0.001-0.8 nM) were used. BIMU8 (10 µM) was used to determine specific binding. To quantify [³H]-GR 113808 bound to WT receptors in cells co-expressing WT and D¹⁰⁰A (or DD) 5-HT₄Rs, experiments were performed in the presence of 10 µM 5-HT, which does not bind to the D¹⁰⁰A or DD mutants. The difference between the total [³H]-GR 113808 binding and the remaining binding measured in the presence of 5-HT corresponded to the [³H]-GR 113808 binding to co-expressed D¹⁰⁰A (or DD) receptors. Protein concentration was determined using the bicinchoninic acid method.

4.6. Data Analysis

The dose-response curves were fitted using GraphPad Prism and the following equation for monophasic dose-response curves: $y = (y_{\max} - y_{\min}) / 1 + [(x / EC_{50})^{n_H}] + y_{\min}$, where EC₅₀ is the concentration of the compound needed to obtain 50% of the maximal effect and n_H is the Hill

coefficient. Competition and saturation experiments were evaluated by non-linear regression analysis using GraphPad Prism. All represented data correspond to the mean \pm SEM of three independent experiments performed in triplicate. Statistical significance was determined with the Student Newman Keul's test using GraphPad Prism.

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Table 1

Drug (10 μ M)	cAMP					
	WT 5-HT _R		5-HT _R -D ¹⁰⁰ A		5-HT _R -T ¹⁰⁴ A	
	R _{max} \pm S.E.M. (%)	EC ₅₀	R _{max} \pm S.E.M. (%)	EC ₅₀	R _{max} \pm S.E.M. (%)	EC ₅₀
Serotonin (5-HT)	88 \pm 5	4.2 \pm 0.8 x 10 ⁻⁹	-	1.9 \pm 0.9 x 10 ⁻⁶	107 \pm 4	7.0 \pm 1.2 x 10 ⁻⁸
BIMU8	100	1.9 \pm 0.3 x 10 ⁻⁸	100	6.3 \pm 0.5 x 10 ⁻⁹	100	8.5 \pm 0.6 x 10 ⁻⁸
AGONISTS						
Indoles and Indole Carboxylates						
HTF919	65 \pm 5	5.0 \pm 0.4 x 10 ⁻⁹	20 \pm 3	1.0 \pm 0.2 x 10 ⁻⁶	57 \pm 5	1.2 \pm 0.2 x 10 ⁻⁷
Benzamides						
Zacopride	91 \pm 4	1.6 \pm 0.4 x 10 ⁻⁷	75 \pm 4	2.1 \pm 0.8 x 10 ⁻⁷	8 \pm 3	2.9 \pm 0.9 x 10 ⁻⁵
SC 53116	72 \pm 3		85 \pm 4		35 \pm 4	
Cisapride	61 \pm 3	7.8 \pm 1.2 x 10 ⁻⁹	72 \pm 5	7.4 \pm 0.5 x 10 ⁻⁹	21 \pm 2	2.0 \pm 0.6 x 10 ⁻⁶
Benzoates						
ML10302	34 \pm 3	1.4 \pm 0.2 x 10 ⁻⁹	60 \pm 2	7.7 \pm 1.0 x 10 ⁻⁹	-	N.D.
Benzodioxanes						
SL 65.0155	22 \pm 3	N.D.	58 \pm 4	N.D.	9 \pm 2	N.D. N.D.
Benzofurans						
Prucalopride	50 \pm 7	2.1 \pm 0.8 x 10 ⁻⁸	83 \pm 6	5.3 \pm 1.1 x 10 ⁻⁸	28 \pm 1	1.0 \pm 0.5 x 10 ⁻⁶
Aryl ketones						
RS 67333	49 \pm 2	8.4 \pm 1.1 x 10 ⁻¹⁰	90 \pm 6	1.2 \pm 0.6 x 10 ⁻⁹	-	N.D.
Naphtalimides						
RS 56532	58 \pm 3	1.4 \pm 0.3 x 10 ⁻⁷	68 \pm 3	1.9 \pm 0.5 x 10 ⁻⁷	20 \pm 5	1.2 \pm 0.7 x 10 ⁻⁵
Benzimidazolones						
BIMU1	64 \pm 5	N.D.	79 \pm 5	N.D.	63 \pm 4	N.D.

Table 2

Drug (10 μ M)	cAMP		
	WT 5-HT _R	5-HT _R -D ¹⁰⁰ A	5-HT _R -T ¹⁰⁴ A
	R _{max} \pm S.E.M. (%)	R _{max} \pm S.E.M. (%)	R _{max} \pm S.E.M. (%)
Serotonin (5-HT)	88 \pm 5	-	107 \pm 4
BIMU8	100	100	100
ANTAGONISTS			
Indoles and Indole			
Carboxylates			
GR 113808	-	50 \pm 4	7 \pm 2
Tropisetron	23 \pm 2	51 \pm 5	38 \pm 4
GR 125487	-	36 \pm 5	-
Pimozide	-	13 \pm 3	-
Benzamides			
SDZ 205557	22 \pm 5	70 \pm 3	-
Benzoates			
ML 10375	9 \pm 3	72 \pm 2	-
Benzoate dioxanes			
SB 204070	29 \pm 4	88 \pm 3	8 \pm 2
Benzoate Aryl Ketones			
RS 39604	19 \pm 3	77 \pm 5	-
RS 100235	20 \pm 2	56 \pm 4	11 \pm 3
RS 67532	25 \pm 2	91 \pm 6	13 \pm 2
INVERSE AGONISTS			
Benzoate dioxanes			
RO 116 2617	--	-	--
RO 116 1148	--	5 \pm 3	6 \pm 2

Table 3

Drug (10 μ M)	IP ₁					
	WT 5-HT _R		5-HT _R -D ¹⁰⁰ A		5-HT _R -T ¹⁰⁴ A	
	R _{max} \pm S.E.M. (%)	EC ₅₀	R _{max} \pm S.E.M. (%)	EC ₅₀	R _{max} \pm S.E.M. (%)	EC ₅₀
Serotonin (5-HT)	82 \pm 7	1.9 \pm 0.3 x 10 ⁻⁷	-	6.2 \pm 0.2 x 10 ⁻¹⁰	60 \pm 7	1.2 \pm 0.9 x 10 ⁻⁵
BIMU8	100	7.8 \pm 1.0 x 10 ⁻⁸	100	2.3 \pm 0.9 x 10 ⁻⁸	100	6.7 \pm 0.6 x 10 ⁻⁷
AGONISTS						
Indoles and Indole Carboxylates						
HTF919	73 \pm 4	6.9 \pm 0.3 x 10 ⁻⁸	-	N.D.	10 \pm 4	1.9 \pm 0.7 x 10 ⁻⁵
Benzamides						
Zacopride	25 \pm 3	1.4 \pm 0.8 x 10 ⁻⁶	71 \pm 4	5.4 \pm 1.0 x 10 ⁻⁷	3 \pm 2	N.D.
Cisapride	-	N.D.	97 \pm 2	2.0 \pm 0.3 x 10 ⁻⁷	14 \pm 3	1.6 \pm 0.5 x 10 ⁻⁵
Benzoates						
ML10302	-	N.D.	50 \pm 4	1.3 \pm 0.6 x 10 ⁻⁷	--	N.D.
Benzofurans						
Prucalopride	-	N.D.	70 \pm 4	4.7 \pm 0.6 x 10 ⁻⁷	-	N.D.
Aryl ketones						
RS 67333	19 \pm 1	2.0 \pm 0.7 x 10 ⁻⁷	38 \pm 3	7.3 \pm 0.7 x 10 ⁻⁸	14 \pm 2	2.3 \pm 1.1 x 10 ⁻⁶
Naphtalimides						
RS 56532	17 \pm 6	2.4 \pm 0.2 x 10 ⁻⁵	59 \pm 3	1.1 \pm 0.4 x 10 ⁻⁶	22 \pm 4	4.3 \pm 0.7 x 10 ⁻⁶

TABLE LEGENDS

Table 1: Agonist potencies (EC_{50}) and maximal response (R_{max}) above basal of the tested 5-HT₄R "agonists" with WT 5-HT₄R, 5-HT₄R-D¹⁰⁰A and 5-HT₄R-T¹⁰⁴A concerning cAMP accumulation.

EC_{50} and R_{max} for cAMP accumulation in COS-7 cells that were transiently transfected with the different 5-HT₄R plasmids (50 ng) following incubation with 5-HT₄R "agonists" (10 μ M for 10 minutes). R_{max} was calculated by removing the basal response and is expressed as a percentage of the BIMU8 maximal response (6.7 \pm 0.3 pmol of cAMP/100,000 cells for WT 5-HT₄R, 5.0 \pm 0.2 pmol for 5-HT₄R-D¹⁰⁰A and 4.4 \pm 0.3 pmol for 5-HT₄R-T¹⁰⁴A). -, no significant effect above basal. Data represent the mean \pm SEM of at least three independent experiments performed in triplicate.

Table 2: Maximal response (R_{max}) above basal of the tested 5-HT₄R "antagonists" and "inverse agonists" with WT 5-HT₄R, 5-HT₄R-D¹⁰⁰A or 5-HT₄R-T¹⁰⁴A concerning cAMP accumulation.

R_{max} for cAMP accumulation in COS-7 cells that were transiently transfected with the different 5-HT₄R plasmids (50 ng) following incubation with 5-HT₄R "antagonists" and "inverse agonists" (10 μ M for 10 minutes). R_{max} was calculated by subtracting the basal response and is expressed as a percentage of the BIMU8 maximal response (6.7 \pm 0.3 pmol of cAMP/100,000 cells for WT 5-HT₄R, 5.0 \pm 0.2 pmol for 5-HT₄R-D¹⁰⁰A and 4.4 \pm 0.3 pmol for 5-HT₄R-T¹⁰⁴A). -, no significant effect above basal. --, inverse agonist effect. Data represent the mean \pm SEM of at least three independent experiments performed in triplicate.

Table 3: Agonist potencies (EC_{50}) and maximal response (R_{max}) above basal of the tested 5-HT₄R ligands with WT 5-HT₄R, 5-HT₄R-D¹⁰⁰A or 5-HT₄R-T¹⁰⁴A concerning IP₁ accumulation.

EC_{50} and R_{max} for IP₁ accumulation in COS-7 cells that were transiently transfected with the different 5-HT₄R plasmids (100 ng) following incubation with 5-HT₄R ligands (10 μ M for 30 minutes). R_{max} was calculated by removing the basal response and is expressed as a percentage of the BIMU8 maximal response (53.8 \pm 6.0 pmol of IP₁/250,000 cells for WT 5-HT₄R, 42.7 \pm 6.9 pmol for 5-HT₄R-D¹⁰⁰A and 40.8 \pm 4.2 pmol for 5-HT₄R-T¹⁰⁴A). Data represent the mean \pm SEM of at least three independent experiments performed in triplicate.

FIGURE LEGENDS

Figure 1: Dose-response curves of IP₁ accumulation following incubation with 5-HT₄R ligands.

COS-7 cells that transiently express WT 5-HT₄R (A, B), 5-HT₄R-D¹⁰⁰A (C, D) or 5-HT₄R-T¹⁰⁴A (E, F) were incubated with the different 5-HT₄R ligands and IP₁ accumulation was expressed as the percentage of the IP₁ production (60.5 ± 2.6 pmol/100,000 cells) induced by 10^{-5} M 5-HT in cells transfected with 100 ng of WT 5-HT₄R plasmid (A, B, E, F) or as a percentage of the IP₁ production (49.6 ± 2.2 pmol/250,000 cells) induced by 10^{-5} M BIMU8 in cells transfected with 100 ng of 5-HT₄R-D¹⁰⁰A plasmid (C, D). Data for each drug correspond to the mean \pm SEM of three independent experiments performed in triplicate.

Figure 2: Characterization of biased 5-HT₄R ligands.

COS-7 cells that transiently express WT 5-HT₄R were incubated with the indicated ligands. (A-C) Dose-response curves for cAMP and IP₁ accumulation. cAMP and IP₁ values were expressed as a percentage of the cAMP or IP₁ production (6.7 ± 0.3 pmol/100,000 cells or 53.8 ± 6.0 pmol/250,000 cells, respectively) induced by 10^{-5} M BIMU8 in cells transfected with 50 ng (cAMP) or 100 ng (IP₁) WT 5-HT₄R plasmid. (D) Competition of BIMU8-induced IP₁ response. IP₁ values were expressed as a percentage of the IP₁ production (42.2 ± 3.7 pmol/250,000 cells) induced by 10^{-5} M BIMU8 in cells transfected with 100 ng WT 5-HT₄R plasmid. Each point in the curve is the mean \pm SEM of three independent experiments performed in triplicate.

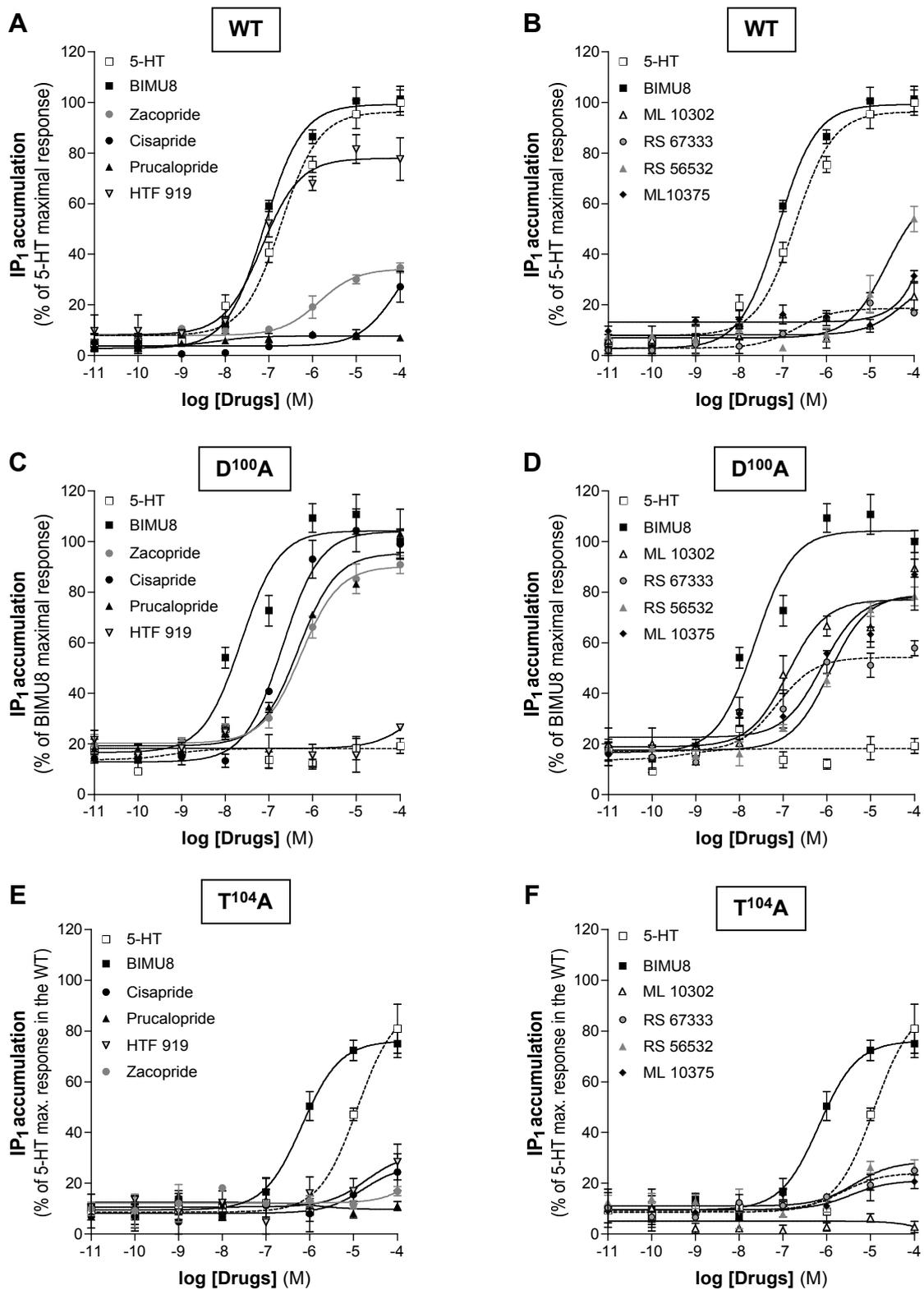


Figure 1

WT

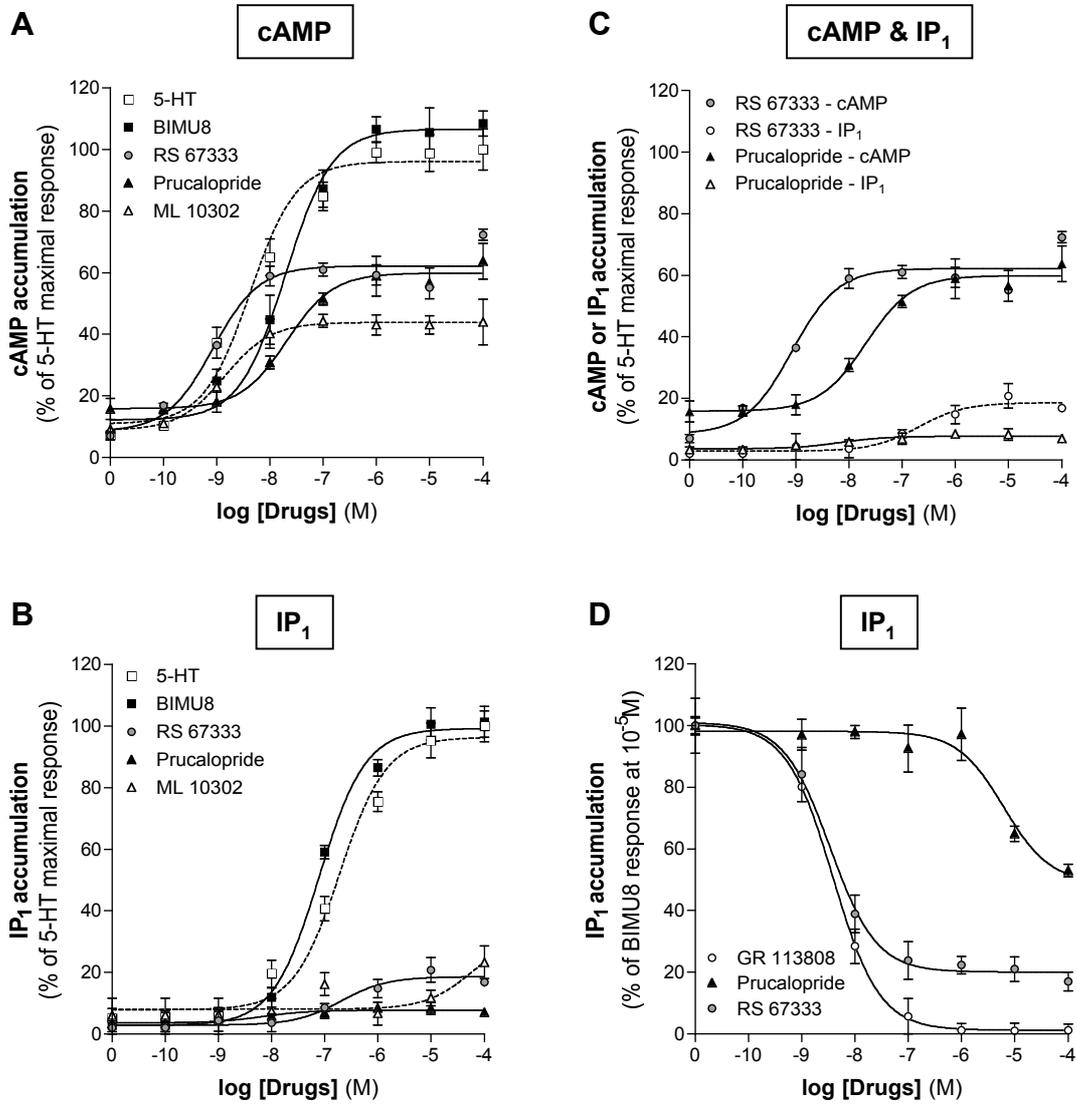


Figure 2

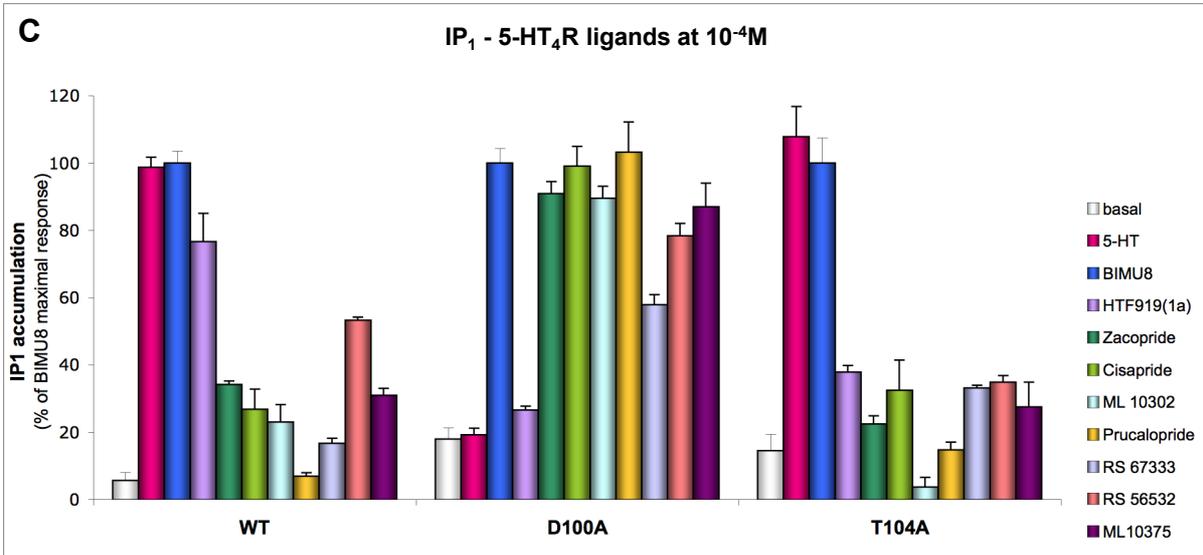
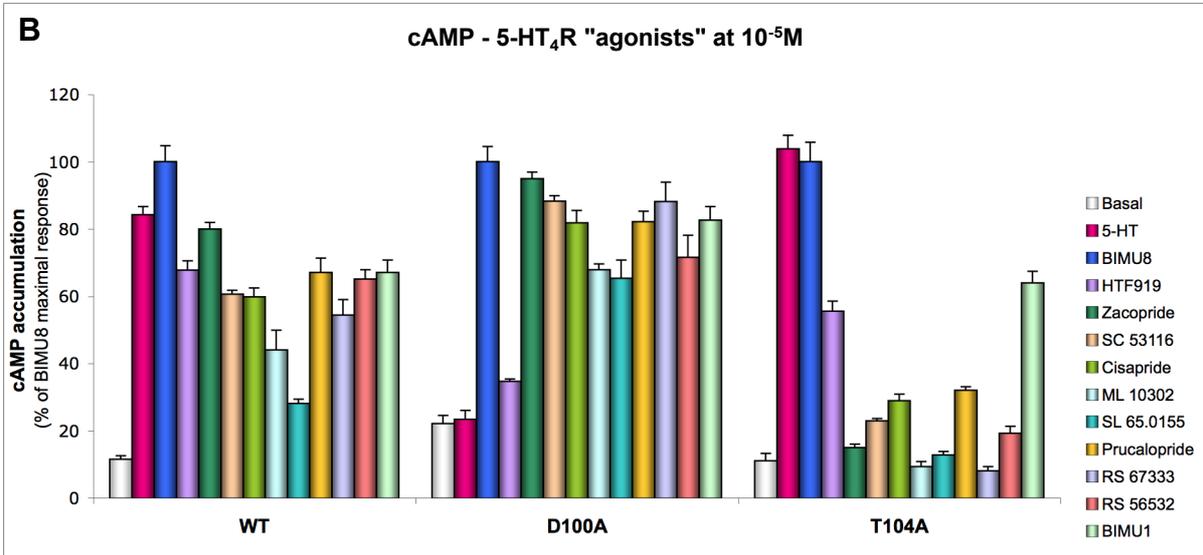
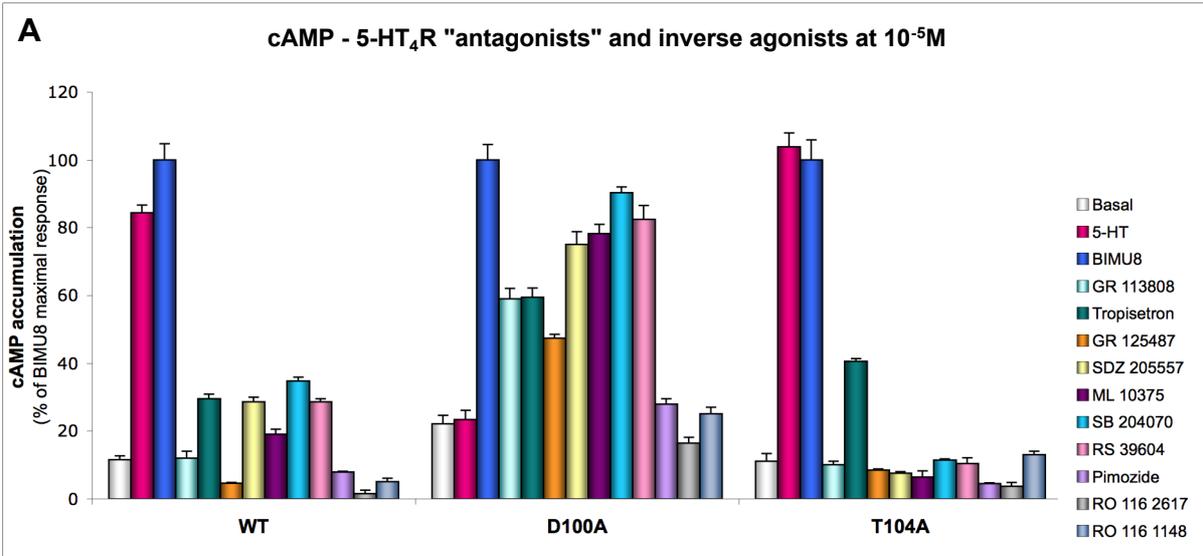
PHARMACOLOGICAL PROFILE OF ENGINEERED 5-HT₄ RECEPTORS AND IDENTIFICATION OF 5-HT₄ RECEPTOR-BIASED LIGANDS

Florence Gaven, Lucie P. Pellissier, Emilie Queffeuilou, Maud Cochet, Joël Bockaert, Aline Dumuis and Sylvie Claeysen

CONTENTS:

Supplemental Figure 1: 5-HT₄R ligand-mediated cAMP and IP₁ maximal responses.

COS-7 cells were transiently transfected with the WT 5-HT₄R (A, B), 5-HT₄R-D¹⁰⁰A (C, D) or 5-HT₄R-T¹⁰⁴A constructs and then (A-B) cAMP accumulation was quantified following incubation with 10⁻⁵M of each 5-HT₄R ligand. Values are expressed as a percentage of the cAMP production (6.7 ± 0.3 pmol/100,000 cells for WT 5-HT₄R, 5.0 ± 0.2 pmol for 5-HT₄R-D¹⁰⁰A and 4.4 ± 0.3 pmol for 5-HT₄R-T¹⁰⁴A) induced by 10⁻⁵M BIMU8 in cells transfected with 100 ng of WT 5-HT₄R plasmid. (C) IP₁ accumulation induced by incubation with 10⁻⁴M ligands. Values are expressed as a percentage of the IP₁ production (53.8 ± 6.0 pmol of IP₁/250,000 cells for WT 5-HT₄R, 42.7 ± 6.9 pmol for 5-HT₄R-D¹⁰⁰A and 40.8 ± 4.2 pmol for 5-HT₄R-T¹⁰⁴A D100A and T104A) induced by 10⁻⁴M BIMU8 in cells transfected with 100 ng of WT 5-HT₄R plasmid. Data for each drug correspond to the mean ± SEM of three independent experiments performed in triplicate.



Supplementary figure 1

