A Single Mutation in the 5-HT\textsubscript{4} Receptor (5-HT\textsubscript{4}-R D100(3.32)A) Generates a G\textsubscript{s}-coupled Receptor Activated Exclusively by Synthetic Ligands (RASSL)*

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Sylvie Claeyesen,\textsuperscript{2} Lara Joubert,\textsuperscript{2} Michèle Sebben, Joël Bockaert,\textsuperscript{2} and Aline Dumuis\textsuperscript{2}
From the Centre National de la Recherche Scientifique, UPR 9023, 141 rue de la Cardonille, 34094 Montpellier, Cedex 5, France

To better understand G-protein-coupled receptor (GPCRs) signaling, cellular and animal physiology, as well as gene therapy, a new tool has recently been proposed. It consists of GPCR mutants that are insensitive to endogenous ligands but sensitive to synthetic ligands. These GPCRs are called receptor activated solely by synthetic ligands (RASSL). Only two examples of such engineered receptors have been described so far: one G\textsubscript{s}-coupled (opioid receptors) and one G\textsubscript{i}-adrenergic receptors. Here, we describe the first RASSL related to serotonin receptors (D100(3.32)A G\textsubscript{s}-coupled 5-HT\textsubscript{4} receptor or 5-HT\textsubscript{4}-RASSL). 5-HT\textsubscript{4}-RASSL is generated by a single mutation, is totally insensitive to serotonin (5-HT), and still responds to synthetic ligands. These ligands have affinities in the range of nanomolar concentrations for the mutant receptor and exhibit full efficacy. More interestingly, two synthetic ligands behave as antagonists on the wild type but as agonists on the 5-HT\textsubscript{4}-RASSL.

G protein-coupled receptors (GPCRs)\textsuperscript{3} are the most numerous and the most diverse type of receptors (1, 2). They transduce messengers as different as odorants, nucleotides, nucleosides, peptides, lipids, or proteins. Eight families of GPCRs are classified according their sequences, their binding domains to ligands, and their ability to activate similar sets of G-proteins. The heterotrimeric (α, β, γ) G proteins are divided into four families based on the nature of their Go subunits (Go\textsubscript{a}, Go\textsubscript{c}, Go\textsubscript{b}, Go\textsubscript{12-13}). Each one couples to a distinct class of receptors and signals through a specific pathway. A series of pathologies have been found to be related to the mutation of GPCRs. These changes lead either to a loss or a gain of function, such as blindness, diabetes insipidus, and hypo- or hyperthyroidism (3). Depressed GPCR signal transduction is related to numerous complex diseases. Heart failure and asthma are associated with a decrease in the G\textsubscript{i}-signaling pathway, whereas an increase in G\textsubscript{s} signaling is a potential cause of dilated cardiomyopathy.

The concept that modified GPCRs could be used as tools to better understand GPCR-controlled signal transduction pathways in a given cell or organ or for gene therapy has recently been proposed (5–7). Several types of modified GPCRs have been developed to prepare such tools. The first, engineered by Conklin and collaborators (8), were named RASSL for “receptor activated solely by synthetic ligands.” The idea is to engineer receptors that would be insensitive to their endogenous ligands but can be fully activated by synthetic ligands. Among the GPCRs, only the κ opioid receptors have been modified so far by Conklin’s group (8) to produce two opioid receptor-RASSLs, Ro1 and Ro2. Ro1 was constructed by substituting the second extracellular loop of the κ opioid receptor with the corresponding portion of the human δ opioid receptor. This substitution induced a lower affinity for the endogenous peptide ligands (including dynorphin) without significantly reducing the response to κ synthetic ligands, like spiradoline (8). The specificity of Ro1 for the synthetic ligands was further enhanced in Ro2 by substituting glutamine for Glu\textsubscript{297} in Ro1.

More recently, a second type of mutant GPCR the “therapeutic receptor-effector complex” (or “TREC”) was proposed to be a biotechnological tool to study GPCR signal transduction (9). The β\textsubscript{2}-adrenergic receptor mutant in 19 positions and fused with Go\textsubscript{αs} was not activated by β-adrenergic agonists, but only by a non-biogenic amine agonist, L156870, although with relatively low potency.

In this report, we describe a third example of RASSL type receptors: a mutant serotonin receptor, the 5-HT\textsubscript{4} receptor. 5-HT\textsubscript{4} receptors are G\textsubscript{s}-coupled receptors expressed in the gastrointestinal tract, human and pig atria, urinary bladder, adrenal medulla, and central nervous system including limbic areas (olfactory tubercles, limbic system, basal ganglia) (10). Over the past 10 years, the pharmacology and structure of 5-HT\textsubscript{4} receptors have been extensively studied (10–12). One of the interesting characteristics of this receptor is its ability to be activated by a wide range of compounds from very different chemical classes (13). Some are used in clinic to treat gastroparesis, dyspepsia gastro-esophageal reflux, or irritable bowel syndromes. The 5-HT\textsubscript{4} agonists are related to tryptamines like 5-HT, to carbazimidas (ITP-B19 or Zelmac) benzamides (metoclopramide or Primapern®), cisapride or “Prepulsid®,” tetrahydrobenzazepines (benzoates SL 10302), benzimidazolones (BIMU8), or ary ketones (for reviews, see Refs. 10 and 13).

The demonstration that tryptamines and benzamides have different pharmacophores (14) prompted us to generate mutant 5-HT\textsubscript{4} receptors with the aim of disrupting the 5-HT recognition site, keeping the recognition site for synthetic 5-HT\textsubscript{4} agonists.

**EXPERIMENTAL PROCEDURES**

Construction of Mutant m5-HT\textsubscript{4}(a) Receptor cDNA—The mutant was generated by exchanging the endogenous residue Asp\textsubscript{100} to Ala in the m5-HT\textsubscript{4}(a)R cDNA sequence with the QuickChange site-directed mutagenesis kit (Stratagene). The sense primer used was: D100A, 5′-ACC TCT CGT CGT CTA CTC ACC-3′.

Cell Culture and Transfection—The cDNAs, subcloned into the pRK5 5′-CAG TCT TCT TCT GAG TCA GCA-3′.
vector, were introduced into COS-7 cells by transfection. In some cases, cells were trypan blue stained, centrifuged, and resuspended in PBS, and resuspended in DMEM (50 mM KCl, 20 mM CH3CO-K, 20 mM KOH, 26.7 mM MgSO4, pH 7.4) with 25–2000 ng of receptor cDNA. The total amount of DNA was kept constant at 15 µg per transfection with pRK5 vector. After 15 min at room temperature, 300 µL of cell suspension (10^6 cells) were transferred to a 0.4-cm electroporation receptacle (Bio-Rad, Ivy sur Seine, France) and pulsed with a gene pulser apparatus (setting 1000 microfarads, 290 V). Cells were diluted in Dulbecco’s modified Eagle’s medium (DMEM; 10^6 cells/mL) containing 10% dialyzed and deamylated fetal bovine serum (fBS) and plated on 15-cm Falcon Petri dishes or into 12-well clusters at the desired density.

**Determination of Cyclic AMP (cAMP) Production in Intact Cells**—Six hours after transfection, the surrounding cell medium was exchanged for DMEM without fBS for 5 h, followed by incubation for 20 h in DMEM without fBS. The cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4 °C for 5 min. The pellets were resuspended in buffer containing 10 mM HEPES, pH 7.4, 4 mM EGTA, 1 mM EDTA, and 0.3 M sucrose and homogenized 10 times with a glass-Teflon potter at 4 °C. The homogenate was centrifuged at 20,000 × g for 20 min. The membrane pellet was resuspended in 50 mM HEPES, pH 7.4 (5 mg of protein in 1 mL of solution) and stored at −80 °C until use. Saturation experiments were performed using unlabelled 5-HT as the competitor. [3H]GR 113808 (113808) at various concentrations ranging from 0.048 to 0.51 nM. The 5-HT receptor binding site density was estimated with [3H]GR 113808 at a saturating concentration (0.5 nM), as described previously (16). 5-HT receptor antagonist binding was determined in the samples with the Bio-Rad protein assay.

**Membrane Preparation and Radioligand Binding Assay**—Membranes were prepared from transiently transfected cells plated on 15-cm dishes and grown in DMEM with 10% fBS for 6 h, followed by incubation for 20 h in DMEM without fBS. The cells were washed twice in PBS, scraped with a rubber policeman, harvested in PBS, and centrifuged at 4 °C for 5 min. The pellets were resuspended in buffer containing 10 mM HEPES, pH 7.4, 4 mM EGTA, 1 mM EDTA, and 0.3 M sucrose and homogenized 10 times with a glass-Teflon potter at 4 °C. The homogenate was centrifuged at 20,000 × g for 20 min. The membrane pellet was resuspended in 50 mM HEPES, pH 7.4 (5 mg of protein in 1 mL of solution) and stored at −80 °C until use. Saturation experiments were performed using unlabelled 5-HT as the competitor. [3H]GR 113808 at various concentrations ranging from 0.048 to 0.51 nM. The 5-HT receptor binding site density was estimated with [3H]GR 113808 at a saturating concentration (0.5 nM), as described previously (16). 5-HT receptor antagonist binding was determined in the samples with the Bio-Rad protein assay.

**Data Analysis**—Competition and saturation experiments were analyzed by non-linear regression curves using the computer program LIGAND (17). Saturation experiments were also analyzed according to Scatchard.

\[ IC_{50} = \frac{S}{K_{d}} + S/K_{d} \]

Data were compared using the Stat-View Student program. Data are presented as the means ± S.E. values of four experiments performed in triplicate.
for F275A, W272A, and S197A, with no change in the maximal 5-HT stimulation (12).

Interestingly, and in contrast to other biogenic amine receptors (9), the mutation of the conserved Asp100(3.32) only moderately affected the specific 5-HT4R radioligand, [3H]GR113808 (KD/H11005 0.17 ± 0.09 nM and 0.38 ± 0.09 nM at WT and D100(3.32)A receptors, respectively) (Fig. 1D). Using [3H]GR 113808 binding, we verified that 5-HT was unable to bind the 5-HT4 D100(3.32)A receptor (Fig 1C).

Fig. 2. Mutation of the conserved Asp100(3.32) in Ala did not impede response and binding of the synthetic 5-HT4 agonist: BIMU8. A, the effects of a range of concentrations of BIMU8 on intracellular cAMP levels were measured in COS-7 cells in which 5-HT4(a) or D100(3.32)A receptors were expressed at equivalent levels (1350 ± 60 and 1580 ± 110 fmol/mg of protein, respectively). Results are expressed as a percentage of the cAMP production in mock-transfected cells. In mock-transfected cells, 0.10 ± 0.04% of [3H]ATP was converted to [3H]cAMP.

TABLE I
5-HT4 receptor drug efficiency on WT and D100(3.32)/A mutant 5-HT4 receptor

| Drugs (10μM)       | 5-HT4(a) (% of max 5-HT response) | 5-HT4D100(3.32%)/A (% of max BIMU8 response) |
|--------------------|-----------------------------------|---------------------------------------------|
| 5-HT                | 100                              | 100                                         |
| BIMU8              | 96 ± 10                          | -                                           |
| HTF 5719           | 66 ± 15                          |                                              |
| Gia001             | 108 ± 5                          | 100 ± 5                                    |
| ML 10375           | 55 ± 9                           | 100 ± 6                                    |
| SB 204070          | 20 ± 3                           | 100 ± 5                                    |
| RS 100(3.32)       | 17 ± 5                           | 70 ± 10                                    |
| RS 124020          | 21 ± 6                           | 75 ± 6                                     |
| RS 97333           | 28 ± 6                           | 85 ± 12                                    |
| RS 47431           | 39 ± 5                           | 100 ± 7                                    |
| GR 113808          | 19 ± 9                           | 78 ± 10                                    |
| ML 10375           | 5 ± 4                            | 80 ± 15                                    |

Fig. 3. D100(3.32)A mutation, in 5-HT4 receptor, resulted in the modification of the property of some antagonists, which became almost full agonists. A and B, the activities of ML 10375 (A) and GR 113808 (B), two potent and specific 5-HT4R antagonists, were tested in COS-7 cells expressing 2700 ± 320 and 2500 ± 280 fmol/mg of 5-HT4 WT/mg of protein and mutant receptors, respectively. Levels of cAMP accumulation were measured after 15-min incubation and expressed as a percentage of basal cAMP production measured in mock-transfected COS-7 cells. The conversion percentage of [3H]ATP to [3H]cAMP in mock-transfected cells was 0.14 ± 0.06% of [3H]cAMP. C, cAMP accumulation was measured in COS-7 cells transiently expressing WT and D100(3.32)A mutant receptors expressed at similar densities as in A and B in the absence and presence of 3 × 10-8 M BIMU8. The abilities of ML 10375 (10-5 M) to inhibit BIMU8-mediated cAMP accumulation were tested in the same COS-7 cells. Data are the means ± S.E. of three experiments performed in triplicate.

5-HT4 D100(3.32)/A receptor (Fig 1C). The binding of [3H]GR 113808 was the first indication that ligands, which include the basic nitrogen of the aromatic ring side chain, in a structured ring, associated with an increase in the distance between this basic nitrogen and the main aromatic ring of the compound, suppressed the requirement of the Asp100(3.32) carboxylic group for ligand binding. This was confirmed when we screened most of the non-tryptamine 5-HT4 receptor agonists for their ability to activate the 5-HT4 D100(3.32)/A receptor.

BIMU8, a specific 5-HT4 agonist structurally very different
from 5-HT, belonging to the azabicycloalanyl benzimidazolone class (21), was found to be a potent agonist. As shown in Fig. 2, this compound remained fully active and even showed higher efficacy and potency on the D100A mutant than on the WT (EC\textsubscript{50} values for cAMP stimulation were 4 ± 1.5 and 1 ± 0.5 nM for WT and D100A, respectively) (Fig. 2A). The affinity of BIMU8 for the 5-HT\textsubscript{4} receptor was also slightly better on the D100A mutant than on the WT. K\textsubscript{D} values for BIMU8, measured by competition with the \([3H]GR 113808\) radioligand, were 30 ± 11 nM and 6.5 ± 3 nM for WT and D100A, respectively (Fig. 2B). Similarly, the benzamides bearing the 2-methoxy-4-amino-5-chloro substitution (tenzapride, 5-zacopride, or cisapride) were equi-effective on the WT and the D100A.22/A mutant (Table I) with nanomolar affinity. A slight decrease in their potency on the mutant D100A receptor was observed. These data suggest that the D100A.22/A mutant could still be activated as long as the agonist could bind the receptor. Furthermore, this mutation had no effect on the receptor expression level.

Two drugs were antagonists on WT and agonists at the D100A.22/A mutant.

Benzamide derivatives bearing the 2-methoxy-4-amino-5-chloro-substitution (ML 10302) (22) and the related aryl ketones (RS 67333, RS 124523, RS 100350, RS 47431) (13) were partial agonists on 5-HT\textsubscript{4} WT receptors. As shown in Table I, they were more efficacious on 5-HT\textsubscript{4} D100A.22/A receptors than on WT. Another benzamide derivative, reported to be either a highly specific 5-HT\textsubscript{4} antagonist (ML 10375) (23) or an inverse agonist on the 5-HT\textsubscript{4} receptor (24), was indeed an antagonist of WT 5-HT\textsubscript{4} receptors (Fig. 3C), which became a full agonist on the 5-HT\textsubscript{4} D100A.22/A mutant (EC\textsubscript{50} = 1 ± 0.6 nM) (Fig. 3, A and C). This encouraged us to screen the putative agonist properties of a series of 5-HT\textsubscript{4} antagonists. We found that the known GR 113808 antagonist (Fig. 3C) used for 5-HT\textsubscript{4} receptor studies (25) was a highly potent (EC\textsubscript{50} = 0.17 ± 0.04 nM) and efficacious agonist on D100A.22/A receptor (Fig. 3D).

Compared with the previously described \(\beta\)-adrenergic G\textsubscript{s}-coupled RASSL (9), the 5-HT\textsubscript{4} RASSL has the advantage of being stimulated by synthetic compounds of much higher affinity (micromolar for \(\beta\)-adrenergic RASSL and nanomolar for 5-HT\textsubscript{4} RASSL).

The structure-activity relationships and the structural analyses of the 5-HT\textsubscript{4} receptor ligands used in this study are consistent with a recent report on comparative receptor mapping of 5-HT4 receptors (25) was a highly potent (EC\textsubscript{50} = 0.6 nM) and efficacious agonist on D100A.22/A receptor (Fig. 3D).

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