G Protein Activation by Serotonin Type 4 Receptor Dimers: evidence that turning on two protomers is more efficient.

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The discovery that class C G protein-coupled receptors (GPCRs) function as obligatorily dimeric entities has generated major interest in GPCR oligomerization. Oligomerization now appears to be a common feature among all GPCR classes. However, the functional significance of this process remains unclear because, in vitro, some monomeric GPCRs, such as rhodopsin and β2-adrenergic receptors, activate G proteins. By using wild type and mutant serotonin type 4 receptors (5-HT4R) (including a 5-HT4R-RASSL) expressed in COS-7 cells as models of class A GPCRs, we show that activation of one protomer in a dimer was sufficient to stimulate G proteins. However, coupling efficiency was 2 times higher when both protomers were activated. Expression of combinations of 5-HT4, in which both protomers were able to bind to agonists but only one could couple to G proteins, suggested that upon agonist occupancy, protomers did not independently couple to G proteins but rather that only one G protein was activated. Coupling of a single heterotrimERIC Gα protein to a receptor dimer was further confirmed in vitro, using the purified recombinant WT RASSL 5-HT4R obligatory heterodimer. These results, together with previous findings, demonstrate that, differently from class C GPCR dimers, class A GPCR dimers have pleiotropic activation mechanisms.

GPCRs have been considered for a long time as monomeric proteins, and the paradigm of “one ligand/one receptor/one G protein” was the driving principle (1). However, a growing number of studies revealed dimerization/oligomerization of GPCRs (4–8) mostly in heterologous cells (homo- or heterodimers) but also in native tissues or in vivo (dimers) (9–13). In line with these observations, a recent report described crystal structures of the chemokine receptor CXCR4 that are consistent with the formation of homodimers (14).

A relatively accepted model proposes that only one protomer in a dimer is fully activated, even when both binding sites are occupied (15–19). The activated protomer interacts with the Gα subunit to accelerate GDP/GTP exchange. This is compatible with recent data showing that a rhodopsin monomer (or a β2-adrenergic receptor monomer) is sufficient to activate its cognate G protein after purification and reconstitution in a phospholipid bilayer (20, 21). However, some observations indicate that the active state of a GPCR dimer is asymmetric (22, 23) and that conformational switches occur between protomers (24, 25). Moreover, occupation of the second protomer of a dimer is probably not “silent” because it can either favor (26) or reduce (22) coupling efficiency. It has also been reported that occupation of both binding sites in 5-HT2C-receptor dimers is mandatory for receptor activation (27).

Collectively, these reports suggest that the second protomer within GPCR homo- or heteromeric assemblies can be involved in different regulatory mechanisms. How such a diversity may be related to the physiological roles of the corresponding receptor and how it can influence signaling efficiency are poorly addressed questions.

Here, we analyzed the functional response elicited by different combinations of wild type (WT) and mutant 5-HT4 receptor (5-HT4R) dimers in a cellular context and in vitro, using purified proteins. These combinations allowed occupancy of one or both binding sites and the control of the coupling of each protomer to G proteins. We considered the receptor dimer-G protein entity as the minimal functional unit (28) because oligomeric entities can be viewed as multiples of dimers. Therefore, throughout we have used the term “dimer” to represent the minimal oligomeric arrangement.

We show that upon agonist occupation of both protomers, 5-HT4R dimers were about twice as efficient in activating G proteins as following occupation of only one binding site. This may suggest that each protomer could be independently coupled to a G protein. However, we provide experimental evi-
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dence suggesting that a single heterotrimERIC G, protein couples to a receptor dimer. These results were confirmed by in vitro experiments showing that activation of a single G, protein was higher when both protomers within a dimer were occupied by an agonist.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Tagged-5-HT₄R cDNA plasmids in pRK5 were generated by adding the c-Myc, HA, FLAG, or RhoTag epitopes to the N-terminal extremity of the receptor using the QuiKChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) as described previously (29). Tagged 5-HT₄R-D100A (D100A), 5-HT₄R-D66N (D66N), 5-HT₄R-D66N/D100A (DD), 5-HT₄R-T104A (T104A), and 5-HT₄R-D330Stop (A329) mutants were generated from tagged 5-HT₄R cloned in pRK5 using the same mutagenesis kit.

Antibodies—Rabbit anti-HA antibody (SG77) was purchased from Cliniscience (Montrouge, France). Rabbit anti-c-Myc antibody (A-14) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-FLAG (M2) and mouse anti-c-Myc (9E10) antibodies were purchased from Sigma. Mouse anti-RhoTag antibody was provided by Dr. S. Costagliola (Institut de Recherche en Biologie Humaine et Nucléaire, Brussels, Belgium) (30). Alexa Fluor 488- and 594-labeled secondary antibodies were purchased from Invitrogen. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from GE Healthcare.

Cell Cultures and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% dialyzed FCS and antibiotics. They were transfected at 60–70% confluence by electroporation as described previously (31) and processed for subsequent experiments.

Cell Surface ELISA—COS-7 cells in 96-well plates were transfected with WT and/or mutant tagged 5-HT₄Rs or GPCR plasmids. Twenty-four hours after transfection, cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 5 min and blocked with phosphate-buffered saline containing 1% fetal calf serum (blocking buffer). Cells were then incubated with the appropriate antibody (anti-HA at 0.6 µg/ml, anti-Myc at 2.2 µg/ml, anti-FLAG at 4.4 µg/ml) in the same buffer for 60 min. After four washes with blocking buffer, cells were incubated with anti-rabbit (1 g/ml) or anti-mouse/HRP conjugate (0.25 µg/ml) (GE Healthcare) for 60 min. After extensive washes, the chromogenic substrate was added (Supersignal® ELISA femto-maximum sensitivity; Pierce). Chemiluminescence was detected and quantified using a Wallac Victor2 luminescence counter.

Fluorescence Resonance Energy Transfer (FRET)—COS-7 cells were transfected with the appropriate plasmids and seeded in 96-well plates (100,000 cells/well). Twenty-four hours after transfection, cells were incubated with the appropriate fluorescent anti-FLAG, -HA, or -Myc antibodies diluted in 50 µl of PBS-KF (20 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 0.1% glucose, 0.1% BSA, 200 mM KF) at 4 °C for 24 h. KF was added to avoid quenching of europium cryptate. Quantification of FRET signals was performed by homogeneous time-resolved fluorescence (HTRF®) using anti-bodies coupled either to europium cryptate as a donor (Cisbio International, Bagnols-sur-Cèze, France) or to various acceptors (Alexa Fluor 647, Molecular Probes; d2, Cisbio International) (32).

Co-immunoprecipitation Experiments and Immunoblotting—COS-7 cells were seeded at 10⁵/150-mm plate 48 h prior to the experiment and then transfected with RhoTag- or Myc-tagged WT and mutant 5-HT₄Rs as indicated in the figure legends. Cross-linking was carried out in phosphate-buffered saline (PBS) completed with 25 mM of dithiobis(succinimidyl propionate) (Pierce) for 30 min, as described previously (33). After 60 min of incubation at 4 °C, samples were processed as described (29), using a 1:1 mixture of Protein A/Protein G-Sepharose beads (GE Healthcare) that were precoupled with 8 µg of anti-Myc antibody (Polyclonal A-14, Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were eluted in Laemmli sample buffer, resolved by SDS-PAGE, and detected by Western blotting.

Determination of cAMP or Inositol Monophosphate (IP1) Production in Transfected Cells—COS-7 cells were transfected with the appropriate plasmids and seeded in 24-well plates (100,000 cells/well for cAMP and 500,000 cells/well for IP1 measurements). Twenty-four hours after transfection, a 10-min (cAMP) or 30-min (IP1) stimulation with the appropriate concentrations of drugs was performed as described previously (29). cAMP or IP1 production 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.

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% dialyzed FCS as described by Claeysen et al. (34). 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 of [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 by the bicinchoninic acid method.

Data Analysis—The dose-response curves were fitted using GraphPad Prism and the following equation for monophasic dose-response curves: y = (yₘₐₓ − yₘᵢₙ) / 1 + (EC₅₀/x) nₜₜ, where EC₅₀ is the concentration of the compound needed to obtain 50% of the maximal effect, and nₜₜ is the Hill coefficient. Competition and saturation experiments were evaluated by non-linear regression analysis using Prism. All represented data corresponded to the mean ± S.E. of three independent
experiments performed in triplicate. Statistical significance was determined with the Student-Newman-Keuls test using Prism.

**Purified WT-D100A 5-HT₄R Dimer Preparation—**5-HT₄R production and refolding were done as described (35). The WT-D100A dimer complex was obtained with a two-step purification process as already done for the BLT1 dimer (36). Briefly, S-tagged (WT) and Strep-tagged (D100A) 5-HT₄Rs were expressed as fusion proteins with KSI (the tag sequence was after the thrombin cleavage site). After removing KSI with thrombin (35), WT and D100A receptors were mixed, refolded, and purified as described (28). Refolded receptors were then immobilized on S-protein-agarose and eluted with 1 mM MgCl₂.

The protein fraction recovered under these conditions was then loaded onto a Streptactin affinity column (5.0 ml) and purified as described (28). Refolded receptors were then immobilized on S-protein-agarose and eluted with 1 mM MgCl₂. The protein fraction recovered under these conditions was then loaded onto a Streptactin affinity column (5.0 ml) and eluted with 2.5 mM dethiobiotin.

**GTPγS-binding Assays—BODIPY FL GTPγS assays were carried out as described (37). Briefly, BODIPY-nucleotide binding to Go subunit was determined in 10 mM Hepes, 1 mM EDTA, and 10 mM MgCl₂, pH 8.0. Fluorescence measurements on a second to minute time scale were made in a 10-mm cell using a Cary Eclipse fluorimeter equipped with an RX.2000 rapid mixing stopped-flow unit (Applied Photophysics). In the association kinetics experiments, 1 μM ligand was added to 20 nM receptor and 200 nM Go,βγ trimer, and then binding was initiated by the addition of the BODIPY-nucleotide. The change in fluorescence was measured over time and normalized to the base-line binding of the fluorescent BODIPY analog in the absence of agonist.

**Fluorescence Measurements—**IANBD (N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine) labeling of 5-HT₄R was carried out as described for the β2-adrenergic receptor (38). Fluorescence emission spectra were recorded at 20°C on a Cary Eclipse spectrophuorimeter (Varian) with an excitation wavelength of 315 nm (bandwidth 2 nm). Receptor concentrations were in the 10⁻⁸ to 10⁻⁷ M range. Buffer contributions were subtracted under the same experimental conditions.

**Chemical Cross-linking and Size Exclusion Chromatography—**The stoichiometry of the receptor-G protein complex was assessed as described for the BLT1 receptor. Briefly, WT dimers, purified as described above and at a concentration of 10⁻⁶ M in the presence of the agonist (serotonin in an agonist/receptor molar ratio of 1:1), and trimeric Go,βγγ (receptor to G-protein molar ratio 1:1:1) were submitted to cross-linking for 5 h, at room temperature, after the addition of dTSP (125 mM stock solution in N,N-dimethylformamide) to a final concentration of 0.5 mM. The reaction was stopped by the addition of glycine to a final concentration of 50 mM. Cross-linked species were submitted to size exclusion chromatography, and the detergent was extracted as described (28). The resulting protein mixture was loaded onto a Superdex S200 HR column (10 × 300 mm; GE Healthcare). The chromatographic retention times were standardized using a gel filtration standard kit (Bio-Rad) comprising IgG (158 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). The protein composition of each peak after elution was assessed by SDS-PAGE under reducing conditions (2-mercaptoethanol).

**RESULTS**

**Pharmacological Properties of the 5-HT₄R Mutants Used in This Study—**To study the functional role of the second protomer of 5-HT₄R dimers, we used different 5-HT₄R mutants. The D100A mutant (supplemental Fig. 1) does not bind to 5-HT but can still be activated by synthetic molecules, such as the full agonist BIMU8 and also ML 10375, which acts as a full antagonist at WT 5-HT₄Rs (Fig. 1) (34). This mutant is one of the recently designed RASSLs (receptors activated solely by synthetic ligands) (34, 39). The D66N mutant poorly activates Go protein, does not display constitutive activity, and does not activate phospholipase C (PLC) (see Fig. 1 for cAMP data and Ref. 40 for cAMP and Ca²⁺/inositol phosphate data). The DD double mutant (D100A/D66N mutation) does not bind to 5-HT and shows impaired Go protein-coupling. (Fig. 1) (40). The T104A mutant is activated by 5-HT and BIMU8 but not by benzamides, such as zacopride (Fig. 1) (41).

**Dimerization of 5-HT₄R at the Cell Surface—**Previous studies reported that 5-HT₄Rs form constitutive dimers in living cells (42). We used TR-FRET tools (32) to confirm that WT 5-HT₄R monomers could form homodimers and also heterodimers with mutant 5-HT₄Rs at the cell surface. To this aim, WT and mutant 5-HT₄Rs were tagged N-terminally with HA or FLAG epitopes and transiently co-transfected in COS-7 cells. As a positive control of constitutive dimerization, we used HA or FLAG-tagged GB₁ and GB₂ GABAB receptor subunits. Similar amounts of 5-HT₄Rs and GABAB₂ receptors were correctly expressed at the cell surface (Fig. 2A). However, the TR-FRET signal detected when HA- and FLAG-5-HT₄R were co-expressed (Fig. 2B) represented only 30% of the signal obtained for GABAB₂ heterodimers. This difference might be explained by the fact that the GABAB² receptors expressed at the cell surface were obligatory heterodimers, whereas HA-5-HT₄R monomers could associate with either HA-5-HT₄R or FLAG-5-HT₄R. Hence, HA-5-HT₄R-FLAG-5-HT₄R dimers, which are the only couples producing FRET, represented only half of the real amount of dimers at the cell surface. One can thus assume that the real signal for 5-HT₄R dimers was around 60% of the GABAB₂ FRET signal. Similarly, all of the 5-HT₄R mutants were correctly expressed at the cell surface (Fig. 2E) and produced FRET signals comparable with those of WT 5-HT₄Rs dimers (Fig. 2F). Formation of 5-HT₄R homo- or heterodimers was further confirmed by co-immunoprecipitation (supplemental Fig. 2).

We then maintained a constant density of HA-5-HT₄R (donors) and increased the density of FLAG-5-HT₄R or FLAG-GB₂ (acceptors). Using WT or DD 5-HT₄Rs, we obtained saturating FRET curves, whereas the signal between 5-HT₄R and GB₂ remained linear and unsaturable (Fig. 2C). These results indicate that 5-HT₄R homodimerization was specific, whereas the 5-HT₄R-GB₂ signal reflected a collisional and nonspecific contact as suggested also by their FRET emission that corresponded to only 8% of the signal for GABAB₂ heterodimers (Fig. 2A).

To further investigate the specificity of 5-HT₄R homodimerization, we performed competition experiments. Constant amounts of HA- and FLAG-5-HT₄R were co-expressed with increasing amounts of competing GPCRs belonging to different receptors.
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A

|       | 5-HT | ML 10375 | BIMU8 | Zacopride |
|-------|------|----------|-------|-----------|
| WT    | 4.2 ± 1.4 10^{-5} | ND      | 1.9 ± 0.8 10^{-5} | 1.2 ± 1.1 10^{-7} |
| D100A | ND   | 6.4 ± 1.2 10^{-5} | 2.2 ± 1.0 10^{-9} | ND         |
| D66N  | 1.9 ± 1.4 10^{-5} | ND      | 4.7 ± 1.8 10^{-5} | ND         |
| D66N-D100A (DD) | ND   | 2.8 ± 2.2 10^{-3} | 4.5 ± 1.2 10^{-5} | ND         |
| T104A | 5.5 ± 1.2 10^{-5} | ND      | 3.1 ± 1.2 10^{-5} | ND         |

B

C

Expression

Cell surface expression (percentage of control, mean ± SD)

Binding

cAMP accumulation

IP1 accumulation

S-HT
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classes: class A (5-HT\textsubscript{7},R and thyroid-stimulating hormone receptor), class B (PAC\textsubscript{1},R), and class C (mGlu\textsubscript{7},R and GABA\textsubscript{B2},R). GABA\textsubscript{B2},R (GB\textsubscript{2}) was unable to compete with 5-HT\textsubscript{4},R homodimerization (Fig. 2D). Thyroid-stimulating hormone receptor, mGlu\textsubscript{7},R, PAC\textsubscript{1},R, and 5-HT\textsubscript{4},R competed with the formation of 5-HT\textsubscript{4},R homodimers to different extents but only at high and probably not physiological concentrations. By contrast, Myc-5-HT\textsubscript{4},R potently reduced the FRET signal and competed with HA- and FLAG-5-HT\textsubscript{4},R for dimer formation.

**Agonist Occupation of Two 5-HT\textsubscript{4},R Protomers Activates the G\textsubscript{s} Signaling Pathway More Efficiently than Occupation of a Single Protomer**—We first verified that the densities of the expressed receptors (assessed by ELISA and radioligand binding assays; see Fig. 1C for WT 5-HT\textsubscript{4},R) were proportional to the quantity of transfected cDNA (up to 250 ng/10\textsuperscript{7} cells). The magnitude of second messenger accumulation (cAMP or IP\textsubscript{1}) was also proportional to the receptors’ densities, a prerequisite to compare the responses obtained with different dimers (Fig. 1C).

To assess whether both protomers of 5-HT\textsubscript{4},R dimers needed to be activated to induce the G\textsubscript{s} signaling pathway, we transfected COS-7 cells with WT 5-HT\textsubscript{4},R and/or the double mutant (DD) that does not bind to 5-HT and does not induce cAMP production (Figs. 1A and 3A). Exposure to 5-HT of cells transfected with the DD mutant alone did not induce cAMP production (Fig. 3B). Conversely, exposure to 5-HT of cells that expressed WT 5-HT\textsubscript{4},R alone or in combination with DD 5-HT\textsubscript{4},R provided overlapping dose-response curves (Fig. 3, A and B). Similar densities of WT or DD 5-HT\textsubscript{4},Rs were measured in cells expressing WT or DD 5-HT\textsubscript{4},R alone (4.3 ± 0.7 and 4.9 ± 1.0 pmol/mg protein, respectively) and in cells that co-expressed the two protomers (4.5 ± 0.6 and 5.6 ± 0.8 pmol/mg protein, respectively). Therefore, the total receptor density was 2 times higher in co-transfected cells than in cells transfected with either WT or DD 5-HT\textsubscript{4},R. Hence, if we arbitrarily set the dimer density in co-transfected cells at 4d, the density in cells transfected with either WT or DD 5-HT\textsubscript{4},R will be 2d (Fig. 3C).

Specifically, in co-transfected cells, three dimer species are theoretically formed (WT-WT, WT-DD, and DD-DD) and their respective densities will be d, 2d, and d (Fig. 3, A and C). If the occupation of one protomer by 5-HT is sufficient to fully activate dimers, WT-WT and WT-DD dimers would be similarly activated by 5-HT, and the total activation would be more elevated in co-transfected cells than in cells expressing WT 5-HT\textsubscript{4},R alone (H1 hypothesis; Fig. 3C). This differed from what we observed experimentally. We therefore reasoned that WT-DD dimers, in which only one protomer is activated by 5-HT, are able to generate cAMP but less efficiently (about 2 times less) than WT-WT homodimers (H2 hypothesis; Fig. 3C). In this hypothesis, the 5-HT dose-response curves in cells
expressing WT 5-HT₄R alone or in combination with DD 5-HT₄R should be comparable, which corresponds to experimental results (Fig. 3D). Further supporting this hypothesis, the D66N/D100A heterodimer occupied by ML 10375, which activates the D100A protomer but not the D66N protomer, was 50% less active than the D100A/H18528 dimer in which both protomers were occupied by ML 10375 (supplemental Fig. 3).

**FIGURE 3.** Agonist occupancy of two 5-HT₄R protomers activates Gₛ signaling more efficiently than occupancy of a single protomer. COS-7 cells were transiently transfected with plasmids encoding WT and D66N/D100A (DD) 5-HT₄Rs (50 ng) alone or in association. A, schematic representation of the theoretical dimer populations. 5-HT activates the WT protomer but not the DD mutant. B, cAMP accumulation following 5-HT stimulation of cells that express WT or/and DD receptors. Each value was expressed as the percentage of the cAMP production (5.1 ± 0.2 pmol/100,000 cells) induced by 10⁻⁵ M 5-HT in cells expressing WT 5-HT₄R. C, theoretical maximal activity (Eₘₐₓ) reached by the different dimer populations according to the H1 and H2 hypotheses (see “Results” for full development of the reasoning). Open ovals, WT protomers; black ovals, DD protomers. Their corresponding density (d) is indicated on the left of the tables. D, comparison between the experimental cAMP accumulation measured upon stimulation of WT receptors alone (2d) and the theoretical cAMP accumulation resulting from stimulation of co-expressed WT (2d) and DD (2d) receptors according to the H1 and H2 hypotheses. Error bars, S.E.

**FIGURE 4.** Comparison between the cAMP productions induced by occupancy of one or two protomers within the same dimer population. A, theoretical dimer populations expected upon expression in COS-7 cells of WT and D100A (100) 5-HT₄Rs at equivalent densities (4.1 ± 0.6 and 4.8 ± 0.9 pmol/mg protein, respectively). 5-HT only activates WT receptors. ML 10375 (ML) is an antagonist at WT receptors and an agonist at D100A receptors. BIMU8 (BIMU) activates both WT and D100A receptors. B, cAMP accumulation induced by stimulating cells with 5-HT, ML 10375, or BIMU8. Each value was expressed as the percentage of the cAMP production (9.9 ± 0.3 pmol/100,000 cells) induced by BIMU8 (10⁻⁵ M). Error bars, S.E.
Then, to compare the maximal cAMP level obtained by activating one or two protomers in the same dimer population, COS-7 cells, in which WT and D100A 5-HT4Rs were co-expressed at equivalent densities (4.1±0.6 and 4.8±0.9 pmol/mg protein, respectively), were incubated with 5-HT, ML 10375, or BIMU8 (5-HT activates only the WT protomer, ML 10375 only the D100A protomer, and BIMU8 both protomers; Fig. 4A). The maximal cAMP level measured upon BIMU8 stimulation (normalized to 100%) was about twice the level obtained following incubation with 5-HT or ML 10375 (52.9±1.1 and 49.4±1.3% of the BIMU8 maximal response, respectively; Fig. 4B). This is consistent with our hypothesis that turning on one protomer within a dimer produces about half of the activity obtained by activating both protomers.

We next modified the equilibrium between 5-HT4R dimers by progressively increasing the number of non-responding protomers (DD) while the amount of responding protomers (WT) was kept constant (Fig. 5, A and B). This resulted in a decrease in WT-WT dimers and a concomitant increment in WT-DD dimers, as indicated by TR-FRET (Fig. 5C) and co-immunoprecipitation experiments (Fig. 5D). However, 5-HT-induced cAMP production remained constant, whatever the amount of DD mutant expressed (Fig. 5E), further supporting our hypothesis that WT-DD heterodimers are less active than WT-WT homodimers (see supplemental Fig. 4 for theoretical results). As expected, cAMP production upon exposure to BIMU8, which activates both WT and DD protomers, increased concomitantly with DD expression, indicating that...
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FIGURE 6. Functional responses induced by dimers obtained by transfection of a constant amount of responding protomers and increasing concentrations of non-responding protomers. A and D, theoretical dimer populations in cells co-transfected with a constant amount (50 ng) of D100A (100) 5-HT₄R plasmid and increasing amounts of D66N (66) 5-HT₄R plasmid (A) and in cells co-transfected with a constant amount (50 ng) of WT 5-HT₄R (WT) plasmid and increasing amounts of T104A (104) 5-HT₄R plasmid (D). ML 10375 (ML) acts as an agonist at the D100A protomer and as an antagonist at the D66N mutant. Zacopride (Zaco) activates WT but not T104A protomers. B, C, E, and F, cAMP accumulation following stimulation (each at 10⁻⁴ M) of the co-expressed receptors with ML 10375 (B), zacopride (E), or BIMU8 (C and F). Each value was expressed as a percentage of the cAMP production (6.4 ± 0.4 pmol/100,000 cells) induced by BIMU8 in cells expressing D100A 5-HT₄R (B and C) or as a percentage of the cAMP production (6.1 ± 0.3 pmol/100,000 cells) induced by BIMU8 in the cells expressing WT 5-HT₄R (E and F). Error bars, S.E.

the cAMP detection system was not saturated (Fig. 5F). Similarly, an increase in cAMP production was observed following expression of increasing amounts of WT 5-HT₄R (supplemental Fig. 5). To ensure that these observations were independent of the mutants and the drugs used, we investigated the functional response generated by different dimer couples. Activation of only the D100A protomer by ML 10375 (34) in D66N-D100A dimers or induction of only the WT protomer by zacopride (41) in WT-T104A dimers did not change the production of cAMP in COS-7 cells in which constant amounts of D100A or WT 5-HT₄Rs were co-transfected with increasing concentrations of D66N or T104A 5-HT₄Rs (Fig. 6).

Protomers of 5-HT₄R Dimers Do Not Independently Couple to G Proteins—Our results suggest that agonist-induced activation of both protomers of 5-HT₄R dimers induces a 2-fold greater activation of cAMP production than occupation of one protomer. We next explored whether simultaneous occupation of the two protomers conferred to 5-HT₄R dimers a structure able to activate more efficiently a single G protein than occupation of a single protomer or whether each occupied protomer independently activated its own G protein.

To discriminate between these two possibilities, we needed a dimer combination in which a fully active protomer (D100A) was associated with a protomer that could bind to a ligand, and thus adopt an “active” conformation, but was “completely inactive” for coupling to G protein (Fig. 7A). Because a protomer that does not activate the Gₛ-cAMP pathway was not available, we took advantage of the capacity of 5-HT₄Rs to activate PLC in COS-7 cells. D66N 5-HT₄R binds to all agonists, including BIMU8 (40), but it did not induce inositol monophosphate (IP₁) production upon activation by BIMU8 (Fig. 7B). When BIMU8 was used as an agonist, the D66N-D100A dimer exhibited a level of activity comparable with that of a dimer (D100A-D100A) in which both protomers can activate the PLC effector pathway (Fig. 7B). This ruled out the hypothesis that each protomer activates its own G protein.

Conformational Switch between Protomers and Faster Activation of G Proteins upon Occupation of both Protomers—To further confirm these observations in vitro, we produced a recombinant WT-D100A 5-HT₄R dimer stabilized in detergent solution, as previously described for the BLT1 receptor (35, 36) (see Fig. 8A for the purification steps). Ligand binding experi-
ments to evaluate the formation of WT-D100A dimers indicated that 5-HT displaced only half of the tritiated antagonist bound to the purified dimeric complex (Fig. 8B).

The stoichiometry of the 5-HT4R dimer assembly was investigated by chemical cross-linking followed by size exclusion chromatography (28). A major species was observed with a chromatographic mobility compatible with that of a complex resulting from the association of a single heterotrimeric Gs protein with a receptor dimer (Fig. 9A). Indeed, the calculated mass for such a complex (181,926 Da) corresponded to the experimental value estimated from the chromatographic mobility of the molecular weight standards (Fig. 9A).

To monitor receptor activation, we mutated all of the dye-accessible cysteines of WT or D100A protomers into serine residues except for cysteine 262, which is located at the cytoplasmic end of TM6 in both protomers (see Supplemental Fig. 1 for Cys262 location). This residue was labeled with the environment-sensitive dye IANBD to selectively monitor ligand-induced changes in the conformation of the modified protomer (38). This modification did not alter their binding properties and functional status (data not shown). As demonstrated for the BLT1 receptor (36), two conformational changes were observed upon agonist activation of one protomer. The conformational change of the unliganded protomer of the dimer was smaller than the change of the agonist-ligated protomer. However, a clear conformational switch between protomers within the dimer was detected upon agonist occupation of only one protomer (Fig. 8C).

We then analyzed GTPγS binding to Goa, induced by activation of one or two protomers in purified WT-D100A dimers. The initial rate of WT-D100A-catalyzed GTPγS binding to Gαs proteins was faster in the presence of BIMU8, which activates both protomers, than in the presence of 5-HT or ML 10375, which activate only one protomer, (WT and D100A, respectively; Fig. 9B). The kinetic constants for receptor-catalyzed GTPγS binding to Gαs were 0.18 ± 0.01 min⁻¹ (with 5-HT), 0.17 ± 0.02 min⁻¹ (with ML 10375), and 0.25 ± 0.02 min⁻¹ (with BIMU8), confirming that the Gαs protein was activated more efficiently when both protomers were turned on.
experiments in which the WT or D100A protomers of purified WT-D100A dimers were labeled with the fluorescence donor (Alexa Fluor 468) at cysteine 262 (see above), and Goα was labeled at its N terminus with the acceptor (Alexa Fluor 568; see “Experimental Procedures”). This modification did not affect Goα functionality, as shown by the similar receptor-catalyzed GDP/GTP exchange rates at unmodified and modified Goα proteins (0.26 and 0.23 min⁻¹, respectively).

When the WT subunit was labeled with the fluorescence donor, challenging with 5-HT (which activates only the WT protomer) produced a much lower FRET signal than following challenge with ML 10375 (which induces the D100A protomer) (Fig. 10A). This indicates that the labeled N terminus of Goα was near the labeled WT protomer when ML 10375 was bound to the dimer and that it was in the inverse orientation (i.e. the N-terminal part of Goα was close to the D100A protomer) following challenge with 5-HT (Fig. 10B). In other words, the C terminus of Goα was close to the activated protomer, consistent with previous reports (43, 44). Moreover, the FRET signal induced by BIMU8, which binds to both protomers, was about half of the signal observed following ML 10375 stimulation and about twice the 5-HT-induced signal (Fig. 10A). These results are consistent with the assumption that the C-terminal part of Goα can move near the WT or D100A protomer, when BIMU8 activates both protomers (Fig. 10B).

Positioning the FRET donor on the D100A (instead of WT) protomer confirmed these results. Indeed, upon 5-HT binding to the WT protomer, the labeled N terminus of Goα moved
Activation of One or Two Protomers in GPCR Dimers

Dimerization has been mostly studied in terms of assembly and intracellular trafficking. Much less is known about the consequences of dimerization on receptor signaling efficiency. Here, we have addressed this question by using 5-HT₄R as a class A GPCR model and by taking advantage of a large variety of mutants, which bind or do not bind to agonists, couple or do not couple to G protein, and can be studied in cellular contexts as well as in vitro (34, 35, 40, 41).

Using FRET and saturation binding experiments, we show that 5-HT₄Rs behave as dimers or oligomers. We then provide evidence consistent with a model in which complete activation of G signaling by 5-HT₄R dimers requires the activation of both protomers. Moreover, our data argue against independent interaction of each protomer of a dimer with a G protein to induce its activation: 1) a dimer, in which both protomers (D66N-D100A) bind to BIMU8 but one (D66N) is unable to couple to G protein, was as active as the (D100A-D100A) dimer composed of two protomers that were both capable of binding to BIMU8 and of interacting with the G protein; 2) purified 5-HT₄R dimers associated with an unique heterotrimic G protein in detergent and formed a pentameric complex, as already proposed for some class A and class C GPCRs (28, 48, 49). Further supporting this pentameric organization, we also demonstrated that the heterotrigemar G protein bound asymmetrically to 5-HT₄R homodimers or heterodimers, as shown previously for the BLT1 and mGlu1 receptors (18, 36).

Allosteric interactions between protomers of dimeric GPCRs and their consequences for G protein activation seem to be pleiotropic. Negative cooperativity, which has been reported for chemokine receptor heterodimers (50) and for glycoprotein hormone receptors (12), is consistent with an activation mechanism in which a single ligand molecule binds to a receptor dimer (at least at low concentration). Javitch and co-workers (22) recently reported maximal activation of the dopamine D2 receptor fused to a Gq5 chimera upon agonist binding to a single protomer. Arcemisbheire et al. (51) found that purified BLT2 receptor monomers in solution activated G₁₂ proteins (GTPγS binding) more efficiently than a solution of dimers at the same receptor concentration. In metabotropic glutamate receptors (mGluRs) (18) as well as in BLT1 receptors (36), only one heptahelical domain is turned on upon activation of these homodimeric receptors when both binding sites are occupied. Conversely, occupation of both protomers is required for full G protein activation by mGluRs (26). Similarly, binding of two ligand molecules is mandatory for 5-HT₂C receptor function (27). Furthermore, each subunit of the yeast α-factor receptor is activated independently by agonists, and the subunits cooperate in activating G proteins (52). Other experiments suggest that interaction of M3 muscarinic receptor dimers with β-Arrestin-1 and the subsequent activation of ERK1/2 require binding of an agonist to each receptor protomer (53). In agreement with these last reports, our study demonstrates that the binding of two agonists to 5-HT₄R dimers is required to provide full G protein activation.

How two 5-HT₄R protomers cooperate to almost double the level of G protein activation in comparison with when a single protomer is activated is unknown. Induction of two subunits leads to a higher GTPγS incorporation rate than following
activation of a single subunit. Further work is without any doubt necessary to determine how 5-HT₄R dimers enhance the GDP/GTP exchange rate. The partial conformational change induced by the activated protomer in the unresponsive protomer observed in biophysical experiments might reflect cross-reactions between both protomers. Thus, when one protomer is occupied by the agonist, the other one adopts a conformation that is different from that of a naive protomer. The hypothesis that binding of a ligand to one protomer might induce a structural change in the other protomer is in agreement with structural data obtained for the chemokine receptor CXCR4 (14). Accordingly, two liganded protomers might form an asymmetric structure when they are occupied by the same agonist (see model in supplemental Fig. 6). Because GPCRs contact both Gα and Gβγ subunits (43), we propose that one 5-HT₄R protomer might contact Gα and the other one might contact Gβγ, as already suggested by Johnston and Siderovski (44). This is consistent with the observations made on family A and C GPCRs (19, 48) and with the present findings, which show asymmetric binding of the G protein to a dimer.

The mono-, di-, or oligomeric structure of rhodopsin molecules is still a matter of debate. As rhodopsin forms dimers, one can easily understand that the possibility to obtain a response upon occupancy of a single protomer is physiologically relevant (54). Despite a high similarity in their secondary structure, GPCRs respond to a wide range of agonists (from photons to neurotransmitters in synapses) via pleiotropic molecular mechanisms. In this context, the possibility to generate a graduated response, depending on the occupation of one or two binding sites in a GPCR dimer, is an adaptive advantage. This might be especially relevant in the brain, where GPCRs are often localized at the periphery of synapses (e.g. group I mGluRs (55)), far from presynaptic releasing sites (e.g. 5-HT receptors (56)).

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Activation of One or Two Protomers in GPCR Dimers
G Protein Activation by Serotonin Type 4 Receptor Dimers: EVIDENCE THAT TURNING ON TWO PROTOMERS IS MORE EFFICIENT
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SUPPLEMENTARY FIGURES LEGENDS
Supplementary Figure 1
Supplementary Figure 2

**A**

**Input**

Immunoblotting

Anti-RhoTag

Dimers

Monomers

**Immunoprecipitation**

Anti-cMyc

**B**

Dimers

NS

Monomers

**C**

Dimers

NS

Monomers

Δ329

Immunoblotting

Anti-cMyc

 WT  WT  WT  WT

 Δ  Δ  Δ  Δ  C

Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Mutants of the 5-HT4 receptor used in this study.
Snake diagram of the 5-HT4 receptor showing the position of the mutants (black circles) and of the IANBD- or Alexa-Fluor 468-labeled cysteine (dark grey circle). Amino acid numbers are indicated using both the classical numbering and Ballestero’s nomenclature (in brackets). Dye-labeling of cysteine 262 is represented by a star.

Supplementary Figure 2: 5-HT4R dimerization assessed by co-immunoprecipitation.
(A) Inputs of the immunoprecipitation experiments. RhoTag-5-HT4R (WT, black flag) was expressed alone or with Myc-5-HT4R (WT, white flag), Myc-D66N-D100A (DD) or Myc-Δ329 (Δ,5-HT4R mutant truncated after the two palmitoylated cysteines and thus devoid of the C-terminal domain) as indicated under panel C. (B) Immunoprecipitation was performed using anti-cMyc antibodies and the receptors were detected using anti-RhoTag antibodies. (C) Immunoprecipitation was performed with anti-cMyc antibodies and receptor detection with anti-cMyc antibodies. Monomers are indicated by a black arrowhead, dimers by a black double arrowhead and Δ329 monomers by a grey arrowhead.

Supplementary Figure 3: Activation of one protomer in 5-HT4R dimers by a ligand that acts as an antagonist at the other protomer.
COS-7 cells were transiently transfected with plasmids (50 ng) encoding D100A (100) or/and D66N (66) 5-HT4Rs. Receptor densities were 4.4 ± 0.7 and 3.9 ± 1.0 pmol/mg proteins in cells expressing D100A or D66N 5-HT4R alone and 4.6 ± 0.8 and 4.1 ± 1.1 pmol/mg proteins, respectively, in co-transfected cells. (A) Schematic representation of the expected theoretical dimer populations in co-expression experiments. ML 10375 (ML) acts as an agonist at the D100A receptor and as an antagonist at the D66N receptor. (B) cAMP accumulation following ML 10375 stimulation of D100A and D66N receptors expressed alone or together. Each value represents the percentage of the cAMP production (3.1 ± 0.2 pmol/100 000 cells) induced by ML 10375 (10^-5 M) in cells expressing D100A. (C) Theoretical maximal activity (E_{max}) reached by the dimer populations according to the H1 and H2 hypotheses (see text for full development of the reasoning). D100A protomers are depicted by 4 open ovals and D66N protomers by 4 black ovals. Their corresponding density (2d) is indicated on the left of the tables. (D) Comparison between the experimentally measured ML 10375-induced cAMP accumulation resulting from the stimulation of D100A receptor alone (2d) and the theoretical ML 10375-induced cAMP accumulation resulting from stimulation of co-expressed D100A (2d) and D66N (2d) receptors according to the H1 or H2 hypothesis.

Supplementary Figure 4: Theoretical responses induced by dimers composed of a WT protomer and a non-responding protomer.
Upper panel: theoretical 5-HT-stimulated cAMP accumulation when a constant amount of responding protomers (WT, open ovals) is co-expressed with increasing amounts of non-responding protomers (DD, black ovals), according to the H1 and H2 hypotheses. Lower panel: theoretical maximal activity (E_{max}) of the different dimer populations according to the H1 and H2 hypotheses. WT protomers are depicted by 4 open ovals and DD protomers by 4 black ovals. Their corresponding density (2d) is indicated on the left of the tables.

Supplementary Figure 5: Functional response induced by increasing densities of HA-WT/FLAG-WT 5-HT4R dimers.
(A) Theoretical expected dimer populations in cells co-transfected with a constant amount (50 ng) of cDNA encoding WT HA-5-HT4R (WT/white box with white flag) and increasing amounts of cDNA encoding WT FLAG-5-HT4R (WT/white box with black flag). 5-HT activates all WT protomers. (B) Cell surface expression of WT HA-5-HT4R co-expressed with increasing amounts of WT FLAG-5-HT4R. ELISA was carried out using anti-HA (white bars) or anti-FLAG (black bars) antibodies in non-permeabilized transfected COS-7 cells. 5-HT4R densities were 4.3 ± 0.7 in cells transfected with HA-5-HT4R alone; and 9.1 ± 0.9; 13.5 ± 1.0 and 21.9 ± 1.2 pmol/mg proteins in cells co-transfected with
HA-5-HT₃R and 50, 100 or 200 ng of WT FLAG-5-HT₄R plasmid, respectively. (C) cAMP accumulation following stimulation with 5-HT of COS-7 cells that express WT HA-5-HT₃R and WT FLAG-5-HT₄R.

Supplementary Figure 6: Gradual activation of G proteins following activation of one or two protomers in 5-HT₃R dimers.
The two protomers of a dimer with empty binding pockets are depicted by deep blue squares. Binding of a first agonist molecule (turquoise square) induces conformational changes that result in: 1) activation of the loaded protomer, 2) coupling of the activated protomer to the G protein (the C-terminus of Gα is located under the loaded protomer), and 3) partial activation of the Gₛ protein (symbolized by one +). Since theses conformational modifications are partially transmitted to the unloaded protomer (arrow), the dimer becomes asymmetric. Binding of a second agonist molecule (if the concentration is sufficient) induces additional conformational movements in both protomers that result in higher activation of the G protein (symbolized ++).