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Running title: 5-HT₄ receptor dimers

Serotonin Type 4 Receptor Dimers

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Abstract

Numerous class A G protein-coupled receptors and especially biogenic amine receptors have been reported to form homodimers. Indeed, the dimerization process might occur for all the metabotropic serotonergic receptors. Moreover, dimerization appears to be essential for the function of serotonin type 2C (5-HT_{2C}) and type 4 (5-HT₄) receptors and required to obtain full receptor activity. Several techniques have been developed to analyze dimer formation and properties. Due to our involvement in deciphering 5-HT₄R transduction mechanisms, we improved and set up new procedures to study 5-HT₄R dimers, by classical methods or modern tools. This chapter presents detailed protocols to detect 5-HT₄R dimers by western blotting and co-immunoprecipitation, including the optimizations that we routinely carry out. We developed an innovative method to achieve functional visualization of 5-HT₄R dimers by immunofluorescence, taking advantage of the 5-HT₄-RASSL (Receptor Activated Solely by

Synthetic Ligand) mutant that was engineered in the laboratory. Finally, we adapted the powerful Time-resolved FRET technology to assess a relative quantification of dimer formation and affinity.

Keywords: dimerization; oligomerization; GPCR; serotonin receptor; dimer visualization

I. Introduction

Serotonin type 4 receptors (5-HT₄Rs) belong to the extended family of serotonin receptors, which counts 15 different types of receptors involved in a wide range of physiological processes (Berger, Gray, & Roth, 2009). All, except 5-HT₃ receptor which is an ionic channel, are G protein-coupled receptors activating G_s-, G_i- or G_q-dependent pathways as well as G protein-independent signaling cascades (Barnes & Sharp, 1999; Bockaert, Claeysen, Becamel, Dumuis, & Marin, 2006; Millan, Marin, Bockaert, & Mannoury la Cour, 2008). Homodimerization of serotonin receptors have been described for the 5-HT_{1A} (Gorinski et al., 2012), 5-HT_{1B/D} (Lee et al., 2000), 5-HT_{2A} (Lukasiewicz, Faron-Gorecka, Kedracka-Krok, & Dziedzicka-Wasylewska, 2011), 5-HT_{2C} (Herrick-Davis, Grinde, & Mazurkiewicz, 2004), 5-HT₄ (Berthouze et al., 2005) and 5-HT₇ (Renner et al., 2012), suggesting that all metabotropic serotonergic receptors form constitutive homodimers. This dimerization process is essential for receptor function. Indeed, the full activity of 5-HT_{2C} and 5-HT₄ receptors has been obtained with the binding of two molecules of ligand and one G protein per dimer (Herrick-Davis, Grinde, Harrigan, & Mazurkiewicz, 2005; Pellissier et al., 2011).

Our team has been involved in the first pharmacological description of 5-HT₄ receptors (Dumuis, Bouhelal, Sebben, Cory, & Bockaert, 1988), in the cloning of some splice variants (Claeysen, Sebben, Becamel, Bockaert, & Dumuis, 1999; Claeysen, Sebben, Journot, Bockaert, & Dumuis, 1996) and in the characterization of several original signaling pathways following the activation of these receptors (Bockaert, Claeysen, Compan, & Dumuis, 2011). For years, we have developed methods and tools to detect 5-HT₄ receptor dimers that we present below. Ranging from classical to more sophisticated methods, we intend to provide here the hints and tips that facilitate 5-HT₄R dimerization study.

We will describe the following procedures:

1. Cell transfection
2. Detection of 5-HT₄R dimers by western blot
3. Detection of 5-HT₄R dimers by co-immunoprecipitation
4. Analysis of 5-HT₄R dimers by immunofluorescence
5. Analysis of 5-HT₄R dimers by Time-Resolved Förster Resonance Energy Transfer (TR-FRET).

II. Materials

1. PBS: Phosphate-buffered saline, pH 7.4, LONZA, DPBS-10X without Ca^{2+} and Mg^{2+} , #BE17-515F.
2. Trypsine EDTA solution 1X, Life Technologies, #2530096
3. EP1X: electroporation mix composed of 50 mM K_2HPO_4 , 20 mM $\text{CH}_3\text{CO}_2\text{K}$, 20 mM KOH and 26,7 mM MgSO_4 , in water. Adjust the pH to 7.4 with acetic acid.
4. 0.4 cm electroporation cuvettes, Eurogentec, #CE-0004-50.
5. Falcon 100 or 150 mm cell culture dishes, cluster of 12, 24 or 96 wells, 12 mL Falcon tubes (BD Biosciences).
6. Microtubes: 1.5mL capacity.
7. DMEM-10% dFBS: DMEM (DMEM 4.5g/L Glucose with L-Glutamine, LONZA, #BE12-604F) supplemented with 10% dialyzed fetal bovine serum (Lonza, #14-810F).
8. Myc-tagged and HA-tagged 5-HT₄ receptors cDNA in plasmid suitable for expression in mammalian cells (e. g. pRK5 or pcDNA3.1). Epitopes are located at the N-terminus of the receptor. The signal peptide (SP) from the metabotropic glutamate receptor type 5 is added before HA-tag whereas no SP is necessary with Myc-tag.
9. Empty plasmid cDNA used as carrier and control (e. g. pRK5 or pcDNA3.1).
10. Rubber policeman: 34mm wide rubber scrapper "Model K" on Saint-Gobain, Verneret Plastic catalog, #V101029, ROGO-SAMPAIC (France) or AUXILAB S.L (Spain).
11. Potter homogenizer: 1 mL capacity PYREX® Potter tissue homogenizer, Corning, #7725T-1.
12. Protease inhibitors: cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Applied Science, #04693132001.
13. Tris-Lysis Buffer: 10 mM Tris-HCl, pH =7.4; 2 mM EDTA; protease inhibitors.
14. Bradford reagent: Quick Start Bradford 1x Dye Reagent, Biorad, #500-0205.
15. Solubilization Buffer: 50 mM $\text{NaHPO}_4/\text{NaH}_2\text{PO}_4$, pH = 7.2; 1 mM EDTA; 1% SDS or 10 mM CHAPS; protease inhibitors.
16. Deglycosylation Buffer: 50 mM $\text{NaHPO}_4/\text{NaH}_2\text{PO}_4$, pH = 7.2; 10 mM EDTA; 1% SDS or 10 mM CHAPS; protease inhibitors.

17. N-glycosydase F: PNGaseF, New England Biolabs, #P0704S.
18. Laemmli Buffer 4X: 200 mM Tris/HCl, pH = 6.8; 8% SDS; 40% glycerol; 20% β -mercaptoethanol; bromophenol blue.
19. TBST: TBS (20 mM Tris/HCl, pH = 7.4; 150 mM NaCl); 0.25% Tween-20.
20. TBST-milk: TBST, 5% milk.
21. TBST-BSA: TBST, 5% BSA (Albumin from bovine serum, Sigma, #A2153)
22. Ms anti-Myc antibody: Mouse anti-c-Myc antibody, Sigma Aldrich 9E10, #M4439.
23. Rb anti-Myc antibody: Rabbit anti-c-Myc antibody, Santa Cruz Biotechnology, sc789, #D1008.
24. Ms anti-HA antibody: Mouse anti-HA antibody, Covance, MMS-101P, #E11AF0013.
25. Rb anti-HA antibody: Rabbit anti-HA antibody, Life technologies, #71-5500.
26. Anti-mouse antibody conjugated with HRP: ECL Mouse IgG, HRP-linked whole A β , GE Healthcare; #NA931.
27. GAR-red: Alexa Fluor® 594 Goat Anti-Rabbit IgG, Life Technologies, #A-11012
28. GAM-green: Alexa Fluor® 488 Goat Anti-Mouse IgG, Life Technologies, #A-11001
29. Pierce ECL Western Blotting Substrate: Thermo Scientific, #32209.
30. DSP: Dithiobis[succinimidyl propionate], Thermo Scientific, #22586. First dilution of DSP at 25 mM in DMSO, then final dilution at 1.25 mM in PBS.
31. Phosphatase inhibitors: 10mM NaF; 2 mM Na⁺vanadate; 1 mM Na⁺pyrophosphate.
32. DDM: n-Dodecyl β -D-maltoside, Sigma, #D5172.
33. DDM-Lysis Buffer: for 10 ml of buffer, 40 mg of DDM, 20 mM HEPES, 150mM NaCl, 1% NP40, 10% Glycerol, phosphatase inhibitors, half a tablet of protease inhibitors, adjust the volume with water.
34. Anti-HA/agarose beads: Mouse anti-HA-tag monoclonal antibody, agarose-conjugated, Sigma, #DMAB8895.
35. Glass slides and coverslips (\varnothing 18 mm).
36. PORN 1X: Poly-L-ornithine hydrobromide (10 mg/L in PBS), Sigma, #P3655.

37. PFA: Paraformaldehyde 16% in water, Euromedex, #RT 15710-S.
38. PBS-Glycine: PBS, 0.1M Glycine (Sigma, #G7126).
39. PBS-Triton: PBS, 0.05% Triton X-100 (Sigma, #T8532).
40. PBS-Gelatin: PBS, 0.2% Gelatin (Sigma, #G9391).
41. 5-HT: 5-Hydroxytryptamine (Serotonin creatinine sulfate monohydrate, Sigma, #H7752)
42. BIMU 8: 2,3-Dihydro-N-[(3-endo)-8-methyl-8--azabicyclo[3.2.1]oct-3-yl]-3-(1-methylethyl)-2-oxo--1H-benzimidazole-1-carboxamide hydrochloride, Tocris Bioscience, #4374.
43. VECTASHIELD: VECTASHIELD Mounting Medium, Vector Laboratories, #H-1000.
44. HBS: 20 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 0.1% glucose, 0.1% BSA.
45. HBS-KF: HSB, 200mM KF.
46. Anti HA-K: Eu³⁺ Cryptate-conjugated mouse monoclonal antibody anti-HA, Cisbio Bioassays, # 610HAKLA.
47. Anti HA-d2: d2-conjugated mouse monoclonal antibody anti-HA, Cisbio Bioassays, # 610HADAA.
48. Anti FLAG M2-K: Eu³⁺ Cryptate-conjugated mouse monoclonal antibody anti-FLAG, Cisbio Bioassays, # 61FG2KLA.
49. Anti FLAG M2-d2: d2-conjugated mouse monoclonal antibody anti-FLAG, Cisbio Bioassays, # 61FG2DLA.

III. Methods

A. Cell transfection

Our protocols are based on transient transfection of COS-7 cells or HEK293 cells by electroporation as described in (Claeysen et al., 1996). Wash cells at 70% confluence once in PBS, trypsinize them and, after centrifugation, resuspend them in EP1X buffer with 25-500 ng of epitope-tagged receptor cDNA and 15 µg empty plasmid that acts as carrier. Transfer 300 µL of cell suspension (10⁷ cells) to a 0.4 cm electroporation cuvette and pulse the cells using a Gene pulser apparatus (settings: 950 µF and

280 V or 270 V for COS-7 or HEK293, respectively). Quickly after the shock, dilute cells in DMEM (10^7 cells/mL) containing 10% dialyzed fetal bovine serum (dFBS) and plate them on 100 or 150 mm Falcon cell culture dishes or into appropriated clusters. 24h post-transfection process the cells to study 5-HT₄R dimers.

B. Detection of 5-HT₄R dimers by western blot

Dimerisation of 5-HT₄ receptors can be evaluated by western blotting in denaturing conditions. Indeed 5-HT₄R dimers form with high affinity and resist to detergents. Four mouse splice variants of the 5-HT₄ receptor have been described that differ in length and composition of their C-terminus: 5-HT_{4(a)}, (b), (e) and (f) with 387, 388, 371 and 363 amino acids, respectively (Claeyssen et al., 1999) (Figure 1A). We used the difference in length of these variants to show that they can interact with each other. We also used a truncated 5-HT₄ receptor at the residue 327: Δ 327 that is devoid of the C-terminus of the receptor (Figure 1A).

Cell lysate and membrane preparation

Dimerization of 5-HT₄ receptors is analyzed on membrane receptor preparations. Plate four electroporations of COS-7 cells (4×10^7 cells) in two 150 mm Falcon dishes, for each condition. Use 500 ng of Myc-tagged 5-HT₄R cDNA per 10^7 cells in single transfection assays and 250 ng of each receptor per 10^7 cells in cotransfection experiments. 24 h post-transfection, wash the cells with ice-cold PBS, then add 5 mL of cold PBS per 150-mm plate and scrape the cells on ice with a rubber policeman. Transfer the content of two dishes in one 12 mL Falcon tube. After centrifugation for 5 min at 2,400 g and at 4°C, resuspend each cell pellet in 500 μ L of Tris-Lysis Buffer and transfer in 1 mL capacity Potter homogenizer. After 20 up and down regular and gentle moves of the pestle in the glass mortar, transfer the cell homogenates in 1.5 mL microtubes and pellet the membranes by 20-min centrifugation at 43,000 g and at 4°C. Resuspend the membrane pellet in 200 μ L of Tris-Lysis Buffer and quantify the protein levels (Bradford reagent) twice with 5 μ L of sample. Aliquot the membrane preparations (200 μ g/aliquot), freeze in liquid nitrogen and store at -80°C for further use.

Solubilization and deglycosylation of the samples

In transfected cells, 5-HT₄ receptors exist in many glycosylated forms resulting in smear bands on SDS-page electrophoresis. To circumvent this problem we add a deglycosylation step of the receptor preparation prior to gel electrophoresis.

Thaw and pellet 400 µg of each sample by 20-min centrifugation at 43,000 g and at 4°C. To solubilize the membrane proteins, resuspend the pellet in 200 µL of Solubilization Buffer and incubate 2 h on a rotating wheel in a cold room at 4°C. Then, pellet the remaining cell fragments by 20-min centrifugation at 43,000 g at 4°C and collect the supernatant. Adjust the EDTA concentration in the sample buffer to increase it to 10 mM EDTA (Deglycosylation Buffer), add 2 µL (1000 units) of N-glycosidase F and incubate the tubes overnight at 37°C. After adding 67 µL of Laemmli Buffer 4X to the sample, load 25 µg of proteins of each sample on an acrylamide gel.

Western blot and detection of the 5-HT₄ receptor dimers

Load the samples on Tris/HCl gels (8% or 10% acrylamide/bis-acrylamide) in denaturing conditions (SDS). To achieve a good separation of the different dimer bands, use 20-cm glass plate systems (Settings: stacking, 1 h at 100V, Separation 5 h at 200V). Transfer the proteins on nitrocellulose using wet-electrophoretic system (Settings: 30V, over-night at 4°C). After transfer, saturate the nitrocellulose membrane for 1h in TBST-milk, then rinse it with TBST and incubate overnight with the anti-Myc antibody diluted 1/1000 in TBST-milk, under gentle agitation at 4°C. Wash the membrane six times, 5 min, in TBST, then incubate it for 1 h with the secondary antibody (e. g. anti-mouse antibody conjugated with HRP, 1/4000) diluted in TBST-milk, under gentle agitation at room temperature. Wash the membrane six times, 5 min, in TBST, then reveal the bands using a chemiluminescent kit according to manufacturer instructions (e. g. Pierce ECL Western Blotting Substrate)

Using this technique we are capable to detect monomers and dimers form for each 5-HT₄ receptor variant (Figure 1A). By co-transfection of a "long" variant with a "short" one e. g. Variant (a) with variant (e), we can discriminate the dimer formation of (a) and (e) protomers (Figure 1B). 5-HT₄

receptor dimers can also be detected using a receptor devoid of its C-terminal domain (Figure 1C) and this truncated receptor is capable to associate with (a) or (b) variants (Figure 1D).

To resume, the key steps in this technique are 1) to start with a membrane preparation of proteins, 2) to apply a deglycosylation step to the samples and 3) to use long-separation gels.

C. Detection of 5-HT₄R dimers by co-immunoprecipitation

Co-immunoprecipitation is a classical technique to demonstrate interactions between proteins. We describe below our protocol to co-immunoprecipitate 5-HT₄ receptors.

Transfect 10⁷ HEK293 cells with Myc-tagged and HA-tagged 5-HT₄ receptors, alone or in combination (500 ng and 300 ng of each construct, respectively, as HA-tagged receptor are expressed more easily) and seed the cells in 150 mm Falcon petri dish in DMEM-10% dFBS. 24 h post-transfection, apply 15-20 mL of the cross-linking agent DSP in PBS for 30 min at 37°C. Stop the reaction by washing the cells twice with PBS. Then, add 1mL of DDM-Lysis Buffer per dish and scrape the cells on ice using a rubber policeman. Collect cell lysates in microtubes. Incubate samples for 1 h at 4°C on a rotating wheel. Centrifuge the samples at 20.000 g for 15 min at 4°C and keep the supernatants containing solubilized proteins. Quantify the protein concentration (e. g. Bradford reagent). 1 mg of solubilized proteins should then be incubated overnight at 4°C with anti-HA/agarose beads on a rotating wheel. Centrifuge at 5.000 g for 1 min at 4°C to pellet the beads and remove the supernatant. Wash the beads 3 times using 1 mL of DDM-Lysis Buffer. Resuspend the beads in 40 µL of Laemmli Buffer 1X to elute the immunoprecipitated proteins. Load 40 µL of each sample on 12% acrylamide/bis-acrylamide gels, resolve proteins by classical SDS-PAGE gel electrophoresis and detect them by western blotting (e. g. use Mini-Protean and Trans-Blot SD Semi-Dry Transfer Cell, Biorad). The immunoblotting protocol described above is used for detection of the receptor bands. Anti-Myc or anti-HA antibodies are diluted in diluted TBST-BSA at 1/1000 (Ms Anti-Myc), 1/400 (Rb Anti-Myc) or 1/500 (Ms Anti-HA), respectively. Typical results obtained using this protocol have been shown in (Pellissier et al., 2011).

D. Analysis of 5-HT₄R dimers by immunofluorescence

The pharmacological properties of the D¹⁰⁰A-5-HT₄ mutant receptors (D¹⁰⁰A) constitute a great advantage to visualize 5-HT₄R dimers by immunofluorescence. The Asp 100 in 5-HT₄R transmembrane domain 3 (D^{3,32} in Ballesteros-Weinstein nomenclature (Ballesteros & Weinstein, 1995)) corresponds to an aspartate residue that is well conserved in GPCRs responding to biogenic amines and involved in their binding site (Strader et al., 1991). D¹⁰⁰A point mutation suppresses the lateral side chain of this aspartate and, consequently, serotonin is unable to bind and activate the mutated 5-HT₄ receptor (Figure 1B) (Claeyens, Joubert, Sebben, Bockaert, & Dumuis, 2003). However, this receptor remains fully activable by highly selective 5-HT₄ R synthetic agonists, such as BIMU 8 (Figure 1B), thus belonging to the RASSL family (Receptor Activated Solely by Synthetic Ligands) (Conklin et al., 2008). 5-HT₄ receptors are rapidly desensitized and internalized by endocytosis after activation by an agonist. Upon 5-HT exposure, D¹⁰⁰A-5-HT₄ receptors stay at the plasma membrane, whereas wild type (WT) 5-HT₄ receptors enter inside the cell. However, if we form dimers between WT and D¹⁰⁰A protomers, these molecular complexes can undergo endocytosis, providing a way to separate and to visualize D¹⁰⁰A dimers from the other populations of 5-HT₄ receptors expressed in the cell.

Prior to cell transfection, place glass coverslips in 12 wells-clusters and incubate them with 300 µL/well of PORN 1X for minimum 30 min at 37°C. Wash the slides twice with 1 mL PBS before seeding the cells. Transfect HEK297 cells (10⁷ cells) with 300 ng of HA- or Myc-tagged 5-HT₄ receptors, alone or in combination. Resuspend them in 20 mL of DMEM-FBSd and plate 1 mL/well (500 000 cell/well). 24 h later, replace the cell culture medium by DMEM alone. 48 h post-transfection, place the clusters at 4°C in a cold room for 15 min. Remove the medium and incubate for 90 min at 4°C with the 500 µL of the primary antibodies (Rb anti-HA, 1/300 and/or Ms anti-Myc, 1/400) diluted in cold DMEM. Wash twice with cold DMEM. Place the clusters back at 37°C in the cell culture incubator under routine parameters for 15 min. Remove cell medium and add 1 mL of stimulation medium (5-HT or BIMU8, 10⁻⁵M in DMEM) or DMEM alone as control for 30 min at 37°C. Add 145 µL of PFA 16% in each well (2% final concentration) and fix the cells 10 min at 37°C. Wash the cells 3 times for 10 min at room temperature with PBS-Glycine. Then, permeabilize the cell

with 500 μ L of PBS-Triton for 5 min at room temperature. Wash 3 times for 5, 10, then 15 min with PBS-Gelatin and incubate with secondary antibodies coupled to fluorophores (e. g. GAR-red, 1/1000 and GAM-green, 1/1000) for 1 h at room temperature in the dark. Wash 3 times for 5, 10, then 15 min with PBS, then mount the coverslips on glass slide using VECTASHIELD. Image the samples using a confocal microscope. A typical experiment is presented in Figure 4.

Under basal conditions, WT-5-HT₄ receptors as well as D¹⁰⁰A-5-HT₄ receptors expressed alone or co-expressed were predominantly located at the cell surface (Fig. 3A, D, G, J, M). A marginal constitutive internalization, reflecting the constitutive activity of 5-HT₄ receptors, was detected for both WT and mutant receptors (for example some dotted labeling in Fig. 3A). Upon activation with an agonist (5-HT or BIMU8), the WT-5-HT₄ receptors were internalized as shown by both an internal dotted labeling and a decrease in cell surface labeling (Fig. 3B, C). In the presence of 5-HT, the D¹⁰⁰A mutant remained at the cell surface (Fig. 3E), whereas it internalized in the presence of BIMU8 (Fig. 3F). However, 5-HT which does not bind to D¹⁰⁰A, induced an internalization of this mutant receptor, when the D¹⁰⁰A mutant was co-expressed with WT receptor (Fig. 3K, 3N). Indeed, 5-HT induced the internalization of WT/WT dimers, (Fig. 3H and Fig. 3N, red dots) as well of WT/D¹⁰⁰A dimers (Fig. 3H, K and Fig. 3N, yellow dots) whereas D¹⁰⁰A/D¹⁰⁰A dimers remained at the cell surface (Fig. 3K and Fig. 3N, green dots). All types of complexes internalized after a 30 min-exposure with BIMU8 (Fig. 3I, L, O). The simplest explanation of these results implicates the existence of a WT/D¹⁰⁰A dimer. When the WT monomer of the WT/D¹⁰⁰A dimer was occupied by 5-HT, both WT and D¹⁰⁰A protomers were internalized.

E. Analysis of 5-HT₄R dimers by TR-FRET

TR-FRET technology provides an easy way to detect the existence of 5-HT₄ dimers and to examine their propensity to form heterodimers with other GPCRs.

Transfect COS-7 cells with the appropriate plasmids (N-terminally tagged with HA or FLAG epitopes) and seed them in 96-well plates (100 000 cells/well). Prepare 12 wells per condition. 24 h after transfection, wash the cells with HBS and incubate them at 4°C for 24 hours with the appropriate fluorescent anti-FLAG or -HA antibodies diluted in HBS-KF (KF is added to avoid quenching of

europium cryptate). In half of the wells, add 50 μ L of 4nM Anti-FLAG M2-K and 50 μ L of 10nM Anti-HA-d2. In the other half of the wells, add 50 μ L of 4nM Anti-FLAG M2-K and 50 μ L of HBS-KF. Quantification of FRET signals is performed by Homogenous Time Resolved Fluorescence (HTRF[®]) settings (Maurel et al., 2004) on appropriate apparatus (see <http://www.htrf.com/readers> for compatible readers). Express the results as the specific signal over background, Delta F, as described in (Maurel et al., 2004).

Figure 5 presents the different type of experiments that can be routinely performed using this simple and convenient technology. HA or FLAG-tagged GB₁ and GB₂ GABA_B receptor subunits are classically used as positive controls of constitutive dimerization. You have to verify that 5-HT₄ and GABA_B receptors are expressed at the cell surface in similar amounts by ELISA quantification for example (Fig. 4A). In these conditions the TR-FRET signal detected when we co-express HA- and FLAG-5-HT₄R (Fig. 4B) represents only 30% of the signal obtained for GABA_BR heterodimers. Indeed, GABA_B receptors expressed at the cell surface are obligatory heterodimers, whereas HA-5-HT₄R monomers could associate with either HA-5-HT₄ or FLAG-5-HT₄ receptors. Therefore, HA-5-HT₄R/FLAG-5-HT₄R dimers, which are the only couples producing FRET, represent only half of the real amount of dimers at the cell surface. Thus, the real signal for 5-HT₄R dimers can be assumed to be around 60% of the GABA_BR FRET signal.

By maintaining a constant density of HA-5-HT₄Rs (donors, use Anti HA-K antibodies) and increasing the density of FLAG-5-HT₄R or FLAG-GB₂ (acceptors, use Anti Flag M2-d2), saturating FRET curves are obtained for WT 5-HT₄ receptor, whereas the signal between 5-HT₄R and GB₂ is linear and unsaturable (Fig. 4C). These type of results indicate that 5-HT₄R dimerization is specific, whereas the low 5-HT₄R/GB₂ signal reflects a collisional and non-specific contact.

Competitions experiments can also be performed to assess specificity of 5-HT₄R dimerization. Co-express constant amounts of HA- and FLAG-5-HT₄R with increasing amounts of competing GPCRs, N-terminally tagged with Myc epitope: GABA_{B2}R, serotonin type 7 receptor (5-HT₇R) or β ₂-adrenergic receptor (β ₂-AR), in Figure 5D. As shown by its corresponding horizontal data-fitting line, GABA_{B2}R (GB₂) is unable to compete with 5-HT₄R dimerization (Fig. 4D). 5-HT₇ receptors compete with the formation of 5-HT₄R homodimers at relatively high and probably not physiological

concentrations. Interestingly, Myc- β_2 -AR strongly reduces the FRET signal as efficiently as Myc-5-HT₄R and competed with HA- and FLAG-5-HT₄ receptors for dimer formation, indicating the possible existence of heterodimers between 5-HT₄ and β_2 -adrenergic receptors.

IV. Discussion

In this chapter, we first described protocols that are classically used in biochemistry of GPCRs such as western blotting and co-immunoprecipitation. This part is important for us, as detecting 5-HT₄ receptors on gel from transient expression in cell lines is a tricky point. Indeed, when this receptor is overexpressed it appears in numerous bands, corresponding to different glycosylation states and maturation. The receptors populations that have reached the plasma membrane are more homogenous in term of sugar maturation, thus starting from a membrane preparation improve the results. One alternative could be to use kits to purify membrane proteins such as Qproteome Plasma Membrane Protein Kit (Qiagen, #37601), which gives good results in our hands, is less time-consuming but more expensive. Adding a deglycosylation step was also a plus to clarify the migration profile of 5-HT₄ receptors. The challenge was to find a replacement to the N-Glycosidase F from Roche that was discontinued. This enzyme was efficient in numerous buffers and the actual replacement enzymes (Peptide-N-Glycosidase F, PNGase F) from different suppliers are not as effective. Migration on long gels is another trick to separate more easily complexes of similar size that helps us to show the possibility of heteromerisation between 5-HT₄R splice variants.

We then described a method to functionally assess the formation of dimers by cross desensitization of the RASSL-5-HT₄ mutant that is unable to bind 5-HT (Claeyssen et al., 2003). In the presence of WT 5-HT₄ receptor and upon 5-HT exposure, the D¹⁰⁰A mutant is co-internalized with the WT receptor, thus providing a nice way to show D¹⁰⁰A/WT dimers. A key step in this procedure is to incubate the cells with the primary antibodies prior to fixation. This procedure avoids the small permeabilization that could be induced by paraformaldehyde. Antibodies in contact with intact cells will label only plasma membrane receptors and not receptors that are in the synthesis pathway. Consequently, the

dimers that are immunolabelled and endocytosed originate without doubt from the plasma membrane. Interestingly, our cross desensitization assay using the D¹⁰⁰A mutant and the WT 5-HT₄ receptor could also be used to assess whether a particular 5-HT₄ mutant named "M" is able to dimerize or not. If the M mutant that has to be tested retains a classic internalization profile upon 5-HT exposure, it will be co-expressed with the D¹⁰⁰A mutant. Upon 5-HT exposure, if RASSL protomers appear to be internalized, one can conclude that the M mutant is capable to dimerize with the D¹⁰⁰A protomers. This technique could thus be used as a dimerization-screening test. 5-HT₄ mutants that have been described to disrupt dimer formation (Berthouze et al., 2007) has to be assayed in our procedure to validate this hypothesis. Moreover, due to the high conservation of an aspartate in the third transmembrane domain of biogenic amine receptors (Strader et al., 1991), corresponding to the D¹⁰⁰ or D^{3.32} position, the point mutation into alanine can easily be transposed in metabotropic serotonergic receptors (Kristiansen et al., 2000) as well as other class A GPCR such as melanocortin-4 receptors (Srinivasan, Santiago, Lubrano, Vaisse, & Conklin, 2007) or histamine H1 receptors (Bakker et al., 2004) conferring similar RASSL properties to the mutated receptors. Consequently, this dimerization-screening test could be extended to other GPCR structurally related to the 5-HT₄ receptors. We finally described a protocol to achieve relative quantification of 5-HT₄R dimer formation using TR-FRET. Using this method we can also assess heteromerization with other GPCRs by competition assay. This technology is simple and reliable and can be improved using covalent binding of the acceptor and donor fluorophores to a small suicide-enzyme inserted N-terminally in the GPCRs instead of the classical tags (Comps-Agrar et al., 2011). Future developments will aim to use permeant substrates of these enzymes to provide intracellular labeling of the dimers and follow their trafficking.

V. Summary

Dimerization process is essential for 5-HT₄ receptor function. We provide here methods to analyze 5-HT₄R dimer formation ranging from classical western blotting and co-immunoprecipitation protocols to cross-internalization screening assay and TR-FRET measurements. We intend to describe in details the experimental procedures with the key points necessary to achieve precise and accurate studies regarding 5-HT₄ receptor dimerization.

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cAMP quantification, FRET measurements and ELISA were carried out using the ARPEGE Pharmacology Screening Interactome facility at the Institute of Functional Genomics (Montpellier, France)

Figure Legends

Figure 1: Mouse 5-HT₄ variants and key mutants receptors used.

A) Scheme of the mouse 5-HT₄ receptor showing the four splice variants differing in length and composition after a common splicing site (vertical bar). Position of $\Delta 327$ mutant, devoid of the C-terminal domain of the receptor, is depicted by a double-head arrow. D¹⁰⁰A mutation is indicated with a dark-blue plain circle. B) Pharmacological profile of the wild-type 5-HT₄ receptor (WT) and of the D¹⁰⁰A mutant. Both receptors are activated by the full agonist BIMU8, whereas only the WT receptor is capable to respond to the endogenous ligand serotonin (5-HT). 5-HT is unable to bind into D¹⁰⁰A mutant.

Figure 2: Analysis of 5-HT₄ receptor dimers by western blot.

All receptors used, N-terminally tagged with c-Myc epitope, have been transiently expressed in COS-7 cells and processed as described in the main text. Transfected variant or mutant receptors are indicated above the blots. A) Transfection of each variant separately show the formation of dimers and monomers of (a), (b), (e) or (f) variants. B) When variants (a) and (e) are co-transfected (right line), a band of an intermediate size between (a) and (e) homodimer bands appears that corresponds to the formation of a dimer between (a) and (e) protomers (indicated with an asterisk). C) Transfection of the $\Delta 327$ mutant devoid of its C-terminal domain shows the presence of monomers, dimers and higher molecular species with a size compatible with "trimers". D) Co-transfections of $\Delta 327$ and (a) or (b) receptors shows the capability of $\Delta 327$ mutant to interact with (a) or (b) variant by the presence of an intermediate size band between $\Delta 327$ and (a) or (b) dimer bands corresponding to a dimer composed of $\Delta 327$ and (a) or (b) protomers (indicated with an asterisk). Legend: Plain triangles, monomers of "long" variants (a) and (b); open triangles, monomers of "short" variants (e) and (e); double-triangles, dimers of "long" variants (plain) or "short" variants (open); arrows, monomers of $\Delta 327$ mutant; double-arrows, $\Delta 327$ dimers; triple-arrows, $\Delta 327$ "trimers"; NS, non-specific band.

Figure 3: Functional evidence of 5-HT₄R dimerization at the cell surface.

Myc-5-HT₄R WT (in red, **A, B, C**), RhoTag-5-HT₄R-D¹⁰⁰A (in green, **D, E, F**), or both (**G-O**) were transiently expressed in HEK-293 cells and visualized by immunofluorescence confocal microscopy. Cells are fixed in basal conditions (**A, D, G, J, M**) or after a 30 min-exposure with 10 μM of either 5-HT (**B, E, H, K, N**) or BIMU8 (**C, F, I, L, O**).

Figure 4: Study of 5-HT₄R dimerization using TR-FRET technology.

(A) COS-7 cells were transiently transfected with plasmids encoding epitope-tagged 5-HT₄R (250 ng/10⁷ cells) and/or GABA_BR (1,000 ng/10⁷ cells). Cell surface expression of 5-HT₄R and GABA_BR expressed alone or in combination was assessed by ELISA using anti-HA (in white) or anti-FLAG (in black) antibodies in non-permeabilized, transfected cells as described in (Barthet et al., 2005). GB₁: GABA_{B1}R; GB₂: GABA_{B2}R. (B) TR-FRET between donor and acceptor fluorophore-labeled antibodies directed against the HA and FLAG tags, respectively, placed at the N-terminus of 5-HT₄R and GABA_BR as exemplified underneath the graph. Tagged-GABA_B receptor subunits GB₁ and GB₂ were used as a positive control of constitutive dimerization. (C) Saturation FRET experiments. A constant amount of WT HA-5-HT₄R (donor) was co-expressed with increasing amounts of FLAG-tagged WT 5-HT₄R or GB₂ (acceptors). The FRET signal was plotted as a function of cell surface expression of the FLAG-tagged receptors determined by ELISA. (D) Competition FRET experiments. A constant amount of WT HA-5-HT₄R and FLAG-5-HT₄R was expressed and the FRET corresponding to their association was determined in the presence of increasing amounts of Myc-tagged GPCRs belonging to different classes. The FRET signal was plotted as a function of cell surface expression of the Myc-tagged receptors determined by ELISA. Challenger GPCRs: 5-HT₇R and 5-HT₄R: serotonin receptor subtypes 7 and 4, respectively; β₂-AR: β₂-adrenergic receptor; GABA_{B2}R: subunit of the GABA_B receptor that reaches the cell surface alone (GB₂).

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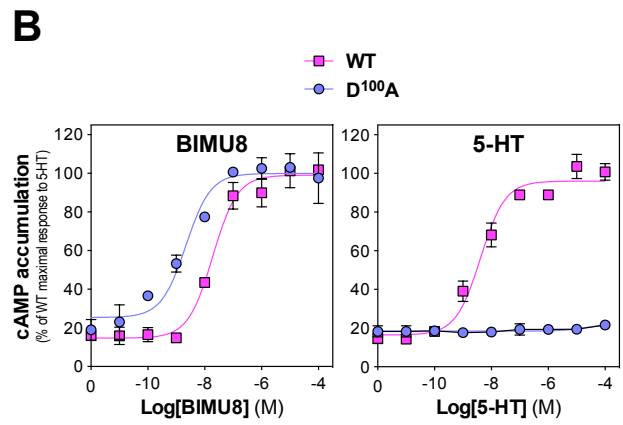
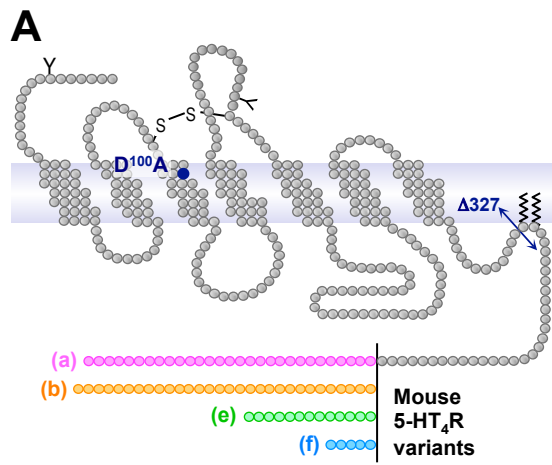


Figure 1

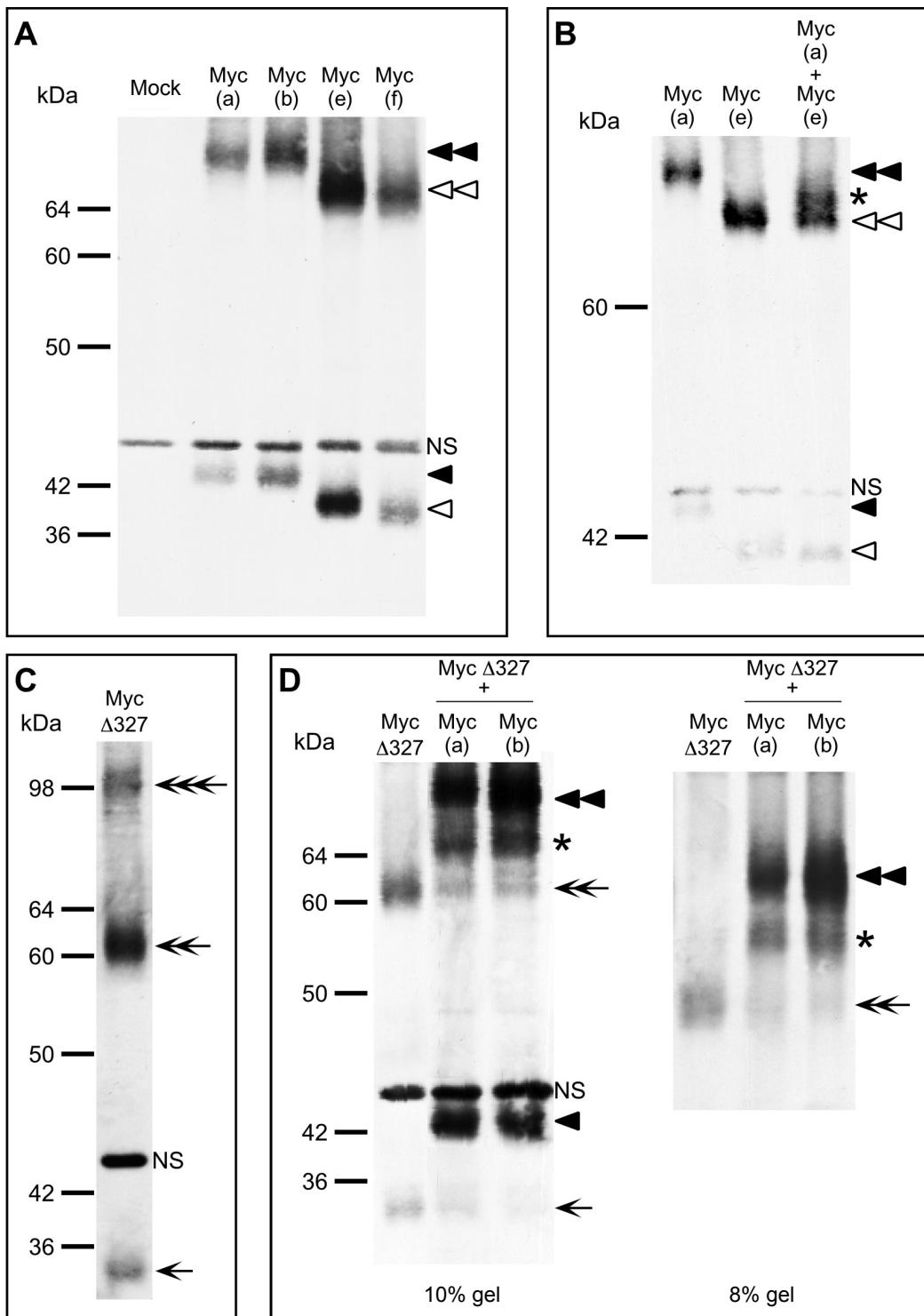


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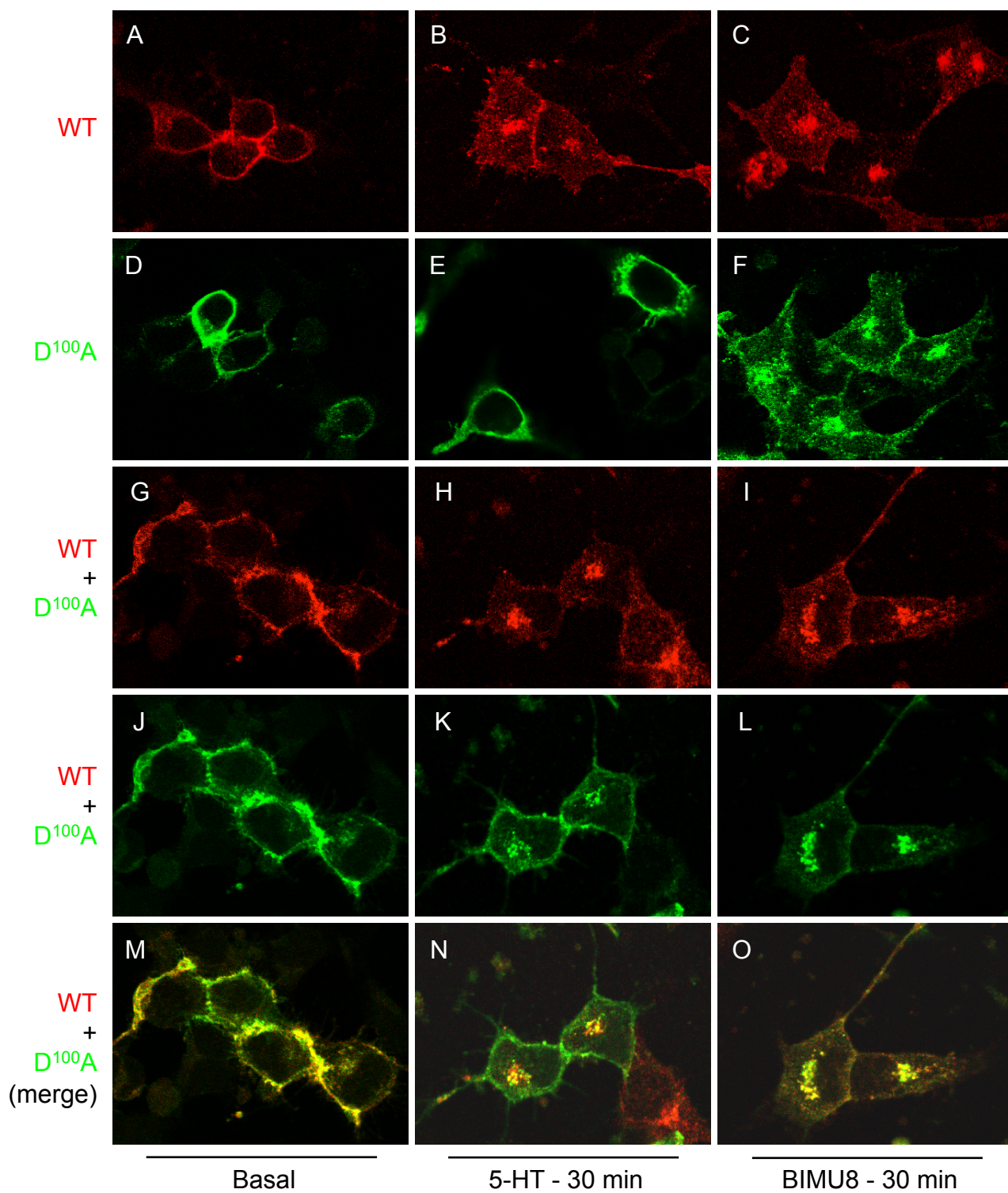


Figure 3

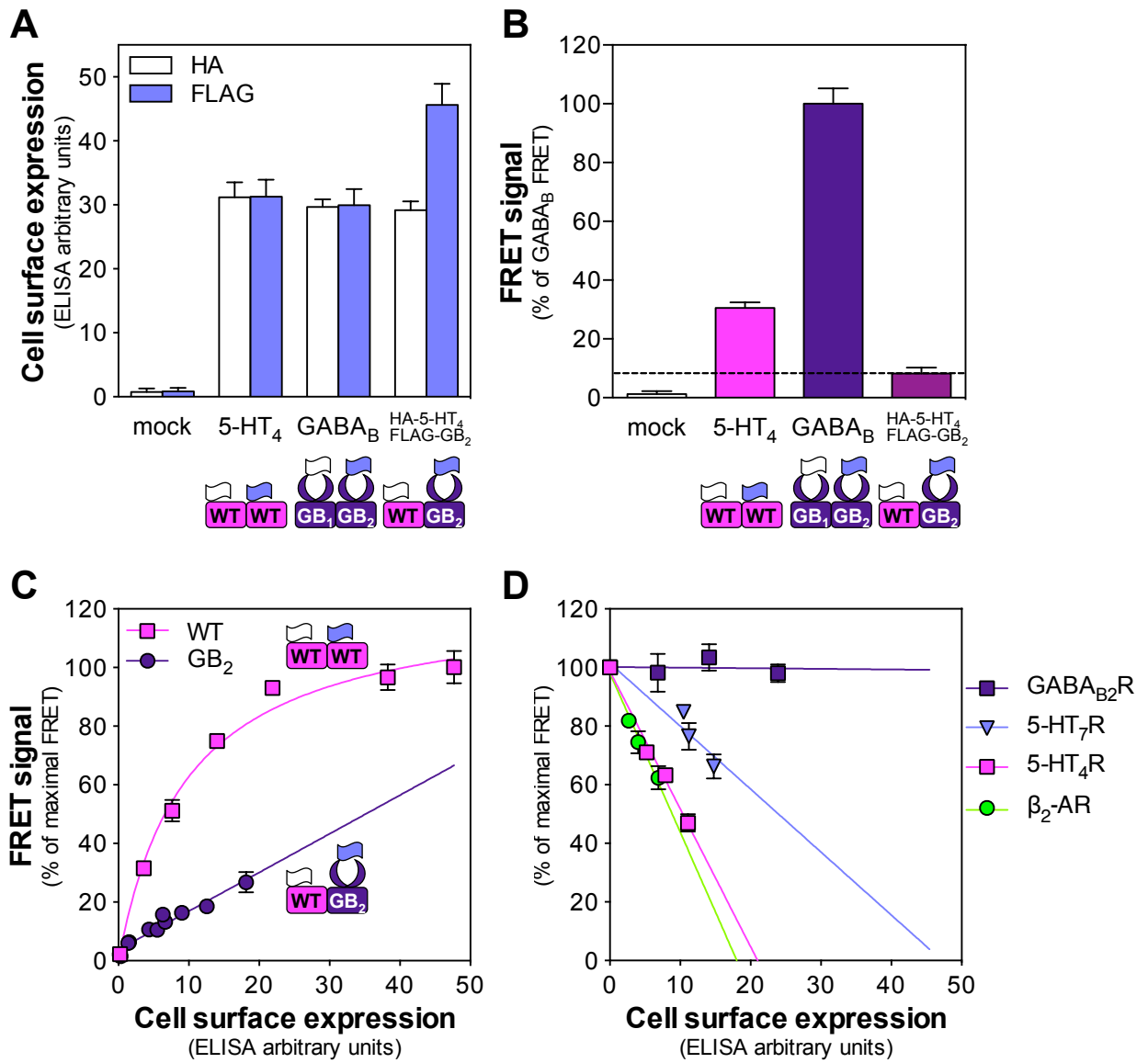


Figure 4