



Heteromerization between α_{2A} adrenoceptors and different polymorphic variants of the dopamine D₄ receptor determines pharmacological and functional differences. Implications for impulsive-control disorders

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ABSTRACT

Polymorphic alleles of the human dopamine D₄ receptor gene (*DRD4*) have been consistently associated with individual differences in personality traits and neuropsychiatric disorders, particularly between the gene encoding dopamine D_{4.7} receptor variant and attention deficit hyperactivity disorder (ADHD). The α_{2A} adrenoceptor gene has also been associated with ADHD. In fact, drugs targeting the α_{2A} adrenoceptor ($\alpha_{2A}R$), such as guanfacine, are commonly used in ADHD treatment. In view of the involvement of dopamine D₄ receptor (D₄R) and $\alpha_{2A}R$ in ADHD and impulsivity, their concurrent localization in cortical pyramidal neurons and the demonstrated ability of D₄R to form functional heteromers with other G protein-coupled receptors, in this study we evaluate whether the $\alpha_{2A}R$ forms functional heteromers with D₄R and whether these heteromers show different properties depending on the D₄R variant involved. Using cortical brain slices from hD_{4.7}R knock-in and wild-type mice, here, we demonstrate that $\alpha_{2A}R$ and D₄R heteromerize and constitute a significant functional population of cortical $\alpha_{2A}R$ and D₄R. Moreover, in cortical slices from wild-type mice and in cells transfected with $\alpha_{2A}R$ and D_{4.4}R, we detect a negative crosstalk within the heteromer. This negative crosstalk is lost in cortex from hD_{4.7}R knock-in mice and in cells expressing the D_{4.7}R polymorphic variant. We also show a lack of efficacy of D₄R ligands to promote G protein activation and signaling only within the $\alpha_{2A}R$ -D_{4.7}R heteromer. Taken together, our results suggest that $\alpha_{2A}R$ -D₄R heteromers play a pivotal role in catecholaminergic signaling in the brain cortex and are likely targets for ADHD pharmacotherapy.

Abbreviations: $\alpha_{2A}R$, α_{2A} adrenoceptor; ADHD, attention deficit hyperactivity disorder; BiLC, bimolecular luminescence complementation; BRET, bioluminescence resonance energy transfer; CODA-RET, complemented donor-acceptor resonance energy transfer; D₂R, dopamine D₂ receptor; D₄R, dopamine D₄ receptor; DA, dopamine; DMT, dexmedetomidine; FK, forskolin; NE, norepinephrine; mBU, milliBRET units; PLA, proximity ligation assay; RLuc, *Renilla* Luciferase; RT, residence time; TAT, HIV-transactivator of transcription peptide; TM, transmembrane; YFP, yellow fluorescent protein.

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L-745
 870 trihydrochloride (PubChem CID:
 49759035)
 Pramipexole dihydrochloride (PubChem CID:
 119569)
 Ropinirole (PubChem CID: 5095)
 Rotigotine hydrochloride (PubChem CID:
 180335)
 RX821002 hydrochloride (PubChem CID:
 11957683)

1. Introduction

In recent years, the role of the so-called dopamine D₄ receptor (D₄R) in the brain is shifting from it being considered a pure dopamine receptor. Several studies indicate that D₄R can indeed respond to other endogenous catecholamines [1–3]. D₄R can be targeted by norepinephrine (NE), and it is able to modulate the function of other catecholamine receptors by heteromerization. Those include pineal α_{1B} and β_1 adrenoceptors ($\alpha_{1B}R$ and β_1R , respectively) [4] and striatal dopamine D₂ receptors (D₂R) [1,5,6]. In addition, heteromerization seems to determine the functional differences of the most frequent products of the *DRD4* gene polymorphisms.

The clearest association between adrenoceptors and D₄Rs occurs in the pineal gland, which contains dense noradrenergic terminals [4,7]. Pinealocytes show a high expression of adrenoceptors of the Gq-coupled $\alpha_{1B}R$ subtype and the Gs-coupled β_1R subtype which play a stimulatory role in the synthesis and release of melatonin and its precursor serotonin [8]. In addition, pinealocytes show a marked circadian expression of D₄R, decreasing very significantly during daylight and being mostly absent at sunset and maximal at sunrise [7]. It was shown that the main effect of D₄R activation in the pineal gland is to decrease melatonin synthesis and release by inhibiting β_1R and $\alpha_{1B}R$ signaling through heteromerization [4].

In the brain, aside from the pineal gland, D₄R are particularly expressed in prefrontal cortical pyramidal glutamatergic neurons, which express D₄R both at the somato-dendritic level and in their striatal terminals [5,6,9–12]. Some studies indicate that, at the terminal level, D₄R forms heteromers with D₂R and that these G protein-coupled receptor (GPCR) complexes play a significant inhibitory control of cortico-striatal glutamatergic transmission [5,6].

The human *DRD4* gene displays a high number of polymorphisms in its coding sequence. The most extensive polymorphism is found in exon 3, which is a region that encodes the third intracellular loop (3IL) of the receptor [13–15]. This polymorphism consists of a variable number of tandem repeats of a 48-base pair sequence, with 2–11 repeats. The two most common polymorphisms contain 4 and 7 repeats (with allele frequencies of about 60% and 20%, respectively) [14] and they encode a dopamine D₄R with 4 and 7 repeats of a proline-rich sequence of 16 amino acids (D_{4.4}R and D_{4.7}R) [13–15]. *DRD4* polymorphisms have been associated with individual differences in personality traits and neuropsychiatric disorders, particularly between the gene encoding D_{4.7}R and attention deficit hyperactivity disorder (ADHD) or substance use disorder (SUD) [13,16–20]. In fact, there is a clear association between D_{4.7}R and impulsivity traits, action impulsivity and choice impulsivity, which are considered endophenotypes of ADHD and SUD [19,21–23].

The question of the functional differences between D_{4.4}R and D_{4.7}R has remained enigmatic until considering GPCR heteromerization. Thus, D_{4.4}R and D_{4.7}R (and other D₄R polymorphic variants) do not show clear functional differences when studied in isolation, but their properties significantly differed when considered as components of D₂R-D₄R heteromers [1,3]. The most remarkable functional difference between D₂R-D_{4.4}R and D₂R-D_{4.7}R was the selective increase in the constitutive activity of D₂R when forming heteromers with D_{4.7}R, but not with D_{4.4}R [1]. This gain of function of the D₂R-D_{4.7}R heteromer could then explain

the increased inhibitory effect of dopamine (DA) on cortico-striatal glutamatergic transmission of mice expressing a D₄R with a humanized 3IL corresponding to the D_{4.7}R [6]. These results suggested that the D_{4.7}R-associated increased vulnerability of ADHD and SUD could be related to an enhanced D₂R-D₄R-mediated dopaminergic control of cortico-striatal transmission.

In addition to the *DRD4* gene, the α_{2A} adrenoceptor ($\alpha_{2A}R$) gene has also been associated with ADHD [24,25]. Although, a large meta-analysis did not find a consistent significant association [18], a more recent study performed at the endophenotype level, established a clear significant association between $\alpha_{2A}R$ gene polymorphisms and action impulsivity [26]. In fact, $\alpha_{2A}R$ localized in pyramidal neurons of the prefrontal cortex [27] are targets of drugs used in the treatment of ADHD such as methylphenidate and the selective $\alpha_{2A}R$ agonist guanfacine [28–30]. In view of the involvement of D₄R and $\alpha_{2A}R$ in impulsivity and ADHD, their concurrent localization in cortical pyramidal neurons and the demonstrated ability of D₄R to form functional heteromers with other adrenoceptors in the pineal gland, we investigated the possibility of $\alpha_{2A}R$ -D_{4.4}R and $\alpha_{2A}R$ -D_{4.7}R heteromerization in mammalian transfected cells and their localization and functional role in the mouse cerebral cortex.

2. Material and methods

2.1. DNA constructs

For bimolecular luminescence complementation experiments, human D_{4.4}R, D_{4.7}R, $\alpha_{2A}R$, $\alpha_{1A}R$, and A₁R were cloned in frame into pcDNA3.1 expressing the amino acid residues 1–229 (nRLuc) or 230–311 (cRLuc) of the RLuc8 protein. For BRET assays, human D_{4.4}R-YFP, D_{4.7}R-YFP, $\alpha_{1A}R$ -YFP, $\alpha_{2A}R$ -RLuc8 ($\alpha_{2A}R$ -RLuc) and A₁R-RLuc constructs were used. For CODA-RET and receptor-G protein BRET assays, the following human G protein constructs were used: G α_{i1} -mVenus and G α_{o1} -mVenus (with the YFP derivative mVenus inserted at position 91), untagged G β_1 and G γ_2 as well as $\alpha_{2A}R$ -nRLuc, $\alpha_{2A}R$ -cRLuc, D_{4.4}R-nRLuc, D_{4.4}R-cRLuc, D_{4.7}R-nRLuc, D_{4.7}R-cRLuc, D_{4.4}R, D_{4.7}R and $\alpha_{2A}R$ -RLuc and unfused D_{4.4}R and D_{4.7}R. For binding and functional assays, $\alpha_{2A}R$ -RLuc was used. All the constructs were confirmed by sequencing analysis. Details of key resources used such as cell lines, animal models, reagents and software appear in Table S1.

2.2. TAT-TM peptides

A peptide derived from the HIV-transactivator of transcription (TAT: YGRKKRRQRRR), was fused to peptides with the amino acid sequences of human D₄Rs or α_{2A} Rs transmembrane (TM) domains 4–7 (Peptide Synthesis Facility, University Pompeu Fabra, Barcelona, Spain), to promote integration of the TM domains in the plasma membrane. Because HIV TAT binds to the phosphatidylinositol-(4, 5)-bisphosphate found on the inner surface of the membrane [31], HIV TAT peptide was fused to the N-terminus of TM4 and TM6 and to the C-terminus of TM5 and TM7 to obtain the right orientation of the inserted peptide. The amino acid sequences were:

TAT-TM4 of D4R: RRRQRRKKRGYGSRRQLLIGATWLLSAAVA

APVLCGL.

TM5-TAT of D4R: YVYSSVCSFFLPCPLMLLLYWATFYGRKKRRQRRR.

TAT-TM6 of D4R: RRRQRRKKRGYAMRVLPVVVGAFLLCWTPFFVVHITQAL.

TM7-TAT of D4R: LVSAVTWLGYNVSNALNPVIYTVFNAYGRKKRRQRRR.

TAT-TM4 of α_{2A} R: RRRQRRKKRGYPRIKAIITVWVISAVISFPPLI.

TM5-TAT of α_{2A} R: YKWYVISSCIGSFAPCLIMILVYVRIYQIAYGRKKRRQRRR.

TAT-TM6 of α_{2A} R: RRRQRRKKRGYFTFVLAVVIGVFVVCWFPPFFFTYTLTAV.

TM7-TAT of α_{2A} R: RTLFKFFFWFGYCNSSLNPVIYTIFNYGRKKRRQRRR.

2.3. Cell culture

HEK-293T cells were purchased from ATCC and kept below passage 20. Cells were maintained in culture with Dulbecco's modified Eagle's medium (DMEM) (Gibco™, ThermoFisher Scientific, MA, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, MEM Non-Essential Amino Acid Solution (1/100), and 5% (v/v) heat-inactivated fetal bovine serum 5% fetal bovine serum (all supplements were from Gibco™, ThermoFisher Scientific) and kept in an incubator at 37 °C and 5% CO₂. HEK-293T cells inducible expressing D4R variants under the control of tetracycline, were obtained with the FLP-In T-Rex system. These cell lines were maintained with hygromycin 50 µg/ml and blasticidin 15 µg/ml and the D4R variant expression was induced for 18–24 h with tetracycline 250 ng/ml.

2.4. BRET saturation experiments and bimolecular luminescence complementation assays

In order to perform the bioluminescence resonance energy transfer (BRET) assays, cells were transiently co-transfected with polyethylenimine (Sigma, St. Louis, MO, USA) with a constant amount of expression vector encoding for receptor fused to RLuc and with increasing amounts of the expression vector corresponding to receptor fused to YFP (0.55 µg of α_{2A} R-RLuc and 0.2–3 µg of D_{4.4}R-YFP or 0.2–3.5 µg of D_{4.7}R-YFP or 0.2–2 µg of α_{1A} R-YFP; 0.1–1 µg of A₁R-RLuc, in order to obtain a constant amount of luminescence emission, and 0.1–8 µg of D_{4.4}R-YFP. Cells were harvested, washed, and resuspended in PBS. For determining Venus expression, 20 µg of protein were distributed in 96-well plates (black plates with a transparent bottom) and the emission at 530 nm after the excitation at 500 nm with a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) was quantified. Protein fluorescence expression was determined as the fluorescence of the sample minus the fluorescence of cells expressing the receptor fused to RLuc alone. In parallel, luminescence and BRET signal were determined as the ratio of the light emitted by Venus (530 nm) over that emitted by coelenterazine H (PJK, Kleinblittersdorf, Germany,) (485 nm) 1 min after the addition of 5 µM coelenterazine H using a Mithras LB940. To quantify receptor-RLuc expression, luminescence readings were also performed after 10 min of adding coelenterazine H. The net BRET was defined as [(530 nm emission)/(485 nm emission)-cf.], where cf. corresponds to [(530 nm emission)/(485 nm emission)] for the receptor-RLuc expressed alone in the same experiment. BRET is expressed as millibRET units (net BRET × 1000). Data were fitted to a nonlinear regression equation with GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

For bimolecular luminescent complementation (BiLC) assays, cells were co-transfected with the cDNA encoding for the receptors of interest fused to RLuc hemiproteins. After 48 h, cells were treated or not with the indicated TAT-TM peptides (4 µM) for 4 h at 37 °C. The quantification of the receptor-reconstituted RLuc expression was measured at 485 nm after 10 min of adding coelenterazine H. Cells expressing the receptor

fused to one hemiprotein showed similar luminescence levels to non-transfected cells.

2.5. CODA-RET and receptor-G protein BRET assays

For complemented donor-acceptor resonance energy transfer (CODA-RET) assays, HEK-293T cells were co-transfected with human G α_{i1} -mVenus or G α_{o1} -mVenus (5 µg), untagged G β_1 (4.5 µg) and G γ_2 (5 µg) and the pair of receptors of interest fused to the corresponding RLuc hemiprotein (1.2 µg of D₄R-nRLuc and 0.3 µg of α_{2A} R-cRuc or 0.83 µg of α_{2A} R-nRLuc and 1.66 µg of α_{2A} R-cRuc). For receptor-G protein BRET assays, cells were co-transfected with human D_{4.4}R or D_{4.7}R (2 µg) fused to RLuc or with α_{2A} R fused (0.5 µg) to RLuc and unfused D_{4.4}R or D_{4.7}R (1 µg), as well as with G α_{i1} -mVenus or G α_{o1} -mVenus (5 µg) and untagged G β_1 (4.5 µg) and G γ_2 (5 µg). The transfections were performed using polyethylenimine in a 1:2 ratio in 100 cm² cell culture plates. All experiments were performed approximately 48 h after transfection. Cells were harvested, washed and resuspended in phosphate-buffered saline. Approximately 200,000 cells/well were distributed in 96-well plates, and 5 µM coelenterazine H was added to each well. 2.5 min after addition of coelenterazine H, agonists were added to each well. Antagonists were added 10 min before coelenterazine H. The fluorescence of the acceptor was quantified (excitation at 500 nm and emission at 540 nm for 1-second recordings) in a Mithras LB940 to confirm the constant expression levels across experiments. In parallel, the BRET signal from the same batch of cells was determined as the ratio of the light emitted by mVenus (530 nm) over that emitted by RLuc (485 nm). G protein-activation was calculated as the BRET change (BRET ratio for the corresponding drug minus BRET ratio in the absence of the drug) observed 10 min after the addition of ligands. E_{max} values were expressed in absolute values or as the percentage of the effect of each ligand over the effect of NE or DA, depending on the experiment. Data were fitted to a nonlinear regression equation, assuming a single-phase dose-response curve with GraphPad Prism 6 software. The transfected amount and ratio among the receptor and heterotrimeric G proteins were tested for optimized dynamic range in drug-induced BRET. All ligands were purchased from either Sigma or Tocris.

2.6. Radioligand binding experiments

Radioligand binding experiments were performed in HEK-293T cells co-expressing the α_{2A} -RLuc and the D4R and in brains of male and female sheep of 4–6 months old freshly obtained from the local slaughterhouse. Brain tissues and cell suspensions, were disrupted with a Polytron homogenizer (PTA 7 rotor, setting 3; Kinematica, Basel, Switzerland) for two 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor cocktail (Sigma). Membranes were obtained by centrifugation twice at 105,000g for 45 min at 4 °C. The pellet was stored at –80 °C, washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Membrane protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard.

Binding experiments were performed with membrane suspensions (0.2 mg of protein/ml) at room temperature in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂. For α_2 R saturation-binding assays, membrane suspensions of HEK-293T cells co-expressing the α_{2A} -RLuc and D_{4.4}R were incubated for 3 h with increasing concentrations of the α_2 R antagonist [³H]RX821002 (PerkinElmer, Boston, MA, USA). Non-specific binding was determined in the presence of 10 µM of the non-radiolabeled antagonist RX821002 (Tocris, Bristol, United Kingdom). For D₄R saturation-binding assays, membrane suspensions of the same cells were incubated for 3 h with increasing concentrations of [³H]YM-09151-2 (PerkinElmer). Nonspecific binding was determined in the presence of 30 µM of dopamine (Sigma), because at this concentration dopamine does not displace the radioligand from sigma receptors.

For competition-binding assays, membrane suspensions were

incubated for 2 h with a constant free concentration of 0.9 nM of the α_2 R antagonist [3 H], or 0.7 nM of the D₂-like receptor antagonist [3 H]YM-09151-2 and free increasing concentrations of each tested ligand.

In dissociation kinetic assays, membranes were pre-incubated at 12 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ in the absence or presence of A-412997 (30 nM). After 30 min, 0.9 nM of the α_2 R antagonist [3 H]RX821002 was added for an additional 1 h period of radioligand association. Dissociation was initiated by the addition of 10 μ M of RX821002. At the indicated time intervals, total binding was measured as described below.

In all cases, free and membrane-bound ligands were separated by rapid filtration of 500 μ L aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold 50 mM Tris-HCl buffer. The filters were incubated with 10 ml of Ultima Gold MV scintillation cocktail (PerkinElmer, Waltham, Massachusetts, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 2800 scintillation counter (PerkinElmer) with an efficiency of 62% [32].

2.7. Binding data analysis

Data were analyzed according to the ‘dimer receptor model’ of Casadó et al. The model assumes GPCR dimers as a main functional unit and provides a more robust analysis of parameters obtained from saturation and competition experiments with orthosteric ligands, as compared with the commonly used ‘two-independent-site model’ [33, 34]. In competition experiments, the model analyzes the interactions of the radioligand with a competing ligand and it provides the affinity of the competing ligand for the first protomer in the unoccupied dimer (K_{DB1}), the affinity of the competing ligand for the second protomer when the first protomer is already occupied by the competing ligand (K_{DB2}) or the radioligand (K_{DAB}), and an index of cooperativity of the competing ligand (D_{CB}). A positive or negative value of D_{CB} implies either an increase or a decrease in affinity of K_{DB2} versus K_{DB1} and its absolute value provides a measure of the degree of increase or decrease in affinity.

Radioligand curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the binding data to the mechanistic dimer receptor model, as described in detail elsewhere [35].

Saturation assays data must be fitted to Eq. (1).

$$A_{\text{bound}} = \frac{(K_{DA2} A + 2 A^2) R_T}{(K_{DA1} K_{DA2} + K_{DA2} A + A^2)} \quad (1)$$

where A represents the free radioligand concentration, R_T is the total amount of receptor dimers, and K_{DA1} and K_{DA2} are the macroscopic equilibrium dissociation constants describing the binding of the first and the second ligand molecule to the receptor homodimer. Nevertheless, in non-cooperative conditions, $K_{DA2}/K_{DA1} = 4$. Then, K_{DA1} is enough to characterize the binding. Therefore, Eq. (1) can be reduced to Eq. (2):

$$A_{\text{bound}} = \frac{2 A R_T}{2 K_{DA1} + A} \quad (2)$$

To calculate the macroscopic equilibrium dissociation constants from competition experiments, the following general equation (Eq. (3)) must be applied:

$$A_{\text{bound}} = \frac{\left(K_{DA2} A + 2 A^2 + \frac{K_{DA2} A B}{K_{DAB}} \right) R_T}{K_{DA1} K_{DA2} + K_{DA2} A + A^2 + \frac{K_{DA2} A B}{K_{DAB}} + \frac{K_{DA1} K_{DA2} B}{K_{DB1}} + \frac{K_{DA1} K_{DA2} B^2}{K_{DB1} K_{DB2}}} \quad (3)$$

where B represents the assayed competing compound concentration. However, in absence of cooperativity of the radioligand and non-allosteric modulation between A and B , Eq. (3) can be simplified due

to the fact that: $K_{DA2} = 4K_{DA1}$ and $K_{DAB} = 2K_{DB1}$ (Eq. (4)):

$$A_{\text{bound}} = \frac{\left(4 K_{DA1} A + 2 A^2 + \frac{2 K_{DA1} A B}{K_{DB1}} \right) R_T}{4 K_{DA1}^2 + 4 K_{DA1} A + A^2 + \frac{2 K_{DA1} A B}{K_{DB1}} + \frac{4 K_{DA1}^2 B}{K_{DB1}} + \frac{4 K_{DA1}^2 B^2}{K_{DB1} K_{DB2}}} \quad (4)$$

For an absence of cooperativity and allosteric modulation between A and B , Eq. (3) can be simplified due to the fact that: $K_{DA2} = 4K_{DA1}$, $K_{DB2} = 4K_{DB1}$ and $K_{DAB} = 2K_{DB1}$ (Eq. (5)):

$$A_{\text{bound}} = \frac{\left(4 K_{DA1} A + 2 A^2 + \frac{2 K_{DA1} A B}{K_{DB1}} \right) R_T}{4 K_{DA1}^2 + 4 K_{DA1} A + A^2 + \frac{2 K_{DA1} A B}{K_{DB1}} + \frac{4 K_{DA1}^2 B}{K_{DB1}} + \frac{K_{DA1}^2 B^2}{K_{DB1}^2}} \quad (5)$$

Dissociation kinetic data were fitted to the following empirical equation (Eq. (6)):

$$A_{\text{total bound}} = \sum_{i=1}^n A_{ei} e^{-t k_i} + A_{\text{nonspecific bound}} \quad (6)$$

where A_{ei} represents the initial radioligand (the α_2 R antagonist [3 H]RX821002) bound at equilibrium for each molecular specie i , t is time, and k_i is the dissociation rate constants for the n different molecular species. For biphasic curves (or complex dissociation kinetics), $n = 2$.

2.8. cAMP production

For cAMP production, homogeneous time-resolved fluorescence energy transfer (HTRF) assays were performed using the Lance Ultra cAMP kit (PerkinElmer), based on competitive displacement of a europium chelate-labeled cAMP tracer bound to a specific antibody conjugated to acceptor beads. HEK-293T cells stably expressing D_{4.4}R or D_{4.7}R were transfected with α_2 A-RLuc receptor. First of all we established the optimal cell density and forskolin concentration for an appropriate fluorescent signal that covered most of the dynamic range of cAMP standard curve. Cells (1200 cells/well) growing in medium containing 30 μ M zardaverine were pretreated with the antagonists or the corresponding vehicle in white ProxiPlate 384-well microplates (PerkinElmer) at 25 °C for 15 min and stimulated with agonists for 15 min before adding 0.5 μ M forskolin (Sigma) or vehicle and incubating for an additional 15 min period. Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab Technologies, Offenburg, Germany).

2.9. ERK1/2 phosphorylation assay

HEK-293T cells stably expressing D_{4.4}R or D_{4.7}R were transfected with α_2 A-RLuc receptor. The day of the experiment, cells were starved by treating them with serum free media for 4 h at 37 °C. After that, cells were incubated with the indicated agonist for 5 min at 37 °C. Then, cells were rinsed with ice-cold phosphate-buffered saline and lysed by adding 200 μ L ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaF, 150 mM NaCl, 45 mM β -glycerophosphate, 1% Triton X-100, 20 mM phenylarsine oxide, 0.4 mM NaVO₄, and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13,000g for 5 min at 4 °C, and the protein was quantified. To determine the level of ERK1/2 phosphorylation, equivalent amounts of protein were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes. Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) was then added, and the membrane was rocked for 90 min. The membranes were then probed with a mixture of a mouse anti-phospho-ERK1/2 antibody (1:2500; Merck, Darmstadt, Germany) and rabbit anti-ERK1/2 antibody that recognizes both phosphorylated and nonphosphorylated ERK1/2 (1:40,000; Merck) overnight at 4 °C. The 42 and 44 kDa bands corresponding to ERK1 and ERK2 were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10,000; Sigma) and IRDye 680

(anti-rabbit) antibody (1:10,000; Sigma) for 2 h and scanned by the Odyssey infrared scanner (LICOR Biosciences). Band densities of western-blot images were quantified using the scanner software and exported to Excel (Microsoft, Redmond, WA). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK1/2 protein band intensities.

2.10. Animals and brain slices preparation

All animals used in the study were maintained in accordance with the guidelines of the National Institutes of Health (NIH) Animal Care, and the animal research conducted to perform this study was reviewed and approved by the NIDA IRP Animal Care and Use Committee (protocol #15-BNBR-73). Procedures using rodents were also approved by the Ethical Committee for Animal Use of the University of Barcelona (OB 408/18 i OB 409/18).

Transgenic D_{4.7}R mice, with a humanized mouse *DRD4* gene containing seven TRs of the human *DRD4* in the homologous region of the mouse gene, which codes for the 3IL [5], were used. Homozygous D_{4.7}R and WT littermates were obtained from a breeding colony of D_{4.7}R heterozygous mice (in a C57Bl/6J background) kept in the National Institute on Drug Abuse, Intramural Research Program (NIDA IRP) breeding facility. Animals were housed (four per cage) and kept on a 12-hour light/12-hour dark cycle with food and water available *ad libitum*. Animals were killed by cervical dislocation. Mice brains were rapidly removed and placed in ice-cold oxygenated (95% O₂/5% CO₂) Krebs-HCO₃⁻ buffer (containing [in mM]: 124 NaCl, 4 KCl, 1.25 KH₂PO₄, 1.5 MgCl₂, 1.5 CaCl₂, 10 glucose, and 26 NaHCO₃, pH 7.4). The brains were sliced coronally at 4 °C. Slices containing cortex (500 µm thick) were kept at 4 °C in this Krebs-HCO₃⁻-buffer during the dissection and were transferred into an incubation tube containing 1 ml of ice-cold Krebs-HCO₃⁻-buffer. The temperature was raised to 23 °C, and after 30 min the medium was replaced by 2 ml of fresh Krebs-HCO₃⁻-buffer (23 °C). The slices were incubated under constant oxygenation (O₂/CO₂: 95%/5%) at 30 °C for 4–5 h in absence or presence of the TM peptides at 4 µM in an Eppendorf Thermomixer (5 Prime, Boulder, Colorado, USA).

For phospho-ERK1/2 determination, the media was replaced by 200 µL of fresh Krebs-HCO₃⁻-buffer and incubated for 30 min before the addition of any agent. Slices were treated or not with the indicated ligand for 5 min at 30 °C. After the indicated incubation period, the solution was discarded, and slices were frozen on dry ice, lysed by the addition of 500 µL of ice-cold lysis buffer and treated as described for cells.

2.11. In situ PLAs in brain tissues and transfected cells

For proximity ligation assays (PLA) in brain tissues, mouse cortex slices (500 µm thick) were incubated or not with TM peptides at 4 µM as described above for phospho-ERK1/2 determination. Slices were then fixed by immersion in 4% paraformaldehyde solution for 1 h at 4 °C. Samples were then washed in 50 mM Tris-HCl, 0.9% NaCl pH 7.8 buffer (TBS), cryopreserved in a 30% sucrose solution for 48 h at 4 °C, and stored at –20 °C until sectioning. 20 µm-thick slices were cut coronally on a freezing cryostat (Leica Jung CM-3000), mounted on slide glass and frozen at –20 °C until use. To perform the PLA, slices were thawed at 4 °C, washed in TBS, permeabilized with TBS containing 0.01% Triton X-100 for 10 min, and successively washed with TBS. For cell-based PLA, HEK-293T were grown on glass coverslips and transfected with 0.01 µg of cDNA corresponding to α_{2A}R, D_{4.4}R or D_{4.7}R, or co-transfected with both α_{2A}R and D₄Rs. 48 h after transfection, cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine, permeabilized with the same buffer containing 0.05% Triton X-100, and successively washed with PBS. In both cases, heteromers were detected using the Duolink *in situ* PLA detection Kit (Sigma) and following the instructions of the supplier. To detect α_{2A}R-D₄R heteromers, a mixture of equal amounts (1:100) of rabbit anti-α_{2A}R antibody

(ab92650) (Thermo Scientific, Fremont, California, USA) and goat anti-D₄R (sc-1439) (Santa Cruz Biotechnology, Santa Cruz, California, USA) antibody were used. Samples were further incubated with anti-rabbit plus and anti-goat minus PLA probes (1:5). Slices were mounted using the mounting medium with DAPI and observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63X oil-immersion objective (N.A. 1.4), and a 405 nm and a 561 nm laser line. For each field of view, a stack of two channels (one per staining) and 9–15 Z stacks with a step size of 1 µm were acquired. Images were opened and processed using ImageJ (<https://fiji.sc/>). In tissue, a quantification of cells containing one or more red spots *versus* total cells (blue nuclei) was determined considering a total of 1500–3000 cells from 4 to 12 different fields (63X) within each region from three different animals. The ImageJ confocal program using the Fiji package was used. Nuclei and red spots were counted on the maximum projections of each image stack. After getting the projection, each channel was processed individually. The nuclei were segmented by filtering with a median filter, subtracting the background, enhancing the contrast with the contrast limited adaptive histogram equalization (CLAHE) plug-in, and finally applying a threshold to obtain the binary image and the regions of interest (ROIs) around each nucleus. Red spot images were also filtered and thresholded to obtain the binary images. Red spots were counted in each of the ROIs obtained in the nuclei images.

2.12. Statistical analysis

In binding assays, goodness of fit was tested according to reduced chi-squared value given by the regression program. The test of significance for two different model population variances was based upon the F-distribution using built-in functions in GraFit software. A probability greater than 95% ($p < 0.05$) was considered the criterion to select a more complex model (cooperativity) over the simplest one (non-cooperativity). Other statistical analyses were performed using GraphPad Prism 6 software. Differences between experimental group pairs were analyzed with unpaired two-sided Student's *t*-test. Differences among more than two groups of results were performed by one-way ANOVA (followed by Dunnett's or Bonferroni's *post hoc* test comparison). In all cases, data were presented as mean ± standard error of the mean (SEM). A level of $p < 0.05$ was considered as critical for assigning statistical significance.

3. Results

3.1. D_{4.4}R and D_{4.7}R form heteromers with α_{2A}R in mammalian transfected cells

First, we analyzed the ability of human D_{4.4}R and D_{4.7}R to form heteromers with human α_{2A}R *in vitro* by using BRET saturation assays. These experiments were performed in HEK-293T cells co-transfected with α_{2A}R cDNA fused to *Renilla* Luciferase (α_{2A}R-RLuc) and increasing amounts of D_{4.4}R or D_{4.7}R cDNAs fused to yellow fluorescent protein (D_{4.4}R-YFP or D_{4.7}R-YFP, respectively). In both cases, BRET saturation curves were hyperbolic, indicating a specific interaction between both fusion proteins (Fig. 1A, B). BRET_{max} values for α_{2A}R-D_{4.4}R were 98 ± 7 milliBRET units (mBU) ($n = 8$) and significantly lower values, 33 ± 2 mBU ($n = 8$), were obtained for α_{2A}R-D_{4.7}R, (non-paired Student's *t*-test: $t(14) = 8.928$, $p < 0.001$). This could indicate a reduced ability of D_{4.7}R to heteromerize with α_{2A}R, as compared with D_{4.4}R, although, most probably it reflects reduced BRET between the intracellularly localized RLuc and YFP due to a hindrance effect related to the large 3IL of D_{4.7}R. Nevertheless, BRET₅₀ values are more indicative of the avidity to heteromerize and were significantly lower (more avidity) for α_{2A}R-D_{4.4}R (14 ± 2) than for α_{2A}R-D_{4.7}R (60 ± 10) (non-paired Student's *t*-test: $t(14) = 4.511$, $p < 0.01$). The specificity of the D₄Rs and α_{2A}R to form heteromers was supported by the observation of a

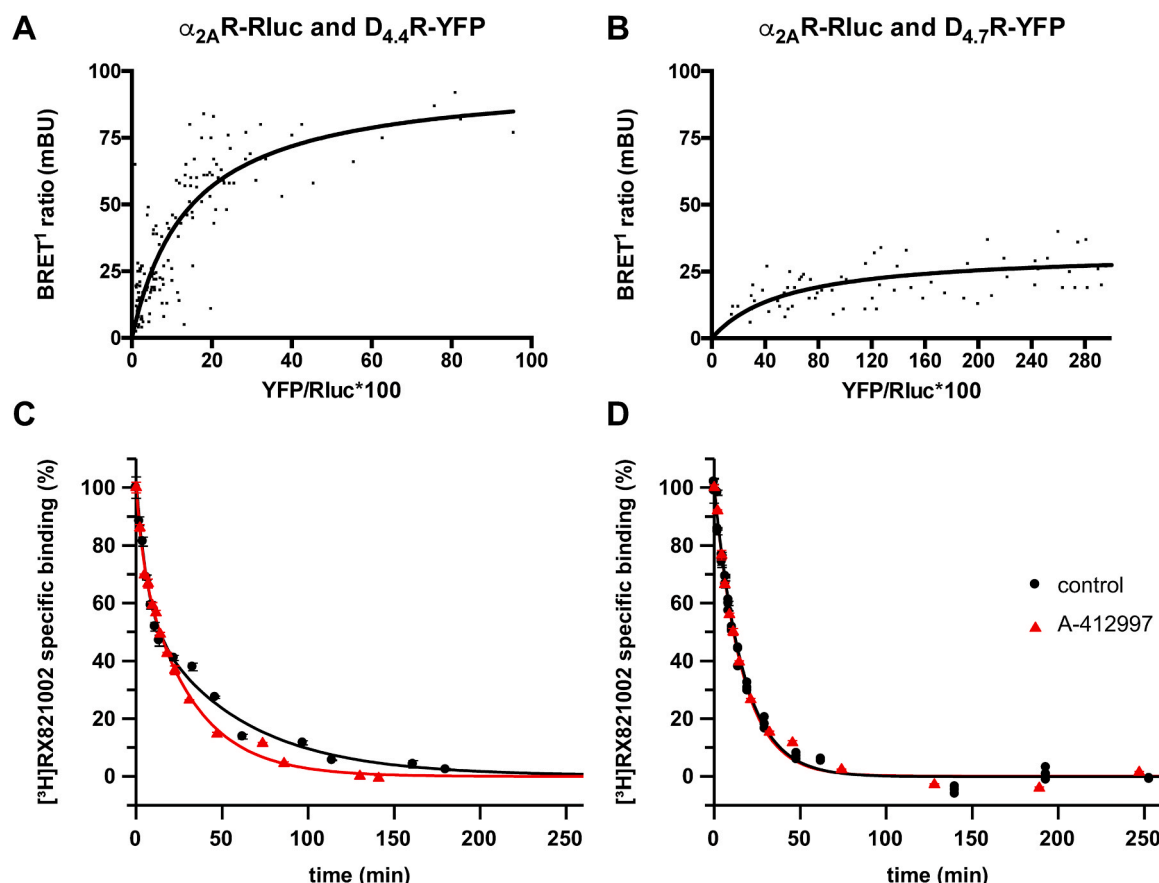


Fig. 1. $D_{4.4}$ R and $D_{4.7}$ R heteromerize with α_{2A} R in HEK-293T cells. (A and B) BRET saturation experiments were performed using HEK-293T cells transiently transfected with α_{2A} R-RLuc cDNA (0.55 μ g) and increasing concentrations of (A) $D_{4.4}$ R-YFP cDNA (0.2–3 μ g) or (B) $D_{4.7}$ R-YFP cDNA (0.2–3.5 μ g). Curves were obtained from 3 to 8 different experiments. mBU: milliBRET units. (C and D) Radioligand dissociation curves of [³H]RX821002 in the absence (black curve) or presence (red curve) of the selective $D_{4.4}$ R agonist A-412997 were performed in membrane preparations from tetracycline-inducible HEK-293T cells expressing $D_{4.4}$ R (C) or $D_{4.7}$ R (D) and transiently transfected with α_{2A} R. Data were fit by the dissociation Eq. (6) that appears in the materials and methods section. Values are means $\pm$ SEM from a representative experiment of 4 different experiments performed in triplicate (see Table 1 for kinetic constant values and statistical comparisons).

linear, non-saturable BRET signal when cells were co-transfected with α_{2A} R-RLuc cDNA and increasing amounts of α_{1A} R-YFP cDNA (Fig. S1A) or adenosine A_1 receptor (A_1 R)-RLuc cDNA and increasing amounts of $D_{4.4}$ R-YFP cDNA (Fig. S1B), indicative of non-specific random collision of the corresponding fused receptors.

3.2. The allosteric modulation of [³H]RX821002 binding to α_{2A} R is dependent on the D_4 R isoform heteromerization

We then evaluated the specificity of different ligands for α_2 Rs, $D_{4.4}$ R and $D_{4.7}$ R, due to the previously described promiscuity of ligands for Gi-coupled dopamine and noradrenergic receptors [3,36]. To this end, we performed binding saturation experiments with the α_2 R antagonist [³H]RX821002 in HEK-293T cells transiently transfected with the human α_{2A} R cDNA and obtained a K_{DA1} value for [³H]RX821002 of 0.62 ± 0.05 nM ($n = 3$) (Fig. S2A). Using the same technique we determined that the affinity (K_{DA1}) of the D_2 -like receptor antagonist [³H]YM-09151-2 in HEK-293T cells stably transfected with $D_{4.4}$ R HEK-293T was 0.06 ± 0.01 nM ($n = 3$) (Fig. S2B). We then performed competitive inhibition experiments using [³H]RX821002 and the putative selective D_4 R agonists PD168077 and A-412997 in membrane preparations from sheep cortex. PD168077 and A-412997 showed a K_{DB1} for α_2 R of 80 ± 10 nM ($n = 3$) and 1400 ± 200 nM ($n = 3$), respectively (Fig. S2C). These affinities were nevertheless much lower than the affinity of the α_2 R agonist dexmedetomidine, which showed a K_{DB1} value of 0.009 ± 0.001 nM and a K_{DB2} of 0.40 ± 0.02 nM ($n = 5$)

(Fig. S2C). Competitive inhibition experiments with [³H]YM-09151-2 versus dexmedetomidine and A-412997 in membrane preparations from HEK-293T cells co-expressing α_{2A} R and $D_{4.4}$ R showed K_{DB1} values of about 2000–6000 nM ($n = 3$) and 1.1 ± 0.1 nM ($n = 3$), respectively (Fig. S2D), while RX821002 did not displace [³H]YM-09151-2 at any concentration up to 10 μ M, indicating that RX821002 does not bind to D_4 Rs (Fig. S2D). Also, in HEK-293T cells co-expressing α_2 R and $D_{4.4}$ R, dexmedetomidine displaced [³H]RX821002 with a K_{DB1} of 5.6 ± 0.8 nM ($n = 6$) (Fig. S2E). Therefore, to study the function of α_2 R- D_4 R heteromers, A-412997 and dexmedetomidine were chosen as selective D_4 Rs and α_2 Rs agonists, respectively. The expression of D_4 R and α_{2A} R in HEK-293T cells stably transfected with either $D_{4.4}$ R or $D_{4.7}$ R and transiently co-transfected with α_{2A} R was about 0.5–1 and 0.3–0.4 pmol/mg protein, respectively.

One of the characteristics of GPCR heteromers is their ability to mediate allosteric modulations between orthosteric ligands of their molecularly different receptor units (protomers). A ligand of one of the protomers can modify the affinity or efficacy of a ligand for the other molecularly different protomer [34]. Experiments of radioligand dissociation can resolve this type of allosteric interactions at the level of ligand binding [37–39]. Significant changes in the dissociation kinetic parameters of an orthosteric ligand by another ligand that does not bind to the same orthosteric site indicate an allosteric modulation [37]. In a GPCR heteromer, the binding of one ligand to the orthosteric site of one protomer can allosterically alter the kinetic properties of the other ligand bound to molecularly different protomer [38]. We first evaluated

the possibility of D₄R ligands to allosterically modulate the binding of [³H]RX821002 in membrane preparations of HEK-293T cells stably expressing D_{4.4}R or D_{4.7}R and transiently expressing α_{2A}R. In cells expressing α_{2A}R and D_{4.4}R, dissociation curves of the α_{2A}R radioligand were biphasic and the D₄R agonist A-412997 significantly modified both the slow and the fast dissociation rate constants of [³H]RX821002 (Fig. 1C, D, and Table 1), indicative of an allosteric modulation between the two orthosteric α_{2A}R and D_{4.4}R ligands and, therefore, an additional demonstration of α_{2A}R-D_{4.4}R heteromerization. The higher dissociation rate constants in the presence of the D₄R agonist A-412997 (lower residence time, RT) indicate a negative allosteric modulation by the D₄R agonist binding to the D_{4.4}R on α_{2A}R antagonist binding. On the other hand, the dissociation curves of [³H]RX821002 in cells expressing α_{2A}R and D_{4.7}R were monophasic and A-412997 was not able to modify the dissociation rate constant of the labeled α_{2A}R antagonist (Fig. 1C, D, and Table 1). These results indicate a functional difference between α_{2A}R-D_{4.4}R and α_{2A}R-D_{4.7}R heteromers, such that D₄R agonists can allosterically modulate α_{2A}R antagonist binding in the α_{2A}R-D_{4.4}R but not in the α_{2A}R-D_{4.7}R heteromer.

3.3. Gi protein activation by α_{2A}R-D₄R heteromers depends on the D₄R polymorphic variant involved

We next sought to study G protein activation by α_{2A}R-D_{4.4}R and α_{2A}R-D_{4.7}R heteromers using CODA-RET assays [40]. In these experiments, two complementary halves of RLuc8 (nRLuc and cRLuc) are separately fused to two receptor molecules putatively able to oligomerize and the mVenus variant of YFP fused the α-helical domain of the Gα_{i1} subunit. Ligand-induced changes in CODA-RET measurements imply, first, a successful complementation of RLuc, confirming the oligomerization of the corresponding protomers and, second, it represents the reading of a specific ligand-induced G protein activation through the GPCR oligomer [40,41]. Using CODA-RET, we determined the potency and efficacy of several adrenergic and dopaminergic ligands (DA and NE, the D₂-like receptor agonists pramipexole, ropinirole and rotigotine, the selective D₄R agonist A-412997 and the α₂R agonists dexmedetomidine, clonidine and guanfacine) at activating Gα_{i1} protein coupled to either the α_{2A}R-α_{2A}R homomer or the α_{2A}R-D_{4.4}R or α_{2A}R-D_{4.7}R heteromers.

HEK-293T cells were co-transfected with α_{2A}R fused to cRLuc, α_{2A}R, D_{4.4}R or D_{4.7}R fused to nRLuc, Gα_{i1} subunit fused to mVenus and untagged Gβ₁, and Gγ₂ subunits (see schematic representation in Fig. 2J–L). The amount of Gα_{i1} subunits (or Gα_{o1} subunits, see below) transfected produced values between 15,000 and 30,000 fluorescence units and the amount of RLuc receptor complementation produced values between 1 and 3 million (arbitrary units). A concentration-response of the ligand-induced change in BRET values allowed determining the potency and the relative efficacy (*versus* NE) of ligand-induced G protein activation by the three oligomers: α_{2A}R-α_{2A}R homomers, α_{2A}R-D_{4.4}R heteromers and α_{2A}R-D_{4.7}R heteromers. EC₅₀ and E_{max} values were obtained by fitting the data to sigmoidal concentration-response curves (Fig. 2A–C, and Table 2). The results show a non-canonical activation of α_{2A}R-α_{2A}R by DA, with lower potency (12 *versus* 6 μM) (non-paired Student's *t*-test: *t*(19) = 3.298, *p* < 0.01) and

efficacy (69%) (*t*(19) = 8.177, *p* < 0.001) than NE (Fig. 2A, and Table 2), as previously described in BRET experiments by analyzing ligand-induced changes in the interaction between G protein subunits [36]. As expected, ropinirole, rotigotine and A-412997 were ineffective in the α_{2A}R-α_{2A}R homomer, but the D₂-like receptor agonist pramipexole activated Gα_{i1} with similar potency and efficacy than DA (Fig. 2A, and Table 2). To our knowledge, this α_{2A}R agonistic activity of pramipexole has not been previously reported. All three synthetic α₂R agonists, dexmedetomidine, clonidine and guanfacine showed high potency (in the nanomolar range) but low efficacy (between 14% and 26% *versus* NE) for the α_{2A}R-α_{2A}R homomer (Fig. 2A, and Table 2).

The potency and efficacy of Gα_{i1} activation by the endogenous neurotransmitters, NE and DA, acting on α_{2A}R-D_{4.4}R or α_{2A}R-D_{4.7}R heteromers looked similar to that of the α_{2A}R-α_{2A}R homomer. The three oligomers showed lower efficacy for DA as compared to NE (about 60–70%). The only significant and striking difference was a five-fold increase in NE potency on the α_{2A}R-D_{4.7}R as compared with the α_{2A}R-D_{4.4}R (Fig. 2B, C, and Table 2). To analyze the contribution of each receptor to the Gi protein activation by NE and DA within each oligomer, cells were pretreated with the α₂R antagonist BRL44408 or the selective D₄R antagonist L745,870. BRL44408 significantly decreased the potency of NE and DA in the α_{2A}R-α_{2A}R homomer and in both α_{2A}R-D₄R heteromers (Fig. 2D–I, and Table 3). Similar results were obtained with the α_{2A}R antagonist yohimbine (Fig. S3). In contrast, L745,870 only decreased significantly the effect of DA and NE in the α_{2A}R-D_{4.4}R heteromer (Fig. 2E, H). The lack of antagonistic effects by L745,870 in the α_{2A}R-D_{4.7}R heteromer suggests an inability of D_{4.7}R to activate Gi upon heteromerization with α_{2A}R (Fig. 2F, I).

To discard a possible artifact related to a loss of function of D_{4.7}R due to its fusion to RLuc hemiproteins, we also evaluated the ability of DA and NE to promote Gi protein activation by binding to D_{4.7}R-D_{4.7}R and also D_{4.4}R-D_{4.4}R homomers (Fig. 3). DA and NE activated with a potency of 0.29 ± 0.07 μM and 230 ± 70 μM the D_{4.4}R-D_{4.4}R homodimer (Fig. 3A) and of 0.13 ± 0.03 μM and 40 ± 20 μM the D_{4.7}R-D_{4.7}R homodimer (Fig. 3B), respectively. The potency of NE to activate α_{2A}R-D_{4.4}R heteromers (10 ± 3 μM; Table 2) was similar to that observed for α_{2A}R-α_{2A}R homodimers (6 ± 1 μM; Table 2), but significantly different to that found to activate D_{4.4}R-D_{4.4}R homodimers (230 ± 70 μM) (one-way ANOVA followed by Tukey's multiple comparison test, *p* < 0.001). Thus, these results indicate that α_{2A}R is mostly responsible for NE-induced Gi activation of α_{2A}R-D_{4.4}R heteromers. Overall, this suggests that the L745,870-induced decrease in the potency of NE for α_{2A}R-D_{4.4}R heteromers is not due to a counteraction of the contribution of D_{4.4}R to Gi signaling, but to cross-antagonism. This is an allosteric mechanism commonly observed in GPCR heteromers, by which an antagonist binding to one protomer can modify the properties of an agonist binding to the other molecularly different protomer in the heteromer [42,43]. However, this allosteric modulation was not observed in the α_{2A}R-D_{4.7}R heteromer, providing an additional pharmacological difference between the two polymorphic variants that depends on the heteromerization with α_{2A}R. In the α_{2A}R-D_{4.4}R heteromer, DA-induced Gi protein activation is most likely mediated by both α_{2A}R and D_{4.4}R, since the potency of DA for the heteromer (7 ± 2 μM; Table 2) represents an intermediate value between the potency for the α_{2A}R-α_{2A}R homodimer (12 ± 1 μM;

Table 1

Effect of a D₄R agonist on dissociation kinetic parameters of the α_{2A}R radiolabeled antagonist. Dissociation kinetic assays were performed at 12 °C on membranes from HEK-293T cells expressing D_{4.4}R or D_{4.7}R and α_{2A}R receptors. *k*_{off} values are means ± SEM from four different experiments performed in triplicate fitted with the dissociation Eq. (6) (see Section 2).

	α _{2A} R-D _{4.4} R				α _{2A} R-D _{4.7} R	
	<i>k</i> _{off1} (min ⁻¹)	RT ₁ (min) ^a	<i>k</i> _{off2} (min ⁻¹)	RT ₂ (min) ^a	<i>k</i> _{off} (min ⁻¹)	RT (min) ^a
Control	0.14 ± 0.03	7	0.020 ± 0.004	50	0.058 ± 0.002	17
+ A-412997	0.25 ± 0.07*	4	0.036 ± 0.007*	28	0.060 ± 0.007	17

**p* < 0.05 *versus* the parameters obtained in the absence of A-412997 (Student's *t*-test).

^a RT: residence time.

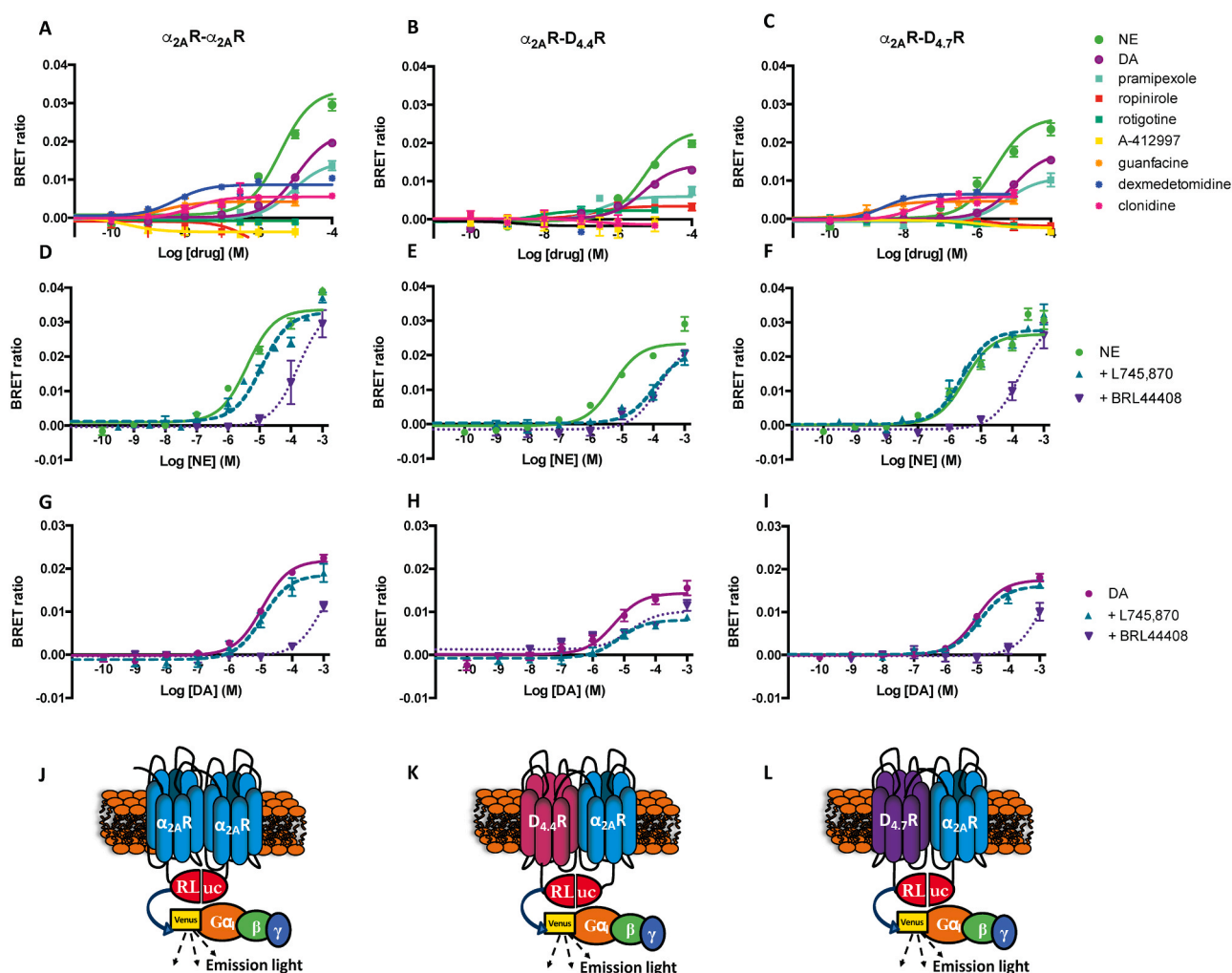


Fig. 2. Ligand-induced changes in the interaction between $G\alpha_{i1}$ protein and $\alpha_{2A}R$ - $\alpha_{2A}R$ homomers, $\alpha_{2A}R$ - $D_{4.4}R$ heteromers and $\alpha_{2A}R$ - $D_{4.7}R$ heteromers. CODA-RET experiments in HEK-293T cells co-transfected with $\alpha_{2A}R$ fused to the C-terminus of RLuc and $\alpha_{2A}R$ (A, D, G, J), $D_{4.4}R$ (B, E, H, K) or $D_{4.7}R$ (C, F, I, L) fused to the N-terminus of RLuc and the $G\alpha_{i1}$ subunit fused to mVenus. Concentration-response experiments of changes in BRET ratio induced by norepinephrine (NE), dopamine (DA), pramipexole, ropinirole, rotigotine, A-412997, guanfacine, dexmedetomidine and clonidine (A–C). Concentration-response experiments of changes in BRET ratio induced by NE (D–F) and DA (G–I) in the presence or absence of the D_4R antagonist L745,870 (10 μ M) or the α_2R antagonist BRL44408 (10 μ M). Data represent the mean $\pm$ SEM of 3–12 different experiments performed in triplicate (see Tables 2 and 3 for EC_{50} and E_{max} values and statistical comparisons). (J–L) Schematic representations of the CODA-RET assay.

Table 2

Ligand-induced changes in the interaction between $G\alpha_{i1}$ protein and $\alpha_{2A}R$ - $\alpha_{2A}R$, $\alpha_{2A}R$ - $D_{4.4}R$ and $\alpha_{2A}R$ - $D_{4.7}R$ dimers. Experiments were performed in HEK-293T cells transiently expressing $\alpha_{2A}R$ -cRLuc and $\alpha_{2A}R$ -nRLuc, $\alpha_{2A}R$ -cRLuc and $D_{4.4}R$ -nRLuc or $\alpha_{2A}R$ -cRLuc and $D_{4.7}R$ -nRLuc, the G protein subunits $G\alpha_{i1}$ -YFP and unfused β_1 and γ_2 . EC_{50} is expressed as mean $\pm$ SEM and E_{max} as mean $\pm$ SEM in % of NE of 5–12 different experiments performed in triplicate.

	$\alpha_{2A}R$ - $\alpha_{2A}R$		$\alpha_{2A}R$ - $D_{4.4}R$		$\alpha_{2A}R$ - $D_{4.7}R$	
	EC_{50} (μ M)	E_{max}	EC_{50} (μ M)	E_{max}	EC_{50} (μ M)	E_{max}
NE	6 $\pm$ 1	100 $\pm$ 3	10 $\pm$ 3	100 $\pm$ 5	1.9 $\pm$ 0.5*	100 $\pm$ 5
DA	12 $\pm$ 1	69 $\pm$ 2*	7 $\pm$ 2	59 $\pm$ 4	11 $\pm$ 2	73 $\pm$ 2**
Pramipexole	9 $\pm$ 2***	46 $\pm$ 2*	0.5 $\pm$ 0.3	28 $\pm$ 3	5.8 $\pm$ 0.6*	48 $\pm$ 5**
Ropinirole	–	0	0.4 $\pm$ 0.2	14 $\pm$ 2	–	0
Rotigotine	–	0	0.007 $\pm$ 0.005	13 $\pm$ 2	–	0
A-412997	–	0	–	0	–	0
Guanfacine	0.008 $\pm$ 0.005	14 $\pm$ 2	–	0	0.014 $\pm$ 0.011	20 $\pm$ 3
DMT	0.011 $\pm$ 0.006	26 $\pm$ 3	–	0	0.003 $\pm$ 0.001	30 $\pm$ 1
Clonidine	0.034 $\pm$ 0.023	18 $\pm$ 2	–	0	0.041 $\pm$ 0.013	26 $\pm$ 4

*p < 0.05, **p < 0.01, ***p < 0.001 versus $\alpha_{2A}R$ - $D_{4.4}R$ (one-way ANOVA followed by Tukey's multiple comparison test).

Table 2) and for the $D_{4.4}R$ - $D_{4.4}R$ homodimer (0.29 $\pm$ 0.07 μ M). Thus, in the $\alpha_{2A}R$ - $D_{4.4}R$ heteromer the decrease in DA efficacy in the presence of L745,870 or BRL44408 is mostly due to their respective competitive antagonism for $D_{4.4}R$ and $\alpha_{2A}R$.

The lack of participation of $D_{4.7}R$ in Gi-mediated signaling in $\alpha_{2A}R$ - $D_{4.7}R$ heteromers is also apparent when comparing the similar results obtained with synthetic ligands on $\alpha_{2A}R$ - $D_{4.7}R$ heteromers and $\alpha_{2A}R$ - $\alpha_{2A}R$ homomers. Nevertheless, these compounds behaved differently on

Table 3

Effect of the antagonists L745,870 or BRL44408 on NE and DA-induced changes in the interaction between $G\alpha_{i1}$ protein and $\alpha_{2A}R$ - $\alpha_{2A}R$, $\alpha_{2A}R$ - $D_{4.4}R$ and $\alpha_{2A}R$ - $D_{4.7}R$ dimers. Experiments were performed in HEK-293T cells transiently expressing $\alpha_{2A}R$ -cRLuc and $\alpha_{2A}R$ -nRLuc, $\alpha_{2A}R$ -cRLuc and $D_{4.4}R$ -nRLuc or $\alpha_{2A}R$ -cRLuc and $D_{4.7}R$ -nRLuc, the G protein subunits $G\alpha_{i1}$ -YFP and unfused β_1 and γ_2 . EC_{50} is expressed as mean $\pm$ SEM and E_{max} as mean $\pm$ SEM in % of NE or DA of 3–12 different experiments performed in triplicate.

	$\alpha_{2A}R$ - $\alpha_{2A}R$		$\alpha_{2A}R$ - $D_{4.4}R$		$\alpha_{2A}R$ - $D_{4.7}R$	
	EC_{50} (μ M)	E_{max} (%)	EC_{50} (μ M)	E_{max} (%)	EC_{50} (μ M)	E_{max} (%)
NE	6 $\pm$ 1	100 $\pm$ 3	10 $\pm$ 3	100 $\pm$ 5	1.9 $\pm$ 0.5	100 $\pm$ 5
NE + L745,870	13 $\pm$ 3	96 $\pm$ 3	130 $\pm$ 40***	86 $\pm$ 7	2.6 $\pm$ 0.5	118 $\pm$ 6
NE + BRL44408	330 $\pm$ 190***	113 $\pm$ 9	160 $\pm$ 20***	105 $\pm$ 10	190 $\pm$ 30***	125 $\pm$ 15
DA	12 $\pm$ 1	100 $\pm$ 3	7 $\pm$ 1.5	100 $\pm$ 6	11 $\pm$ 2	100 $\pm$ 3
DA + L745,870	11 $\pm$ 2	90 $\pm$ 10	8 $\pm$ 3	61 $\pm$ 5**	13 $\pm$ 1	90 $\pm$ 5
DA + BRL44408	> 1000***	95 $\pm$ 5	40 $\pm$ 20*	67 $\pm$ 8**	> 1000***	120 $\pm$ 10

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the corresponding cells not treated with antagonists (one-way ANOVA followed by Dunnett's *post-hoc* test).

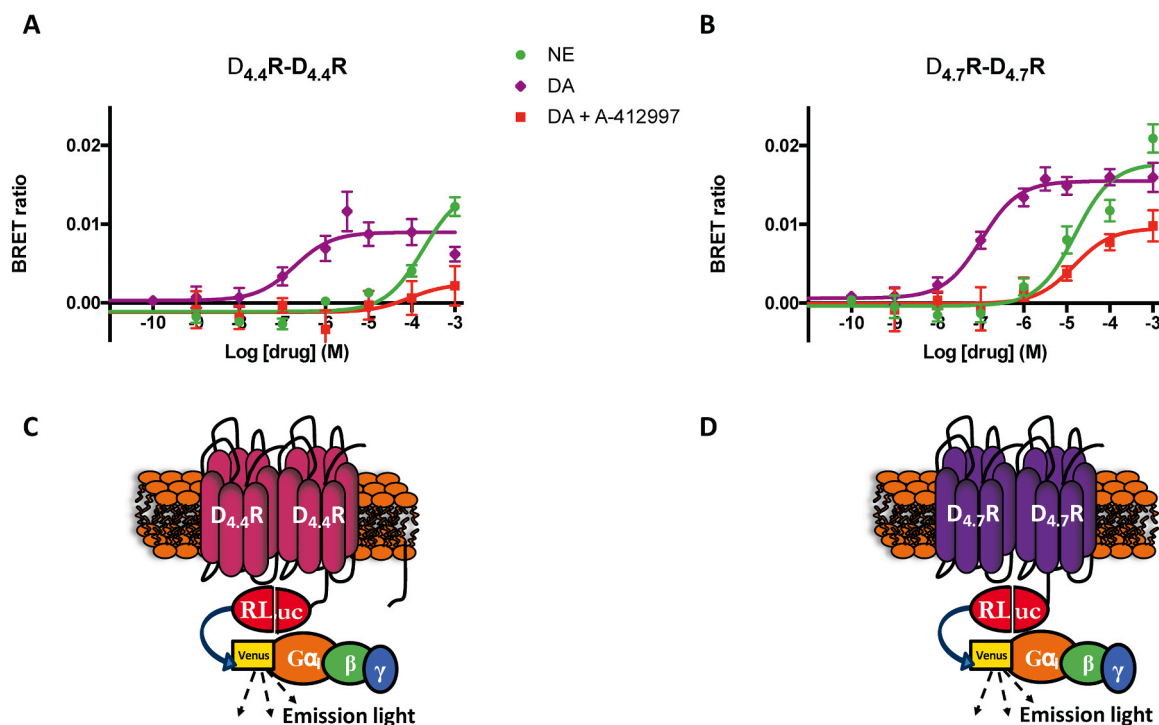


Fig. 3. Ligand-induced changes in the interaction between $G\alpha_{i1}$ protein and $D_{4.4}R$ - $D_{4.4}R$ or $D_{4.7}R$ - $D_{4.7}R$ heteromers. CODA-RET experiments in HEK-293T cells co-transfected with either $D_{4.4}R$ (A and C) or $D_{4.7}R$ (B and D) fused to the C-terminus and N-terminus of RLuc and the $G\alpha_{i1}$ subunit fused to mVenus. Concentration-response experiments of changes in BRET ratio induced by norepinephrine (NE), dopamine (DA) and DA in the presence of the D_4R antagonist A-412997 (1 μ M) in the interaction of $D_{4.4}R$ - $D_{4.4}R$ (A) or $D_{4.7}R$ - $D_{4.7}R$ (B) dimers with $G\alpha_{i1}$ protein. BRET values are expressed as absolute values and represent the mean $\pm$ SEM of 5–14 different experiments performed in triplicate.

$\alpha_{2A}R$ - $D_{4.4}R$ heteromers (Fig. 2, Table 2). This type of difference agrees with the recently reported distinct ligand-induced G_i protein activation of the $D_{2S}R$ - $D_{2S}R$ homomer and the $D_{2S}R$ - $D_{4.4}R$ and $D_{2S}R$ - $D_{4.7}R$ heteromers [1]. In those experiments, the profile of G_i activation by pramipexole and ropinirole with the $D_{2S}R$ - $D_{2S}R$ homomer was different from that observed with the $D_{2S}R$ - $D_{4.4}R$ heteromer, but indistinguishable from the profile of the $D_{2S}R$ - $D_{4.7}R$ heteromer [1]. In the present experiments, pramipexole showed a significantly higher potency for the $\alpha_{2A}R$ - $D_{4.4}R$ heteromer as compared with the $\alpha_{2A}R$ - $\alpha_{2A}R$ homomer and $\alpha_{2A}R$ - $D_{4.7}R$ heteromer, albeit with lower efficacy (Fig. 2A–C, and Table 2). Ropinirole and rotigotine produced a small $G\alpha_{i1}$ protein activation in $\alpha_{2A}R$ - $D_{4.4}R$ transfected cells, while they did not produce any response within the $\alpha_{2A}R$ - $\alpha_{2A}R$ homomer nor the $\alpha_{2A}R$ - $D_{4.7}R$ heteromer (Fig. 2A–C, and Table 2). On the other hand, the three α_2R agonists, dexmedetomidine, clonidine and guanfacine showed a complete lack of efficacy for the $\alpha_{2A}R$ - $D_{4.4}R$ heteromer, while for the $\alpha_{2A}R$ - $D_{4.7}R$ heteromer they showed the same response as for the $\alpha_{2A}R$ - $\alpha_{2A}R$ homomer (Fig. 2A–C, and Table 2). The selective D_4R agonist A-412997 also failed

to activate $G\alpha_{i1}$ protein by $\alpha_{2A}R$ - $D_{4.4}R$ (Fig. 2B, and Table 2). Altogether, the results using the synthetic compounds confirmed that the heteromerization of $\alpha_{2A}R$ with $D_{4.7}R$, but not with $D_{4.4}R$, blunts the ability of dopaminergic ligands to promote D_4R -mediated G_i protein activation. On the other hand, heteromerization with $D_{4.4}R$, but not with $D_{4.7}R$, seems to blunt the ability of exogenous, but not endogenous, adrenergic ligands to promote $\alpha_{2A}R$ -mediated G_i protein activation.

The lack of efficacy of the potent and selective D_4R agonist A-412997 not only on $\alpha_{2A}R$ - $D_{4.7}R$ but also on the $\alpha_{2A}R$ - $D_{4.4}R$ heteromer did not fit with our initial interpretation of selective blunting of D_4R -mediated G_i protein activation in the $\alpha_{2A}R$ - $D_{4.7}R$. Therefore, we also tested its ability to promote activation of G_i protein with $D_{4.4}R$ - $D_{4.4}R$ and $D_{4.7}R$ - $D_{4.7}R$ homomers in CODA-RET experiments. In fact, A-412997 behaved as a competitive antagonist of DA in both homomers, producing a pronounced shift to the right of the concentration-response curve of DA-mediated G_i protein within $D_{4.4}R$ - $D_{4.4}R$ and $D_{4.7}R$ - $D_{4.7}R$ homomers (Fig. 3).

3.4. Go protein activation by $\alpha_{2A}R$ -D₄R heteromers depends on the D₄R polymorphic variant involved

The inability of A-412997 to promote Gi activation by any D₄R homomer and heteromer is at odds with its previously described full agonist profile in mammalian transfected cells and in laboratory animals [44–46]. Since recent findings indicate that some ligands show functional selectivity for different inhibitory G α subunits [36], we sought to compare the ability of A-412997 to activate G α_{i1} versus G α_{o1} subunits, by analyzing BRET changes in cells transfected with D_{4,4}R or D_{4,7}R fused to RLuc and G α_{i1} or G α_{o1} fused to YFP (Fig. 4A–C). As in D_{4,4}R-D_{4,4}R and D_{4,7}R-D_{4,7}R homomers (Fig. 3), A-412997 did not promote D_{4,4}R- or D_{4,7}R-mediated G α_{i1} activation, but it did promote significant G α_{o1} activation with both D₄R variants (Fig. 4A, B). In CODA-RET experiments (Fig. 4D–F), A-412997 promoted G α_{o1} activation by $\alpha_{2A}R$ -D_{4,4}R heteromer but not by the $\alpha_{2A}R$ -D_{4,7}R heteromer, in agreement with the selective blunting of signaling of D_{4,7}R by endogenous or exogenous ligands when it heteromerizes with $\alpha_{2A}R$ seen in Fig. 2.

Although dexmedetomidine, clonidine and guanfacine are known $\alpha_{2A}R$ agonists [47,48], they failed to activate G α_{i1} in the $\alpha_{2A}R$ -D_{4,4}R heteromer and showed only a weak intrinsic efficacy on the $\alpha_{2A}R$ - $\alpha_{2A}R$ homomer and the $\alpha_{2A}R$ -D_{4,7}R heteromer (Fig. 2A–C). Therefore, we

evaluated the ability of dexmedetomidine to activate α_{2R} coupled to G α_{o1} with BRET experiments in cells transfected with $\alpha_{2A}R$ fused to RLuc and G α_{o1} fused to YFP and co-transfected with either D_{4,4}R or D_{4,7}R (Fig. 4G–I). We found that dexmedetomidine promotes a significant $\alpha_{2A}R$ -mediated G α_{o1} activation in the presence of either D_{4,4}R or D_{4,7}R (Fig. 4G, H). A-412997 produced a significant 2- to 3-fold decrease in the potency of dexmedetomidine in the cells co-transfected with D_{4,4}R but not with D_{4,7}R (Fig. 4G, H). This decrease is indicative of a selective negative allosteric crosstalk between the two agonists in $\alpha_{2A}R$ -D_{4,4}R heteromers coupled to G α_{o1} . Finally, CODA-RET experiments showed that dexmedetomidine was also able to activate G α_{o1} in both the $\alpha_{2A}R$ -D_{4,4}R and $\alpha_{2A}R$ -D_{4,7}R heteromers (Fig. 4D, E).

3.5. D₄R modulation of $\alpha_{2A}R$ -mediated signaling in mammalian transfected cells depends on the D₄R isoform heteromerization

Since the BRET experiments demonstrated the functional and pharmacological differences between $\alpha_{2A}R$ -D_{4,4}R and $\alpha_{2A}R$ -D_{4,7}R heteromers and their distinct coupling to G α_{i1} or G α_{o1} subunits, we decided to identify possible differences in the signaling of $\alpha_{2A}R$ -D_{4,4}R and $\alpha_{2A}R$ -D_{4,7}R heteromers in a mammalian cell line without transfection of exogenous G α proteins. It is well known that most G α subunits are

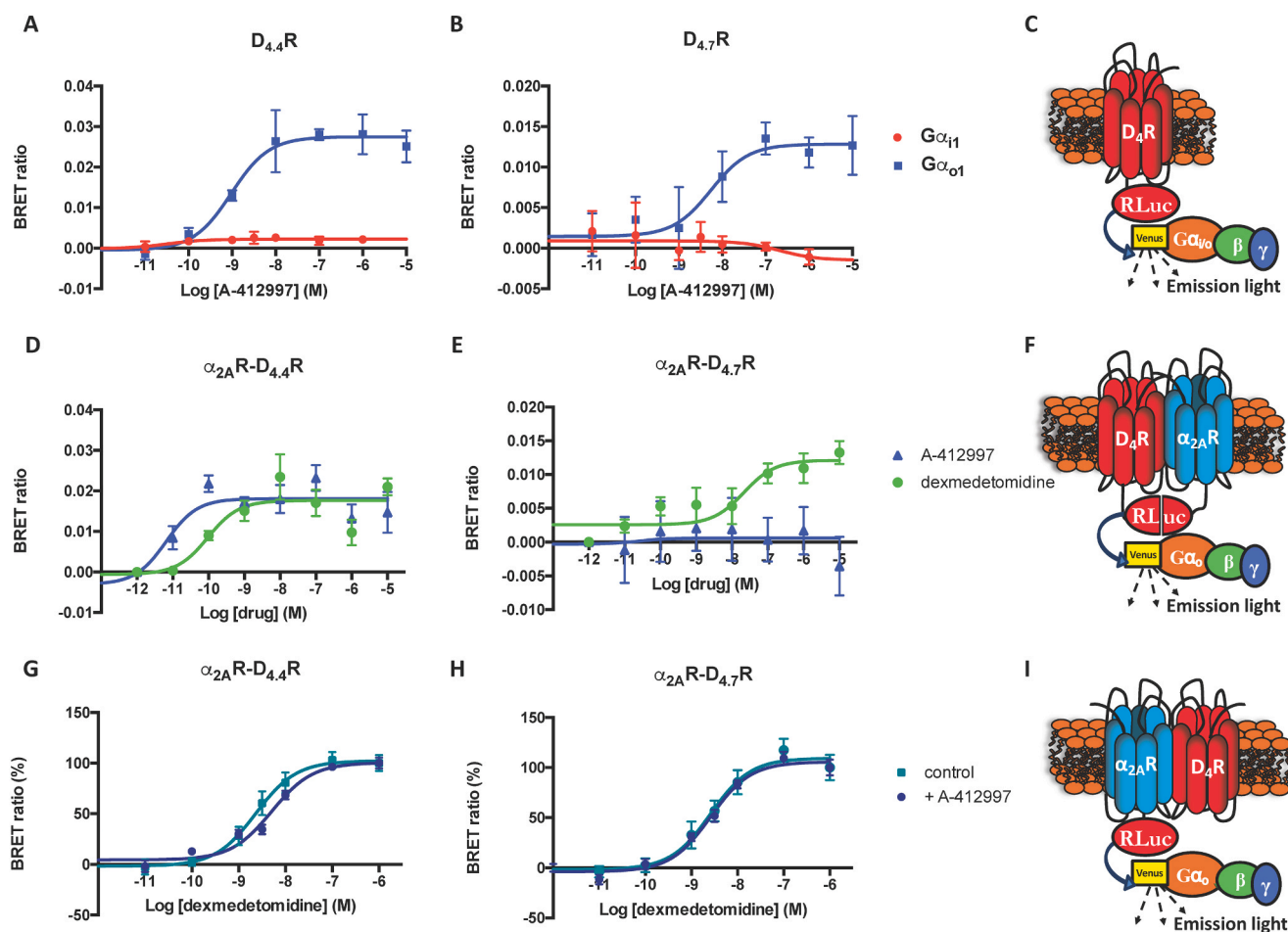


Fig. 4. Functional selectivity of $\alpha_{2A}R$ and D₄R ligands dependent on the α subunit of the G protein and the D₄R polymorphic variant. (A–C) BRET experiments in HEK-293T cells co-transfected with either D_{4,4}R (A) or D_{4,7}R (B) fused to RLuc and G α_{i1} or G α_{o1} subunits fused to mVenus. Concentration-response experiments of changes in BRET ratio induced by the D₄R agonist A-412997. (D–F) CODA-RET experiments in HEK-293T cells co-transfected with $\alpha_{2A}R$ fused to the C-terminus of RLuc and D_{4,4}R (D) or D_{4,7}R (E) fused to the N-terminus of RLuc and the G α_{o1} subunit fused to mVenus. Concentration-response experiments of changes in BRET ratio induced by A-412997 and the $\alpha_{2A}R$ agonist dexmedetomidine. (G–I) BRET experiments in HEK-293T cells co-transfected with $\alpha_{2A}R$ fused to RLuc and unfused D_{4,4}R (G) or D_{4,7}R (H) and the G α_{o1} subunit fused to mVenus. Concentration-response experiments of changes in BRET ratio induced by dexmedetomidine with and without A-412997. BRET values are expressed in absolute values (A, B, D, E) or as the percentage of the maximal effect of dexmedetomidine alone (G and H) and represent the mean $\pm$ SEM of 5–8 different experiments performed in triplicate. (C, F, I) Schematic representations of the different BRET assays.

ubiquitous and each cellular subtype can express at least four or five G α subtypes in different proportions [49]. First, we studied adenylyl cyclase signaling, measuring the ability of the α_2 AR and D $_4$ R agonists, alone or in combination, to counteract forskolin-induced cAMP accumulation in HEK-293T cells stably transfected with either D $_{4.4}$ R or D $_{4.7}$ R and transiently co-transfected with α_2 AR (α_2 AR-D $_{4.4}$ R and α_2 AR-D $_{4.7}$ R cells) (Fig. 5A). Although both agonists inhibited cAMP accumulation, A-412997 was more effective than dexmedetomidine in α_2 AR-D $_{4.4}$ R cells. Since A-412997 showed to be ineffective at activating α_2 AR-D $_{4.7}$ R heteromers (either by coupling with G α_{i1} or G α_{o1} proteins) (Figs. 2C and 4E), its efficacy in α_2 AR-D $_{4.7}$ R cells most probably reflects the activation of D $_{4.7}$ R not forming heteromers with α_2 AR. As mentioned above, the transfected stable cell lines had an excess of D $_4$ R *versus* α_2 AR. In addition, in the α_2 AR-D $_{4.7}$ R cells, the effect of dexmedetomidine plus A-412997 was additive and significantly different from dexmedetomidine alone (Fig. 5A). On the other hand, the results obtained in α_2 AR-D $_{4.4}$ R cells were not additive and the combined treatment did not produce a significantly different effect compared with dexmedetomidine alone, indicating a negative crosstalk (Fig. 5A).

Similar to cAMP levels, both agonists induced significant ERK1/2 phosphorylation, which was additive in α_2 AR-D $_{4.7}$ R but not in α_2 AR-D $_{4.4}$ R cells, where the coactivation with both agonists was not significantly different from A-412997 alone (Figs. 5B and S4). Again, relative to the effect of dexmedetomidine, A-412997 was more effective in α_2 AR-D $_{4.4}$ R cells than in α_2 AR-D $_{4.7}$ R cells. Altogether, these experiments reproduced the results obtained with dexmedetomidine and A-412997 on α_2 AR-D $_{4.4}$ R and α_2 AR-D $_{4.7}$ R heteromer-mediated Go protein activation, with a specific inefficacy (or lower efficacy) of A-412997 in the α_2 AR-D $_{4.7}$ R and a negative crosstalk in the α_2 AR-D $_{4.4}$ R heteromer, but not in α_2 AR-D $_{4.7}$ R.

3.6. α_2 AR-D $_4$ R heteromers are expressed in the cerebral cortex of wild type and hD $_{4.7}$ R knock-in mice

To determine whether α_2 AR and D $_4$ R could form heteromers in native brain tissue, we used the PLA technique, designed to detect *in situ* two different proteins only when located in close proximity [50] (schematic representation appears in Fig. 6J). Using specific anti-D $_4$ R and anti- α_2 AR antibodies, we analyzed the expression of putative α_2 AR-D $_4$ R complexes in the cortex of knock-in hD $_{4.7}$ R mice, which express a D $_4$ R with a

humanized 3IL corresponding to the D $_{4.7}$ R, and WT littermates, which express a shorter third intracellular loop, comparable to the human D $_{4.2}$ R [5]. We observed a great number of cells containing red dots (% of positive cells in Fig. 6A–C), compatible with the presence of α_2 AR-D $_4$ R complexes in the cortex of mice of both genotypes, as compared with the staining in the corpus callosum (taken as negative control brain area) (Fig. 6D). Similar % of positive cells than in corpus callosum were obtained in cortical slices in which one of the primary antibodies of the PLA was missing (Fig. 6E). The specificity of the method to detect α_2 AR and D $_4$ R complexes was validated by immunocytochemistry, comparing the labeling in cells transfected with the corresponding receptors and controls (Fig. S5).

The heteromer disrupting peptide technique was then applied to demonstrate that α_2 AR-D $_4$ R complexes identified by PLA in the mouse brain cortex represent bona fide α_2 AR-D $_4$ R heteromers. This technique has been successfully used to identify GPCR heteromers both in mammalian transfected cells and in native tissues (see, for instance [38, 51]). In order to assess which TM domains are forming the interface between the two protomers, we fused synthetic peptides with the amino sequence of several TM domains of the α_2 AR and D $_4$ R to the HIV-transactivator of transcription peptide (TAT-TM peptides). We tested the ability of TM4, TM5, TM6 and TM7 of D $_4$ R and α_2 AR to disrupt BiLC of α_2 AR fused to cRLuc and D $_{4.4}$ R or D $_{4.7}$ R fused to nRLuc (see Fig. 7A). Only TM4 of both D $_4$ R and α_2 AR were able to significantly decrease the levels of luminescence, indicating that these TMs form part of the heteromeric interface (Fig. 7B). Cells transfected with α_1 AR-nRLuc and α_2 AR-cRLuc or with D $_{4.4}$ R-nRLuc and A $_1$ R-cRLuc were used as negative controls (Fig. 7B). In PLA experiments, in complete correlation with the BiLC experiments, TM4 (but not TM5 or TM7) of D $_4$ R also decreased the number of cells expressing α_2 AR-D $_4$ R complexes detected in the cerebral cortex of WT mice (Fig. 6F–I), indicating that these complexes correspond to α_2 AR-D $_4$ R heteromers.

We next sought to examine the functional significance of α_2 R-D $_4$ R heteromers in the brain cortex. If a significant population of α_2 AR in the mouse cerebral cortex forms heteromers with D $_4$ R and preferentially couple to G α_o proteins, we should be able to observe the same qualitative differences in the pharmacological profile of dexmedetomidine and A-412997 in cortical tissue from knock-in hD $_{4.7}$ R mice *versus* WT mice as those we previously described in α_2 AR-D $_{4.7}$ R cells *versus* α_2 AR-D $_{4.4}$ R cells (Fig. 5B). Dexmedetomidine and A-412997 administered alone

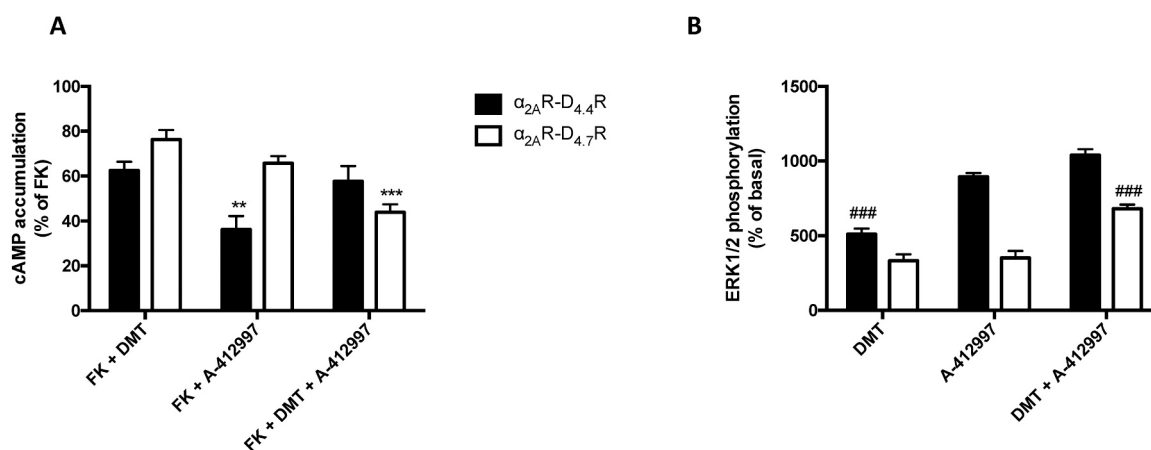


Fig. 5. Differential signaling in α_2 AR-D $_{4.4}$ R and α_2 AR-D $_{4.7}$ R cells. Agonist-induced adenylyl cyclase inhibition and ERK1/2 phosphorylation assays in tetracycline-inducible HEK-293T cells expressing D $_{4.4}$ R (black bars) or D $_{4.7}$ R (white bars) and transiently transfected with α_2 AR. (A) For cAMP determination, cells were treated with the α_2 AR agonist dexmedetomidine (DMT, 100 nM), the D $_4$ R agonist of A-412997 (100 nM) or both agonists in the presence of forskolin (FK, 0.5 μ M). Values are expressed as means $\pm$ SEM of the percentage of cAMP concentration relative to the effect of FK alone of 7 independent experiments performed in triplicates. (B) For ERK1/2 phosphorylation assays, cells were treated with DMT (300 nM), A-412997 (300 nM) or both agonists. Values are expressed as means $\pm$ SEM of the percentage of phosphorylation relative to basal levels in not treated cells of 5–7 independent experiments performed in triplicates. Representative western-blot are shown in Fig. S4. ** p < 0.01, *** p < 0.001 *versus* DMT treatment; ### p < 0.001 *versus* A-412997 treatment (two-way ANOVA followed by Tuckey's *post-hoc* test).

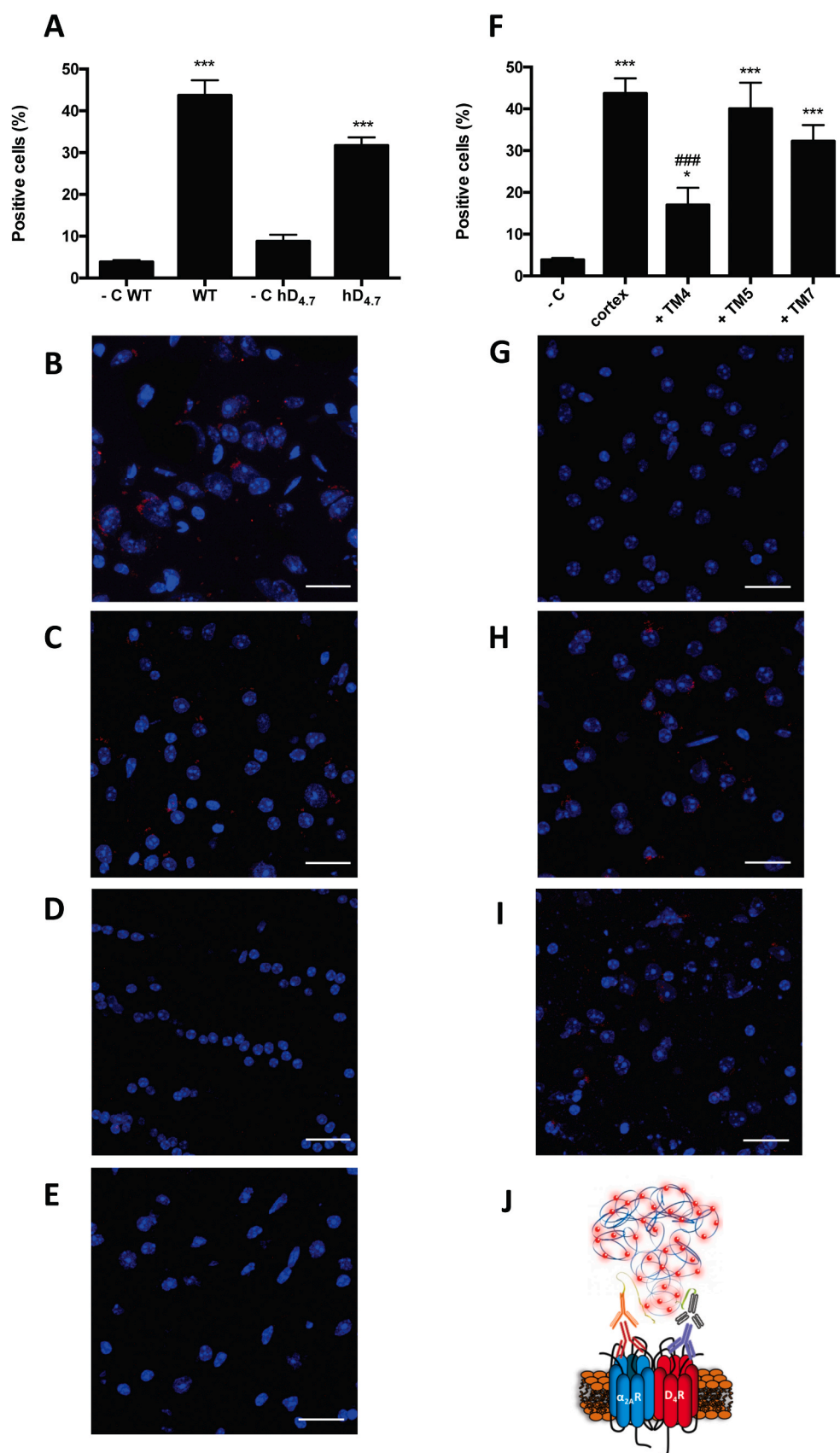


Fig. 6. Expression of $\alpha_{2A}R$ -D₄R heteromers in cortical tissue from WT and knock-in hD_{4.7}R mice. (A) Quantification of cells showing $\alpha_{2A}R$ -D₄R complexes with the PLA assay from cortical brain slices of WT and knock-in hD_{4.7}R mice. Representative confocal microscopy images from cortex of WT (B) or knock-in hD_{4.7}R (C) mice, from corpus callosum of WT mice (D) or from cortex of WT mice in which primary antibody against $\alpha_{2A}R$ is missing (E) (superimposed sections). (F) Quantification of cells showing $\alpha_{2A}R$ -D₄R complexes with the PLA assay from cortical brain slices of WT mice in the absence or presence of TM peptides of the D₄R (TM4, TM5 and TM6). Representative confocal microscopy images from cortex of WT in the presence of D₄R TM4 (G), TM5 (H) or TM7 (I) (superimposed sections). (J) Schematic representation of the PLA assay. The $\alpha_{2A}R$ -D₄R complexes appear as red spots. Cell nuclei are stained with DAPI (blue). Scale bars = 20 μ m. Cortical slices were treated for 4 h with vehicle or with 4 μ M of the corresponding TM peptide before performing the PLA assay. The number of positive cells (containing one or more red spots) is expressed as the percentage of the total number of cells (% of positive cells) and data represent the mean $\pm$ SEM of counts in 4–8 different fields of 3 different experiments. * $p < 0.05$, *** $p < 0.001$ versus the respective negative control in the corpus callosum (–C); ### $p < 0.001$ versus cortex WT, Student's *t*-test (A); one-way ANOVA followed by Dunnett's *post-hoc* test (F).

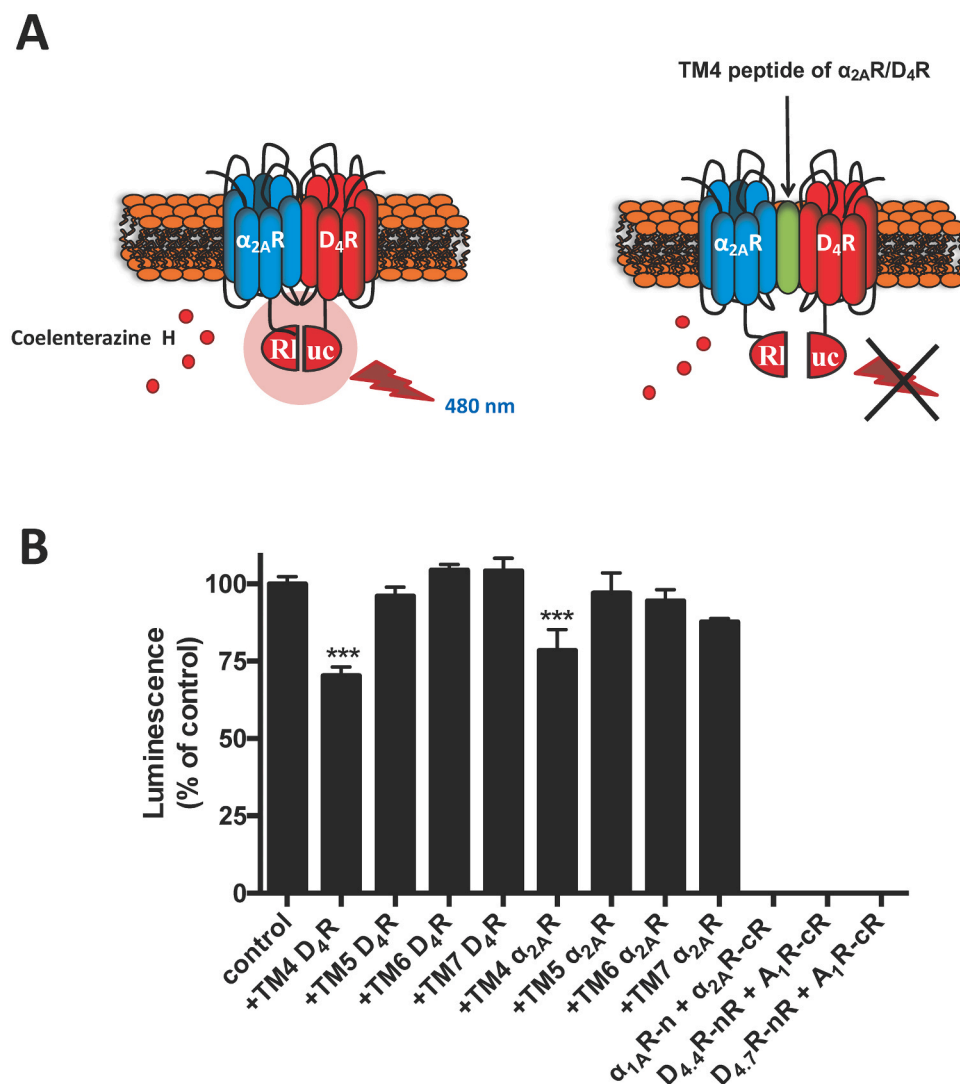


Fig. 7. Disturbing effect of TM peptides on α_{2A} R-D₄R heteromerization in bimolecular luminescence complementation experiments. (A) Schematic representation of the effect of transmembrane peptides disturbing α_{2A} R-D₄R heteromerization and, therefore, RLuc reconstitution of bimolecular luminescence complementation. (B) Quantification of luminescence due to RLuc complementation in HEK-293 cells co-expressing D_{4.4}R-nRLuc and α_{2A} R-cRLuc in the absence or presence of TM4, TM5, TM6 and TM7 peptides of α_{2A} R and D₄R. Cells were treated for 4 h with vehicle or with the corresponding TM peptide (2 μ M) before performing the complementation assay. Values (in % of control) represent means $\pm$ SEM from 5 to 10 different experiments performed in triplicate. Control luminescence was $1,000,000 \pm 100,000$ arbitrary units. Negative controls were obtained with the pairs α_{1A} R-nRLuc and α_{2A} R-cRLuc or D₄R-nRLuc and A₁R-cRLuc, which showed luminescence values lower than 1% of control. Statistical significance was calculated by one-way ANOVA followed by Dunnett's *post-hoc* test. *** $p < 0.001$ versus cells non-treated with the corresponding TM.

produced a similar degree of MAPK activation in cortical slices from WT mice, while A-412997's effect was significantly weaker than of dexmedetomidine in hD_{4.7}R mice (Fig. 8A, B). In addition, in WT mice, we found a negative crosstalk between dexmedetomidine and A-412997 evidenced by a significantly lower MAPK activation than that observed when either drug was administered alone (Fig. 8A). This negative crosstalk could be attenuated with the application of TM4 of the D₄R, which selectively disrupts α_{2A} R-D₄R heteromerization, but not with TM6 of the D₄R (Fig. 8C–E). Thus, in WT mice, in the presence of TM4, but not TM6, of the D₄R, the combined treatment of dexmedetomidine and A-412997 produced about the same level of MAPK activation than either drug when administered alone. The same level of MAPK activation induced by dexmedetomidine alone or combined with A-412997 was observed in hD_{4.7}R mice (Fig. 8B). Therefore, in contrast with the results obtained in transfected cells (Fig. 5B), the combined effects of α_{2A} R and D₄R agonists did not exceed that of the activation of α_{2A} R alone, even in the presence of D_{4.7}R. This difference could be explained by a ceiling effect occurring in brain tissue. Altogether, the evident negative crosstalk observed in α_{2A} R-D₄R heteromers and the D_{4.7}R-dependent decrease in the effect of A-412997 indicate the existence of a significant population of functional α_{2A} R-D₄R heteromers in the mouse cerebral cortex.

4. Discussion

The results presented in this study demonstrate the presence of functional α_{2A} R-D₄R heteromers in mouse cerebral cortex. In addition, we show a functional difference in the function of the two most frequent human *DRD4* gene polymorphisms, D_{4.4}R and D_{4.7}R [13–15]. Synthetic peptides with the amino acid sequence of TM domains of GPCRs have become a very useful tool for the demonstration of GPCR oligomers, the identification of their putative oligomeric (homomeric and heteromeric) interfaces and their demonstration in native tissues (for recent studies, see [52–55]). In the present study, the application of synthetic peptides in experiments with PLA and α_{2A} R and D₄R agonist-induced MAPK activation, allowed to demonstrate that α_{2A} R-D₄R heteromers constitute a significant functional population of α_{2A} R and D₄R in the mouse cerebral cortex. The functional differences described here might help to explain the association of human polymorphisms of α_{2A} R and D₄R genes with impulsivity and vulnerability to ADHD [13,16–20,26].

We used BRET assays to study the *in vitro* effect of endogenous ligands, DA and NE, and several synthetic ligands on their ability to promote activation of G $\alpha_{i/o}$ proteins by α_{2A} R- α_{2A} R homomers and α_{2A} R-D₄R heteromers. The drugs showed not able pharmacological

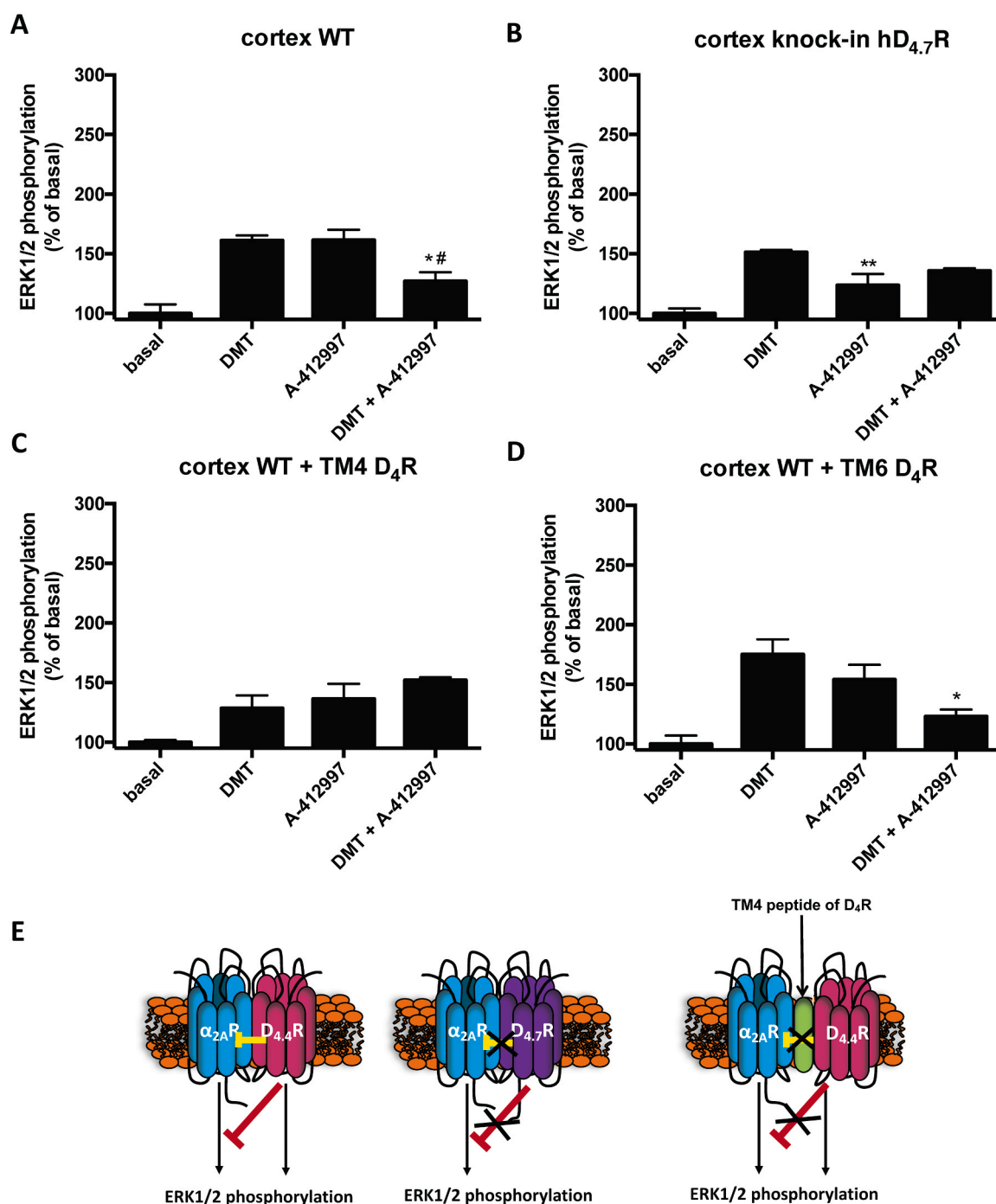


Fig. 8. Differential signaling of α_2 R-D₄R heteromers expressed in cortical tissue from WT and knock-in hD_{4.7}R mice. ERK1/2 phosphorylation in cortical brain slices from WT (A, C, D) and knock-in hD_{4.7}R (B) mice determined by western-blot. Cortical slices were treated for 4 h with vehicle (A and B) or with 4 the D₄R interference peptides TM4 (C) or TM6 (D) (4 μ M in both cases) before treatment with the α_2 R agonist dexmedetomidine (DMT, 1 μ M), the D₄R agonist A-412997 (1 μ M) or both agonists. Values are expressed as mean $\pm$ SEM of phosphorylation relative to basal levels in not-treated slices of 3–5 independent experiments performed in quadruplicate. Representative western-blot are shown in Fig. S4. * $p < 0.05$, ** $p < 0.01$ versus 1 μ M DMT; # $p < 0.05$ versus 1 μ M A-412997 (one-way ANOVA followed by Bonferroni's *post-hoc* test). (E) Schematic representation of the allosteric interactions within the α_2 R-D₄R heteromers and the effect of transmembrane peptide TM4 of both receptors disturbing the heteromer formation and, therefore, the inter-protomer allosteric modulations in WT mice.

differences that depended on the G protein subtype ($G\alpha_{i1}$ or $G\alpha_{o1}$) and on the polymorphic variant of the D₄R. Recent results obtained with such assays allowed to extend the concept of functional selectivity, which generally implies the selectivity of some ligands in promoting biased G protein-dependent or G protein-independent signaling [56], to a G protein subunit-dependent functional selectivity [36,57]. More commonly, instead of a complete functional selectivity, this phenomenon implies that different ligands demonstrate different potencies and

efficacies at activating different G α protein subunits [36,57].

In the present study, we report a G protein-subtype dependent functional selectivity of a D₄R ligand, A-412997, which behaved as a potent and efficacious agonist at activating $G\alpha_{o1}$, whilst it behaved as a competitive antagonist at activating $G\alpha_{i1}$ with both D_{4.4}R and D_{4.7}R. From a physiological point of view, $G\alpha_o$ proteins seem to be predominant in the brain, which would agree with the previously reported efficacy of A-412997 in animals [44–46]. In addition, to our knowledge,

here we report for the first time a G protein-dependent functional selectivity that is not only dependent on receptor heteromerization, but on the polymorphic variant of one of the GPCR protomers of the heteromer. The endogenous ligands, NE and DA, were able to activate $G\alpha_{i1}$ by binding to the $\alpha_{2A}R$ forming $\alpha_{2A}R$ homomers and $\alpha_{2A}R$ -D₄R heteromers, as demonstrated by the counteracting effect of an $\alpha_{2A}R$ antagonist. On the other hand, the exogenous ligands dexmedetomidine, guanfacine and clonidine were unable to activate $G\alpha_{i1}$ with the $\alpha_{2A}R$ -D_{4.4}R heteromer. Yet, dexmedetomidine was effective at activating $G\alpha_{o1}$ with both $\alpha_{2A}R$ -D_{4.4}R and $\alpha_{2A}R$ -D_{4.7}R heteromers.

Another remarkable pharmacological difference between $\alpha_{2A}R$ -D_{4.4}R and $\alpha_{2A}R$ -D_{4.7}R heteromers was the loss of efficacy of any D₄R agonists to promote activation of any G protein within the $\alpha_{2A}R$ -D_{4.7}R heteromer. Hence, the D_{4.7}R appears to be a silent partner when present in the $\alpha_{2A}R$ -D_{4.7}R heteromer. Nevertheless, $\alpha_{2A}R$ -D_{4.7}R heteromerization significantly increased the potency of NE, by five times, representing a gain of function of the $\alpha_{2A}R$. This is reminiscent of the pharmacological properties of the D₂R-D_{4.7}R heteromer, which is also associated with an increase in the potency of DA and a gain of function of the D₂R (increase in constitutive activity; see Section 1 and Sánchez-Soto et al. [1]). In contrast, D_{4.4}R can be activated by NE and DA when present in the $\alpha_{2A}R$ -D_{4.4}R heteromer, regardless of the G protein subtype. In addition, it promotes a negative crosstalk between $\alpha_{2A}R$ and D_{4.4}R agonists. Here, we showed the ability of A-412997 to selectively decrease signaling induced by dexmedetomidine in HEK-293T cells expressing $\alpha_{2A}R$ and D_{4.4}R, but not $\alpha_{2A}R$ and D_{4.7}R. Together with the significant A-412997-induced signaling in $\alpha_{2A}R$ -D_{4.4}R cells and its attenuation in $\alpha_{2A}R$ -D_{4.7}R cells, these results also indicated that $G\alpha_o$ rather than $G\alpha_i$ proteins are primarily involved.

The results obtained in cortical tissue from WT and hD_{4.7}R knock-in mice recapitulated the phenomena observed in cultured cells. In addition, the selective counteraction of the negative crosstalk with the $\alpha_{2A}R$ -D₄R heteromer disruptive peptide, demonstrated the existence of a significant population of $\alpha_{2A}R$ -D₄R heteromers in mouse cerebral cortex. Moreover, it indicated that in native tissue the heteromer is likely coupled to $G\alpha_o$ proteins. This led to two obvious questions: first, what is the functional role of $\alpha_{2A}R$ -D₄R heteromers and the different functional role of $\alpha_{2A}R$ -D_{4.4}R and $\alpha_{2A}R$ -D_{4.7}R heteromers; and second, how these differences relate to the human D₄R variants and their associations with impulsivity, ADHD and SUD and their respective pharmacological treatment. A recent large meta-analysis [20] indicated that D_{4.7}R is an ADHD risk factor in European-Caucasian populations and is linked to a poor treatment efficacy of methylphenidate, while D_{4.4}R is a significant protective factor in European-Caucasian and South American populations and is also associated to a positive response to methylphenidate.

Previous studies have shown similar potencies for NE and DA at activating D_{4.4}R or D_{4.7}R when coupled to $G\alpha_{o1}$, while higher potencies for NE than DA are found for $\alpha_{2A}R$ [3,36]. Hence, both $\alpha_{2A}R$ and D_{4.4}R protomers can be activated by NE and DA, at least with the $\alpha_{2A}R$ -D_{4.4}R heteromer. Our heuristic hypothesis is that the $\alpha_{2A}R$ -D_{4.4}R heteromer represents a fine-tune device that senses different concentrations of NE. Low NE concentrations activate $\alpha_{2A}R$ and higher NE concentrations or the simultaneous presence of DA also activate the D_{4.4}R, which exerts a negative crosstalk promoting a negative feedback. This inverted u-shaped function might provide a buffer mechanism responsible for resilience to the development of ADHD and for a therapeutic response to methylphenidate in the presence of the $\alpha_{2A}R$ -D_{4.4}R, which is not as effective when acting on $\alpha_{2A}R$ -D_{4.7}R heteromers.

In summary, we demonstrate that $\alpha_{2A}R$ can form oligomeric complexes with D_{4.4}R and D_{4.7}R in transfected cells, that these heteromers constitute a significant functional population of $\alpha_{2A}R$ and D₄R in mouse cerebral cortex and that their pharmacological and functional profiles depend on the D₄R polymorphic variant involved. Taken together, our data indicate that $\alpha_{2A}R$ -D₄R heteromers need to be considered as novel targets for the development of therapeutic agents for ADHD and other impulsivity-related neuropsychiatric disorders and that the therapeutic

benefits and strategies of these drugs must consider the differential properties of the most common human D₄R variants (such as D_{4.4}R and D_{4.7}R) present in the $\alpha_{2A}R$ -D₄R heteromers.

Author contributions

V.C.-A., E.M., A.C., E.-I.C., S.F., V.C. participated in the research design. V.C.-A. conducted all the experiments. E.M. aided with BRET, PLA and ERK phosphorylation experiments. M.S.-S. performed and aided with CODA-RET assays. J.B. aided with mice experiments. P.H.-R. aided with BRET experiments. A.C., V.C. aided with binding assays. N.-S.C., M.R. contributed research materials. V.C.-A., E.M., M.S.-S., J.B., S.F., V.C. contributed to the data analyses and interpretation. E.-I.C., S.F., V.C. acquired funding. V.C.-A., E.M., M.S.-S., J.B., M.R., S.F. and V.C. wrote or contributed to the writing and revision of the manuscript. All authors contributed to and have approved the final manuscript.

Declaration of Competing Interest

The authors declare that no competing interests exist.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper or the [Supplementary materials](#).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.phrs.2021.105745](https://doi.org/10.1016/j.phrs.2021.105745).

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