Published in final edited form as:

Pharmacol Res. 2019 January ; 139: 337–347. doi:10.1016/j.phrs.2018.11.032.

Reinterpreting anomalous competitive binding experiments within G protein-coupled receptor homodimers using a dimer receptor model

Verónica Casadó-Anguera^{a,b}, Estefanía Moreno^{a,b}, Josefa Mallol^{a,b}, Sergi Ferré^c, Enric I. Canela^{a,b}, Antoni Cortés^{a,b}, and Vicent Casadó^{a,b,*}

^aCentro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

^bInstitute of Biomedicine of the University of Barcelona (IBUB), and Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

^cNational Institute on Drug Abuse, I.R.P., N.I.H., D.H.H.S., Baltimore, MD 21224, USA

Abstract

An increasing number of G protein-coupled receptors (GPCRs) have been reported to be expressed in the plasma membrane as dimers. Since most ligand binding data are currently fitted by classical equations developed only for monomeric receptors, the interpretation of data could be misleading in the presence of GPCR dimers. On the other hand, the equations developed from dimer receptor models assuming the existence of two orthosteric binding sites within the dimeric molecule offer the possibility to directly calculate macroscopic equilibrium dissociation constants for the two sites, an index of cooperativity (D_c) that reflects the molecular communication within the dimer and, importantly, a constant of radioligand-competitor allosteric interaction (K_{DAB}) in competitive assays. Here, we provide a practical way to fit competitive binding data that allows the interpretation of apparently anomalous results, such as competition curves that could be either bell-shaped, monophasic or biphasic depending on the assay conditions. The consideration of a

*Corresponding author: Vicent Casadó. Laboratory of Molecular Neurobiology, Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain. Tel.: (34) 934039279, fax: (34) 934021559, vcasado@ub.edu, vcasadoanguera@gmail.com, (V. Casadó-Anguera), fifa877@hotmail.com, (E. Moreno), jmallol@ub.edu, (J. Mallol), sferre@intr.nida.nih.gov, (S. Ferré), ecanela@ub.edu, (E.I. Canela), antonicortes@ub.edu, (A. Cortés), vcasado@ub.edu, (V. Casadó).

Chemical compounds studied in this article:

CGS21680, PubChem CID: [3086599](#)

DPCPX, PubChem CID: [1329](#)

R-PIA, PubChem CID: [93205](#)

SCH23390, PubChem CID: [22450127](#)

SCH442416, PubChem CID: [10668061](#)

SKF81297, PubChem CID: [1218](#)

Competing interests

The authors declare that there are no competing interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

radioligand-competitor/ allosteric interaction allows fitting these curve patterns both under simulation conditions and in real radioligand binding experiments, obtaining competitor affinity parameters closer to the actual values. Our approach is the first that, assuming the formation of receptor homodimers, is able to explain several experimental results previously considered erroneous due to their impossibility to be fitted. We also deduce the radioligand concentration responsible for the conversion of biphasic to monophasic or to bell-shaped curves in competitive radioligand binding assays. In conclusion, bell-shaped curves in competitive binding experiments constitute evidence for GPCR homodimerization.

Graphical abstract

Keywords

G protein coupled receptor; pharmacological parameter; allosteric modulation; protein-protein interaction; receptor homodimer; dimer receptor model

1. Introduction

G protein-coupled receptors (GPCRs) comprise the largest protein superfamily in mammalian genomes, covering around 3% of the human proteome [1]. They are involved in 80% or more of the signal transduction processes that occur across cell membranes, exerting their intracellular effects in response to a wide array of extracellular ligands, including single photons, neurotransmitters, hormones and peptides [2]. Their critical role in the mediation of transmembrane signal transduction makes GPCRs therapeutic targets in a large number of diseases, either due to their direct role in the pathophysiology of specific disease or due to their ability to modulate a set of signaling cascades implicated in a specific disease [1, 3]. Therefore, GPCRs are the most important class of membrane proteins in clinical medicine, accounting for about 40% of all current marketed drugs. Moreover, 20% of recently Food and Drug Administration (FDA) approved drugs act through modulating GPCR functions [4–6], providing treatments for CNS disorders, cardiac dysfunction, cancer, diabetes, obesity, inflammation, and pain [7].

The long perceived notion that GPCRs only function in monomeric form [8–13] has been changed in the last decades by the description of a number of GPCRs of classes A, B and that are found as homodimers, heterodimers and higher order oligomers (for review, see [14–26]). In many cases, oligomeric structures of GPCRs are essential for receptor maturation, ligand pharmacology, signal transduction, and cellular trafficking [22, 27–31]. Recently, several pharmacological tools have been developed to detect homodimers *in vitro*, *ex vivo* and *in vivo* such as bivalent and bitopic ligands. Interestingly, the bitopic ligand SB269652

was described as the first allosteric molecule able to distinguish between dopamine D₂ receptor (D₂R) monomers and homodimers [32, 33]. It has also been recently reported a true bivalent ligand that is able to bind simultaneously to both orthosteric sites of the D₂R homodimer [34]. On the other hand, based on single molecule tracking assays, some authors have reported that GPCR dimerization is a dynamic process in which GPCRs can associate, dissociate and re-associate and that agonists, bitopic and bivalent ligands can alter this dynamics, stabilizing preexisting dimers prolonging their lifetime [35–40]. However, an equilibrium clearly displaced towards the formation of oligomeric complexes has been proposed for the adenosine A_{2A} receptor-D₂R heterotetramer [41, 42], which forms functional pre-coupled complexes with their cognate Gs and Gi proteins and with subtype 5 of the adenylyl cyclase [43].

Radioligand binding is widely used to characterize receptors and determine their anatomical distribution. Saturation, competition and kinetic assays are the three types of experiments more commonly used. In saturation experiments, tissue sections, cultured cells, or homogenates are incubated with an increasing concentration of a radioligand, which is usually a radiolabeled synthetic drug. The subsequent analysis using nonlinear regression programs measures the affinity of the labeled ligand for the receptor (equilibrium dissociation constant K_D), and the receptor density (B_{max}). In kinetic experiments, samples are incubated with a constant radioligand concentration and, measuring its binding along the time, the rate of association (k_+ or k_{on}) to or dissociation (k_- or k_{off}) from a receptor is calculated. However, in pharmacological studies, competition binding assays are the most widely used to determine the affinity and selectivity of an unlabeled ligand to compete for the binding of a fixed concentration of radiolabeled ligand to a receptor. Quantitative autoradiography and positron emission tomography (PET) image analysis are other sensitive techniques to detect low levels of radiolabeled ligands and to determine the anatomical distribution of receptors in tissue slices or in whole organs [44].

Monomeric GPCRs have a single binding site and drug–receptor interactions are driven by the law of mass action, where a drug bounds to a particular receptor at a rate that depends on the concentration of both the drug and the receptor [45]. Therefore, at equilibrium, in saturation-binding studies, the B_{max} expressed by the sample must be the same for all radioligands and should be independent of assay conditions. In competitive binding assays, all ligands should fully compete with each other, and dissociation constants determined in saturation and competition studies must be the same [45]. In this context of monomeric receptors, complex saturation or competition curves are interpreted considering two independent receptor populations that are not in equilibrium: one bound to G protein and the other not. In this scenario, the receptors coupled to G proteins have higher affinity (K_{DH}) for the agonist. These receptors could then be converted into low affinity (K_{DL}) receptors with the addition of GTP, which uncouple the G protein from the receptor [46].

However, given that GPCRs are oligomers, it would be expected that this could alter the properties of the receptor, with equilibrium and kinetic binding assays exhibiting cooperativity [47]. In fact, there is a growing list of receptors that have been found to form homomers, such as adenosine A₁ [48, 49], A_{2A} [42, 43, 49–51] and A₃ [52], dopamine D₁ [53, 54], D₂ [41–43, 55, 56] and D₃ [54, 56], serotonin 5HT_{1A} [50], 5HT_{2A} [53], 5HT_{2C}

[57–59] and 5HT₇ [60], adrenergic α_{1B} [53], β_1 [61,62] and β_2 [53, 61, 63, 64], cannabinoid CB₁ [65], angiotensin AT₁ [66], metabotropic glutamate mGlu₂ [67], muscarinic M₁ [53, 68], M₂ [53, 68,69] and M₃ [68,70], δ [71–73], κ [74] and μ [75] opioid, neurotensin 1 [76], melatonin MT₂ [77], niacin [78], and chemokine CXCR4 [79] receptors. Analysis of complex radioligand binding curves, such as upward concave nonlinear Scatchard plots in saturation experiments and as biphasic curves in agonist-antagonist competitive experiments, have contributed to this notion. To deal with receptor homodimers, dimeric models have been developed [80–85]. These models consider an allosteric communication through the two protomers that allows negative cooperativity, meaning that the binding of a ligand to the first protomer decreases the affinity of the same ligand for the second protomer. In these cases, when handling with complex radioligand-binding curves, the use of the traditional two-independent-site model generates values for the equilibrium dissociation constants and for the number of receptors that vary significantly depending on the concentration of the radioligand employed [86, 87]. This lack of robustness of the two-independent-site model indicates that it is inappropriate and that the parameters must be estimated using a dimer receptor model [24, 25]. In other cases, when working with dimeric receptors and using monomeric models, dissociation constants determined in saturation and competition experiments do not match [47, 88]. Moreover, concave-downward Scatchard plots, i.e. positive cooperativity [88], only can be explained by dimeric models. In addition, mismatches in constant values obtained when dissociation is performed by dilution vs. by an excess of unlabeled ligand [48, 89–91] cannot be explained by monomeric receptor models either.

Here, we focus on anomalous competitive binding experiments, when curves appear as biphasic, monophasic or bell-shaped depending on the assay conditions. Previously, Wreggett and Wells [92], working with cardiac muscarinic receptors, reported that non-hydrolysable guanyl nucleotides induced bell-shaped curves in competitive radioligand binding assays which would only be interpretable assuming tetrameric receptors and a modulation between agonist and radioligand. Albizu et al. [88] also showed some competition curves exhibiting an increase of the radioligand binding to vasopressin receptors for low concentrations of competitors, suggesting a cooperative binding process. Later, we provided experimental evidence showing the effect of radioligand concentration on the conversion of biphasic to monophasic curves [93]. Now, we demonstrate that the existence of a radioligand-competitor allosteric interaction deduced from our dimer receptor model allows the fitting of bell-shaped curves, typically considered as anomalous or erroneous results. We also deduce the radioligand concentration responsible for the conversion of biphasic to monophasic or to bell-shaped curves in competitive radioligand binding assays. These curves are only interpretable assuming the formation of receptor homodimers.

2. Materials and Methods

2.1. Membrane preparation and protein determination

Membrane suspensions from sheep brain striatum were processed as described previously [94, 95]. Tissue was disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for three 5 s-periods in 10 volumes of 50 mM Tris-HCl

buffer, pH 7.4 containing a protease inhibitor cocktail (1:1000, Sigma, St. Louis, MO, USA). Cell debris were eliminated and membranes were obtained by centrifugation at 105,000 x g (60 min, 4°C), and the pellet was resuspended and recentrifuged under the same conditions. The resulting pellet was stored at -80°C and washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard.

2.2. Radioligand binding experiments

For radioligand competition experiments, membrane suspensions (0.2 mg protein/ml) were incubated 2 h in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and 0.2 U/ml (1 µg/ml) desalted bovine adenosine deaminase (EC 3.5.4.4; Roche, Basel, Switzerland; only in adenosine A_{2A} receptor binding experiments) with the indicated free concentration of the adenosine A₁ receptor (A₁R) antagonist [³H]DPCPX (PerkinElmer, Wellesley, MA, USA), the adenosine A_{2A} receptor (A_{2A}R) agonist [³H]CGS21680 (PerkinElmer, Wellesley, MA, USA) or the dopamine D₁ receptor (D₁R) antagonist [³H]SCH23390 (PerkinElmer) and increasing concentrations of the A₁R agonist R-PIA, the A_{2A}R antagonist SCH442416, or the D₁R agonist SKF81297 (triplicates of 10–13 different concentrations from 0.1 nM to 50 µM; Tocris, Ellisville, MO, USA), respectively. Incubations were carried out at 25°C, except for A_{2A}R binding assays that were at 12°C. For radioligand saturation experiments of [³H]SCH23390, membranes were incubated in the same buffer, containing MgCl₂, with increasing free concentrations (26 pM to 2.2 nM) of the radioligand.

For radioligand dissociation kinetic experiments, membrane suspensions of sheep striatum (0.5 mg protein/ml) were incubated at 25°C for 90 min in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and 0.17 nM of the D₁R antagonist [³H]SCH23390 in the absence or presence of 5 nM SKF81297 or 0.5 nM SKF83566. Dissociations were initiated by the addition of 50 volumes of the binding buffer (the infinite dilution method) in the presence or absence of the corresponding modulator at the same concentration as for the association. At the indicated time intervals, total binding was measured as described below.

Nonspecific binding was determined in the presence of an excess of the corresponding unlabeled antagonist and confirmed that the value was the same as calculated by extrapolation of the competition curves. Free and membrane bound radioligands were separated by rapid filtration of 500 µl aliquots (for the competition assays) or 10 ml (for the dissociation assays) 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 Tris-HCl buffer. The filters were incubated with 10 ml of Ultima Gold MV scintillation cocktail (PerkinElmer, Boston, MA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 2800 scintillation counter (PerkinElmer, Boston, MA, USA) with an efficiency of 62% [95].

2.3 Binding data analysis using the dimer receptor model

Radioligand saturation curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the specific binding

data to our dimer receptor model [25, 83, 93]. To calculate the macroscopic equilibrium dissociation constants involved in the binding of the radioligand, the following equation deduced by Casadó et al. [83] was considered:

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

where A represents the free radioligand (the A₁R antagonist [³H] DPCPX, the A_{2A}R agonist [³H] CGS21680, or the D₁R antagonist [³H]SCH23390) concentration, R_T is the total amount of receptor dimers and K_{DA1} and K_{DA2} are the macroscopic dissociation constants describing the binding of the first and the second radioligand molecule (A) to the receptor homodimer.

Radioligand competition curves were also analyzed by nonlinear regression fitting experimental data to the following equation deduced from our dimer receptor model [83]:

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

where A represents the radioligand (the A₁R antagonist [³H]DPCPX, the A_{2A}R agonist [³H]CGS21680, or the D₁R antagonist [³H]SCH23390) concentration, R_T is the total amount of receptor dimers and K_{DA1} and K_{DA2} are the macroscopic dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed competing compound (the A₁R agonist R-PIA, the A_{2A}R antagonist SCH442416, or the D₁R agonist SKF81297) concentration and K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B; K_{DAB} can be described as a hybrid equilibrium radioligand-competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

Since the radioligand A (the antagonist [³H]DPCPX, the agonist [³H]CGS21680, or the antagonist [³H]SCH23390) showed non-cooperative behavior ([83, 93] and results not shown), Eq. (2) was simplified to Eq. (3) due to the fact that K_{DA2} = 4K_{DA1} (see [83]):

$$A_{\text{bound}} = \frac{\left(4K_{DA1}A + 2A^2 + \frac{4K_{DA1}AB}{K_{DAB}}\right)R_T}{4K_{DA1}^2 + 4K_{DA1}A + A^2 + \frac{4K_{DA1}AB}{K_{DAB}} + \frac{4K_{DA1}^2B}{K_{DB1}} + \frac{4K_{DA1}^2B^2}{K_{DB1}K_{DB2}}} \quad (\text{Eq. 3})$$

The dimer cooperativity index for the radioligand A or the competing ligand B was calculated as (see [83]):

$$D_{CA} = \log \left(4 \frac{K_{DA1}}{K_{DA2}} \right) \quad D_{CB} = \log \left(4 \frac{K_{DB1}}{K_{DB2}} \right) \quad (\text{Eq. 4})$$

where 0 means non-cooperative value, positive values indicate positive cooperativity, whereas negative values imply negative cooperativity.

In the experimental conditions when both the radioligand A and the competitor B show non cooperativity, it results that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$, and Eq. (2) and (3) were simplified to:

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

The radioligand-competitor allosteric index can be calculated according to the equation:

$$D_{AB} = \log \left(2 \frac{K_{DB1}}{K_{DAB}} \right) \quad D_{BA} = \log \left(2 \frac{K_{DA1}}{K_{DBA}} \right) \quad (\text{Eq. 6})$$

where 0 means that the presence of the radioligand does not affect the binding of the competitor to the empty protomer within the dimer, and vice-versa. Positive or negative values of D_{AB} indicate that the presence of the radioligand increases or decreases, respectively, the affinity of the competitor for binding to the empty protomer within the dimer. D_{AB} and D_{BA} are correlated because $K_{DBA} = K_{DAB} K_{DA1} / K_{DB1}$ [48].

For comparison, data were also fitted to the classical one-site receptor model when monophasic competition curves were observed and to the classical two-independent-site receptor model when biphasic competition curves were obtained, using respectively the equations:

$$A_{\text{bound}} = \frac{R IC_{50}}{IC_{50} + B} \quad (\text{Eq. 7})$$

$$A_{\text{bound}} = \frac{R_H IC_{50H}}{IC_{50H} + B} + \frac{R_L IC_{50L}}{IC_{50L} + B} \quad (\text{Eq. 8})$$

where R , R_H and R_L are the specific binding in the absence of competing ligand. IC_{50} , IC_{50H} and IC_{50L} of the B compound are related with the respective equilibrium dissociation constants K_D , K_{DH} and K_{DL} according with Cheng and Prusoff equation [96]:

$$K_{DH} = \frac{IC_{50H}}{1 + \frac{A}{K_{DA}}} \quad K_{DL} = \frac{IC_{50L}}{1 + \frac{A}{K_{DA}}} \quad (\text{Eq. 9})$$

Dissociation kinetic data were fitted to the following empirical equation:

$$A_{total_bound} = \sum_{i=1}^n A_{ei} \cdot e^{-t \cdot k_i} + A_{nonspecific_bound} \quad (\text{Eq. 10})$$

where A_{ei} represents the initial radioligand (the D_1 antagonist [3H]SCH23390) specifically 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 monophasic curves $n=1$, and for biphasic curves $n=2$.

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program. The test of significance for two different model population variances was based upon the F distribution (see [94], for details). Using this F test, a probability greater than 95% ($p < 0.05$) was considered the criterion to select a more complex model (cooperativity in Eq. (3) or two-sites in Eq. (8)) over the simplest one (non-cooperativity in Eq. (5) or one site in Eq. (7)). Results are given as parameter values $\pm$ SEM of three independent experiments performed with different striatum homogenates of 3–4 animals.

3. Results

3.1 Adenosine A_1 receptors show slightly bell-shaped competitive curves

Working with A_1R , competition experiments are usually performed with an antagonist radioligand, such as [3H]DPCPX, displaced by a non-labeled agonist, such as R-PIA. Sometimes the curves obtained show a slight increase in the radioligand binding at low concentrations of the displacer R-PIA. This small bell-shaped pattern cannot be fitted by classical mathematical models based on monomeric receptors, such as the commonly used two-independent site model of radioligand binding to receptors. Data showed in Figure 1A are an example of a competition experiment of 0.009 nM [3H]DPCPX vs. R-PIA. Parameter values obtained by fitting these data to the classical two-independent-site model (Eq. (8)) appear in Table 1 and the fitting curve is represented in Figure 1A. The affinity values of R-PIA deduced from Cheng and Prusoff equation (Eq. 9) [96] are much higher than those obtained from saturation assays that were in the low nM range [48]. As we can see, the points showing enhanced radioligand binding are left out. However, if we use our dimer receptor model (see Methods), and the points showing enhanced radioligand binding have low experimental dispersion, the bell-shaped curve can be accurately fitted by Eq.(3) using $K_{DA1}=0.038$ nM [48] (see Figure 1B). The parameter values obtained also appear in Table 1.

Our dimer receptor model is able to explain the enhanced radioligand binding at low displacer concentrations due to the existence of an allosteric interaction between the two orthosteric ligands, radioligand ($[^3\text{H}]\text{DPCPX}$) and competitive displacer (R-PIA), which is measured by the K_{DAB} parameter (see Methods and Table 1). By including this parameter in the fit, the two affinity values obtained for cooperative R-PIA are closer to the expected values from saturation assays [48].

However, if the points showing enhanced radioligand binding have high experimental dispersion, the inclusion of a new parameter (K_{DAB}) does not significantly improve data fit to the dimer receptor model. In this case, the points showing enhanced radioligand binding are commonly underestimated and the fit to the dimer model overlaps with that obtained using the two independent-site model.

3.2 Adenosine A_{2A} receptors show moderate bell-shaped competitive curves

Next, we moved to competitive radioligand binding assays with the $A_{2A}\text{R}$ due to our expertise with the displacement of the $A_{2A}\text{R}$ agonist $[^3\text{H}]\text{CGS21680}$ by unlabeled competitive antagonists of this receptor. With these ligands, the curves obtained frequently show a more evident bell-shaped pattern despite that the experimental dispersion with this radioligand is high. In this particular experiment, we used 24 nM $[^3\text{H}]\text{CGS21680}$ as free radioligand and increasing concentrations of SCH442416 as competitor. In the conditions pointed out in Figure 2, we always obtained a bell-shaped curve when the concentrations of competitor (from 0.1 nM to 30 nM) clearly increased the binding of $[^3\text{H}]\text{CGS21680}$, significantly more than in Figure 1. When these data are fitted by the classical one site or two-independent site models (Eq. (7) or (8)), the first points are not correctly fitted, and the points showing enhanced radioligand binding are left out (Figure 2A, dashed line). When fitting binding data to our dimer receptor model, we can consider the competitor SCH442416 as non-cooperative and only use the K_{DB1} parameter (Eq. (5) with $K_{\text{DAB}}=2K_{\text{DB1}}$). The values obtained, using $K_{\text{DA1}}=41$ nM [51], appear in Table 2 and the regression curve (solid line) roughly overlaps with the dashed line in Figure 2A. In contrast, if we consider that the antagonist shows cooperativity, we must include a second parameter, K_{DB2} (Eq. (3) with $K_{\text{DAB}}=2K_{\text{DB1}}$). In this case, the fit is not better and overlaps with the solid line. Only when we consider the presence of an allosteric modulation between radioligand and competitor (K_{DAB} parameter), we obtain a bell-shaped curve and all points are correctly fitted (Eq. (5)) (see Figure 2B). All parameter values obtained also appear in Table 2. Importantly, only the high affinity value obtained for SCH442416 (K_{DB1}) by fitting the bell-shaped curve with K_{DAB} is according with the high affinity value reported for this antagonist [97, 98], and this K_{DB1} value is significantly lower ($p<0.05$) than the obtained without K_{DAB} (see Table 2). Moreover, the fit with the dimer receptor model including the K_{DAB} parameter is statistically better ($p<0.05$) than the fit not including the K_{DAB} (Figure 2). These results again show that the radioligand-competitor allosteric interaction measured by K_{DAB} is responsible for obtaining bell-shaped curves in competitive binding assays.

3.3 Dopamine D_1 receptors clearly show bell-shaped competitive curves

Then, we wanted to test our model with competitive curves working with $D_1\text{R}$ because we previously reported the existence of a great radioligand-competitor allosteric interaction

between [^3H]SCH23390 and the agonist SKF81297 and because of the low experimental dispersion when working with this radioligand. The K_{DAB} value was 2 ± 1 nM, two times lower than the K_{DB1} value (7 ± 1 nM), pointing out a positive effect of [^3H]SCH23390 on the SKF81297 affinity ($D_{\text{AB}} = +0.7 \pm 0.1$, according to Eq. (6)) (see Methods section and [93]). This inter-ligand allosteric interaction was higher than between [^3H]SCH23390 and SKF38393, where D_{AB} value was $+0.3 \pm 0.1$. Here, first of all, we developed saturation curves of [^3H]SCH23390 being aware of the light sensitivity of this radioligand. In Figure 3A we show saturation experiments of [^3H]SCH23390 non-exposed to light, fitted to Eq. (1). This radioligand did not show cooperativity ($D_{\text{CA}} = 0$), as expected for an antagonist (Eq. (4)), and the affinity value (K_{DA1}) was 0.27 nM. Surprisingly, when we developed competition curves of [^3H]SCH23390 vs. SKF81297 at high (1.4 nM, Figure 3B, red line) or at low (0.15 nM, Figure 3B, blue line) radioligand concentration, we obtained two different curve patterns: always biphasic (red line) or always bell-shaped (blue line) curves when fitting to Eq. (5) of the dimer receptor model for high or low radioligand concentration, respectively. Considering cooperativity in B binding (Eq. (3)), the fit was statistically not better (see Methods). In Figure 3C we show a magnification of a representative bell-shaped curve obtained at low radioligand concentration, also fitted to monomeric models (dotted line) according to Eq. (7). The fit to the dimer model was significantly better than to the monomeric model ($p < 0.05$). The parameter values obtained with Eq. (5) appear in Table 3.

3.4 Simulating biphasic, monophasic and bell-shaped competitive binding curves with the dimer receptor model

Previously, we reported that competition experiments between [^3H]SCH23390 and SKF81297 showed biphasic curves even in the absence of any cooperativity of the radioligand or the competitor [93]. Assuming a marked positive radioligand-competitor modulation ($D_{\text{AB}} \gg 0$), our dimer receptor model predicted the evolution from biphasic to monophasic patterns when decreasing the radioligand concentration in the assay [93]. Now, we simulate a set of competition curves considering the general competitive Eq. (5) and a set of parameters in the range of the [^3H]SCH23390/SKF81297 system: $K_{\text{DA1}} = 1$ nM ($K_{\text{DA2}} = 4$ nM, $D_{\text{CA}} = 0$), $K_{\text{DB1}} = 10$ nM ($K_{\text{DB2}} = 40$ nM, $D_{\text{CB}} = 0$) and $K_{\text{DAB}} = 2$ nM ($D_{\text{AB}} = 1$), and $R_{\text{T}} = 0.5$ pmol/mg protein. Importantly, decreasing the radioligand concentration (A) from 10 nM to 0.2 nM, curves evolve not only from biphasic to monophasic but also to bell-shaped patterns (Figure 4A). Next, we investigate the effect of K_{DAB} values at high (A = 5 nM, Figure 4B) and low radioligand concentrations (A = 1 nM, Figure 4C). As showed in Figure 4B, at high radioligand concentrations, when K_{DAB} values are low (with $D_{\text{AB}} \gg 0$, i.e. positive allosteric modulation between ligands), curves are clearly biphasic, and when K_{DAB} values are high ($D_{\text{AB}} \ll 0$, i.e. negative allosteric modulation) curves abruptly decay; when $K_{\text{DAB}} \sim 2K_{\text{DB1}}$ ($D_{\text{AB}} \sim 0$) curves are monophasic. On the other hand, at low radioligand concentration (see Figure 4C), low K_{DAB} values ($D_{\text{AB}} \gg 0$) generate bell-shaped curves. Again, high K_{DAB} values ($D_{\text{AB}} \ll 0$) generate abruptly decreasing curves and with $K_{\text{DAB}} \sim 2K_{\text{DB1}}$ ($D_{\text{AB}} \sim 0$) curves show a normal monophasic pattern. The main conclusion of our simulation with the dimer receptor model is that there is a difference between the experiments performed at high or at low radioligand concentration when there is positive radioligand-competitor allosteric modulation ($D_{\text{AB}} \gg 0$). In this case, curve pattern can be biphasic (for high radioligand concentration) or bell-shaped (for low radioligand concentration).

At this point, the question was: what concentration of radioligand causes competition curves to have a biphasic pattern? To answer this question, we analyzed Figure 4B, which shows a set of curves for high radioligand concentrations. These curves clearly show a pinched point, i.e. a common point for all curves. At this competitor concentration (B), all the curves (and all potential K_{DAB}) have the same radioligand binding (A_{bound}). The next question to answer was: what is this concentration of competitor? The mathematical expression of competitive binding used to generate the simulated curves when A and B are non-cooperative (Figure 4B) is Eq. (5) (see Methods). Then, for any two curves n and m of this figure, the radioligand bound is:

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

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

where all parameters are the same except for K_{DABn} and K_{DABm} (in red in the equations). At the B concentration of the competitor corresponding to the singular pinched point, all curves have the same radioligand bound and, then, $A_{bound\ n} = A_{bound\ m}$. Therefore, equating the two expressions and solving the system, we obtain the quadratic equation (11):

$$\frac{K_{DA1}^2}{K_{DB1}^2} B^2 + \frac{4 K_{DA1}^2}{K_{DB1}} B + 4 K_{DA1}^2 - A^2 = 0 \quad (\text{Eq. 11})$$

Only one solution is possible (positive):

$$B = \frac{(A - 2 K_{DA1}) K_{DB1}}{K_{DA1}} \quad (\text{Eq. 12})$$

but just if $A > 2 K_{DA1}$. In any other case, the concentration of B would be negative i.e. unreal. The B concentration at the pinched point considering the simulated parameter values is 30 nM, with $A_{bound} = RT$, i.e. with the dimers semi-occupied, exactly as deduced from Figure 4B.

Which graph pattern is obtained when $A = 2 K_{DA1}$? To answer this question, we simulated this scenario in Figure 4D, obtaining only monophasic curves. Thus, for the parameters

employed in the simulations of the Figure 4, the critical concentration of the radioligand is $A=2$ nM.

The last question is: what radioligand concentration (A) is responsible for obtaining bell-shaped curves? The answer to this question can be found determining the slope of the curve corresponding to Eq. (5). The derivative of A_{bound} with respect to competitor concentration (B), when B tends to zero, must be positive to observe a bell-shaped curve, and its value must be negative for obtaining decreasing monophasic curves. When the derivative is zero, we obtain the quadratic expression (Eq. 13):

$$\frac{1}{K_{DAB}}A^2 + \frac{2K_{DA1}}{K_{DB1}}A + \frac{4K_{DA1}^2}{K_{DB1}K_{DAB}}(K_{DAB} - K_{DB1}) = 0 \quad (\text{Eq. 13})$$

Solving the equation (13), we find the maximum critical A concentration that allows the obtaining of a bell-shaped curve:

$$A = \frac{2K_{DA1}}{K_{DB1}}(K_{DB1} - K_{DAB}) \quad (\text{Eq. 14})$$

It is easily deduced that A must be lower than $2K_{DA1}$ and that it tends to $2K_{DA1}$ value when K_{DAB} is much lower than K_{DB1} (high positive radioligand-competitor allosteric interaction). In addition, K_{DB1} must be higher than K_{DAB} . We can also deduce the competitor B corresponding to the maximum of the bell-shaped curve (equation not shown), being always $B \geq 2K_{DB1} - 2K_{DAB}$ and tending to $2K_{DB1} - 2K_{DAB}$ for low radioligand concentrations (A tending to 0).

In conclusion, if A and B are non-cooperative and there is a positive radioligand-competitor interaction (positive D_{AB}), when using radioligand concentrations higher than $2K_{DA1}$, the curves will be biphasic in competition experiments (Figure 4B). In contrast, when $A=2K_{DA1}$, monophasic curves are always obtained (Figure 4D). Finally, when $A < 2K_{DA1}$, we can obtain bell-shaped curves if $K_{DAB} < K_{DB1}$ and A specifically lower than $2K_{DA1} (K_{DB1} - K_{DAB}) / K_{DB1}$ (Figure 4C) or, in any other cases, curves will be always monophasic.

3.5 Bell-shaped competitive curves with dopamine D1 receptors can be modulated according with the radioligand concentration used

We proved the hypothesis deduced above from our dimer receptor model, i.e. that competitive curve patterns experimentally vary depending on the radioligand concentration employed in the assays and the different data sets can be properly fitted with our model. Effectively, the bell-shaped curve (Figure 3B, blue line) was obtained at a low free radioligand concentration of 0.15 nM, lower than $2K_{DA1}=0.54$ nM. Under these conditions the above simulation predicts that the existence of a radioligand-competitor interaction ($K_{DAB}=2.5$ nM) can generate bell-shaped competition curves. The same experiment showed

a biphasic pattern (Figure 3B, red line) when developed at a high free radioligand concentration of 1.4 nM, higher than $2K_{DA1}=0.54$ nM.

However, the same experiment performed at an intermediate free radioligand concentration, near to 0.54 nM, should generate monophasic curves. For this reason, we developed [3 H]SCH23390 vs. SKF81297 competition curves at an intermediate free radioligand concentration (0.4 nM) and curves fitted to Eq. (3) were now monophasic, as showed in Figure 3B (black line). These results experimentally demonstrate that the pattern of the competitive curves can vary according to the radioligand concentration employed, due to the existence of a positive radioligand-competitor interaction ($D_{AB}>0$) in a receptor homodimer and, for the first time, this fact is taken into account by our dimer receptor model with the inclusion of the K_{DAB} parameter. The affinity values of SKF81297 are highly robust at the three concentrations of radioligand used (from 3 to 9 nM, not significantly different), and are in the range of previously reported values [93]. Moreover, it should be noted that not only the affinity values, but also the number of receptors deduced using the dimer receptor model including the K_{DAB} parameter (Eq. 5) are robust (from 0.39 to 0.44 pmol/mg protein, not significantly different) and do not depend on the concentration of the radioligand employed (see Table 3). Conversely, both the affinity and the number of receptors obtained by the monomeric one-site (Eq. 7) or two-site (Eq. 8) model considerably vary depending on the radioligand concentration (Table 3), pointing out that the monomer-based model is unable to explain our experimental results.

Finally, we also verified the existence of D_1R homodimers by dissociation kinetic experiments of [3 H]SCH23390 in the absence or presence of other D_1R orthosteric ligands. In all cases, monophasic curves were obtained; therefore, we fitted the experimental data to equation (10) with $n=1$. Values of K_{off} appear in Table 4. The agonist SKF81297 but not the antagonist KF83566 significantly affected the dissociation kinetic constant of the radioligand demonstrating D_1R homodimerization and also the existence of an allosteric interaction between the antagonist ([3 H]SCH23390) and the agonist (SKF81297) represented by the K_{DAB} parameter, which accounts for the bell-shaped curves in Figure 3B–C. We also show in Table 4 the dissociation kinetic parameters obtained with $A_{2A}Rs$ and previously reported by us [41, 42], which also demonstrated $A_{2A}R$ homodimerization and the existence of an allosteric interaction between radioligand and competitor (agonist vs. antagonist) represented by the high value of D_{AB} (+1.9); this parameter is shown in Table 2 and is responsible for the marked bell-shaped curve of Figure 2B.

4. Discussion

When analyzing competitive binding experiments, we sometimes obtain the surprising findings of a biphasic curve using an antagonist ligand or a monophasic curve using an agonist ligand, as competitors. Even more anomalous are those curves showing a bell-shaped pattern, where increasing concentrations of a competitor produce an increase of the radioligand binding before causing the expected displacement. This increase is usually from low to moderate; for example, when working with vasopressin receptors, increases in radioligand binding were frequently observed which did not exceed 20% of the binding in the absence of competitor [88, 99]. Now, we demonstrate that the existence of a radioligand-

competitor allosteric interaction (K_{DAB}), deduced from our dimer receptor model [93] (see Figure 5), allows the fitting of bell-shaped curves, typically considered as anomalous or erroneous results. We also deduce the radioligand concentration responsible for the conversion from biphasic to monophasic or to bell-shaped curves in competitive radioligand binding assays. Moreover, according to the K_{DAB} definition, these bell-shaped curves are only interpretable assuming the formation of receptor homodimers and, therefore, obtaining bell-shaped curves in competition experiments is a clear evidence for receptor dimerization *ex-vivo*, working with membrane preparations from tissues. These results are according with those obtained by radioligand dissociation kinetic assays. It is well established the use of this method to detect cooperative interactions between two distinct binding sites and thus, to determine the presence of homodimers and heteromers [100]. Concretely, with this method it was demonstrated the presence of homodimers of A_2AR *ex vivo* in sheep striatum membranes [42] and of A_1R in CHO transfected cells [52]. Here, by dissociation kinetic experiments, we have also demonstrated the presence of D1R homodimers in striatal membranes, which is according with the detection of a K_{DAB} parameter indicating homodimerization in the competitive binding assays now reported.

In agreement with our results, we can decrease the radioligand concentration under its affinity value ($2K_{DA1}$ or lower) to obtain bell-shaped competition curves. This is a demonstration of the existence of positive radioligand-competitor interaction ($D_{AB}>0$), denoted by a low K_{DAB} value. In this scenario, if $K_{DAB}<K_{DB1}$, we will obtain bell-shaped curves but, if K_{DAB} is between K_{DB1} and $2K_{DB1}$, we will obtain monophasic curves. Working with high affinity radioligands, radioligand concentration much higher than their K_{DA1} values are usually used, which precludes the observation of bell-shaped curves. In addition, if the value of D_{AB} is low, in order to observe the bell-shaped curves, the radioligand concentration should be significantly reduced. Otherwise, the bound ligand would be low and the experimental dispersion could mask the bell-shaped pattern of the competitive curves, as showed in Figure 1. The use of radioligand concentrations under their $2K_{DA1}$ values is more common when working with low affinity radioligands. In these cases, competition experiments similar to those shown in Figure 2 exhibit evident bell-shaped patterns, if D_{AB} is clearly positive (especially higher than +0.5).

It is interesting to note that, although there is clear evidence of the dimerization of GPCRs, currently, radioligand binding data are still fitted to equations deduced for one site or for two-independent site models. This is because experimental data usually fit equally to these simpler models or to the dimer receptor model. The experiments showed here, i.e. bell-shaped curves in competition assays, only can be explained considering homodimeric/oligomeric receptors and fitting data to a dimer receptor model. Chidiac et al. [80] also suggested that a bell-shaped pattern cannot be obtained from a system of independent sites. Likewise, the conversion of the curve pattern from biphasic to monophasic [93] and to bell-shaped curves, decreasing the radioligand concentration in the competition assay, only can be interpreted by dimeric or oligomeric models. Our model explains all these cases due to the existence of a radioligand-competitor allosteric interaction, only explainable if receptors form homodimers. Our dimer receptor model can overcome scenarios where different affinity values for a competitive drug are obtained, when different radioligands are used and monomeric models are applied [45]. This is because our model takes in account that each

pair of radioligand-competitor has a particular K_{DAB} value according to the magnitude of its allosteric interaction. If this K_{DAB} value is not included in the mathematical fitting model, the affinity values of the competitor obtained with the monomeric one-site and two-independent-site models will not be robust, but inconsistent and erroneous. Furthermore, when bell-shaped curves are obtained in competitive radioligand binding assays, the affinity values of the competitor obtained from equations including K_{DAB} are closer to the actual values. On the contrary, the K_D values obtained from classical monomeric models are usually much higher.

5. Conclusion

In summary, by assuming the formation of receptor homodimers, our study is the first that can explain several experimental results previously considered erroneous due to their impossibility to be well fitted. Furthermore, our model is easy to use, in contrast to the over-parameterization of other dimer receptor models, and it can successfully predict the pattern of competition curves according to the experimental conditions. Our findings have potential therapeutic interest because GPCR homodimers are the actual and better target for drug development, and the agonist-antagonist allosteric interaction within GPCRs must be taken into account when designing new therapeutic strategies.

Acknowledgments

Funding

This work was supported by “Ministerio de Economía y Competitividad” and European Regional Development Funds of the European Union (Grant SAF2014-54840-R and SAF2017-87629-R), “Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas” (Grant CB06/05/0064), the “Fundació La Marató de TV3” (Grant 20140610), “Generalitat de Catalunya” (Grant 2017-SGR-1497) and intramural funds of the National Institute on Drug Abuse.

Abbreviations:

A	radioligand concentration
A₁R	adenosine A ₁ receptor
A_{2A}R	adenosine A _{2A} receptor
A_{bound}	specific radioligand binding
B	competitor concentration
D₁R	dopamine D ₁ receptor
D_{AB}	dimer radioligand-competitor modulation index
D_{CA}	dimer cooperativity index for the ligand A binding
D_{CB}	dimer cooperativity index for the ligand B binding
GPCR	G protein-coupled receptor

K_{DA1}	macroscopic dissociation constant describing the binding of the first radioligand molecule (A) to the receptor homodimer
K_{DA2}	macroscopic dissociation constant describing the binding of the second radioligand molecule (A) to the receptor homodimer
K_{DB1}	macroscopic dissociation constant describing the binding of the first competitor molecule (B) to the receptor homodimer
K_{DB2}	macroscopic dissociation constant describing the binding of the second competitor molecule (B) to the receptor homodimer
K_{DAB}	hybrid equilibrium radioligand-competitor dissociation constant
R_T	total amount of receptor dimers

References

1. Cvicek V, Goddard WA, Abrol R (2016) Structure-Based Sequence Alignment of the Transmembrane Domains of All Human GPCRs: Phylogenetic, Structural and Functional Implications. *PLOS Comput Biol* 12:e1004805 10.1371/journal.pcbi.1004805 [PubMed: 27028541]
2. Wells GJ (2014) Allosteric modulators of G protein-coupled receptors. *Curr Top Med Chem* 14:1735–7 [PubMed: 25176126]
3. Lefkowitz RJ (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol Sci* 25:413–22. 10.1016/j.tips.2004.06.006 [PubMed: 15276710]
4. Garland SL (2013) Are GPCRs still a source of new targets? *J Biomol Screen* 18:947–66. 10.1177/1087057113498418 [PubMed: 23945874]
5. Katritch V, Cherezov V, Stevens RC (2013) Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol* 53:531–56. 10.1146/annurev-pharmtox-032112-135923 [PubMed: 23140243]
6. Wang C-IA, Lewis RJ (2013) Emerging opportunities for allosteric modulation of G-protein coupled receptors. *Biochem Pharmacol* 85:153–62. 10.1016/j.bcp.2012.09.001 [PubMed: 22975406]
7. Hauser AS, Attwood MM, Rask-Andersen M, et al. (2017) Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* 16:829–842. 10.1038/nrd.2017.178 [PubMed: 29075003]
8. Chabre M, le Maire M (2005) Monomeric G-protein-coupled receptor as a functional unit. *Biochemistry* 44:9395–403. 10.1021/bi050720o [PubMed: 15996094]
9. Ernst OP, Gramse V, Kolbe M, et al. (2007) Monomeric G protein-coupled receptor rhodopsin in solution activates its G protein transducin at the diffusion limit. *Proc Natl Acad Sci U S A* 104:10859–64. 10.1073/pnas.0701967104 [PubMed: 17578920]
10. Whorton MR, Bokoch MP, Rasmussen SGF, et al. (2007) monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A* 104:7682–7. 10.1073/pnas.0611448104 [PubMed: 17452637]
11. Whorton MR, Jastrzebska B, Park PS-H, et al. (2008) Efficient coupling of transducin to monomeric rhodopsin in a phospholipid bilayer. *J Biol Chem* 283:4387–94. 10.1074/jbc.M703346200 [PubMed: 18033822]
12. habre M, Deterre P, Antonny B (2009) The apparent cooperativity of some GPCRs does not necessarily imply dimerization. *Trends Pharmacol Sci* 30:182–7. 10.1016/j.tips.2009.01.003 [PubMed: 19269046]
13. Kuszak AJ, Pitchiaya S, Anand JP, et al. (2009) Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2. *J Biol Chem* 284:26732–41. 10.1074/jbc.M109.026922 [PubMed: 19542234]

14. Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* 2:274–86. 10.1038/35067575 [PubMed: 11283750]
15. Devi LA (2001) Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. *Trends Pharmacol Sci* 22:532–7 [PubMed: 11583811]
16. Hiller C, Kühhorn J, Gmeiner P (2013) Class A G-Protein-Coupled Receptor (GPCR) Dimers and Bivalent Ligands. *J Med Chem* 56:6542–6559. 10.1021/jm4004335 [PubMed: 23678887]
17. Ferré S (2015) The GPCR heterotetramer: challenging classical pharmacology. *Trends Pharmacol Sci* 36:145–52. 10.1016/j.tips.2015.01.002 [PubMed: 25704194]
18. Gomes I, Ayoub MA, Fujita W, et al. (2016) G Protein-Coupled Receptor Heteromers. *Annu Rev Pharmacol Toxicol* 56:403–25. 10.1146/annurev-pharmtox-011613-135952 [PubMed: 26514203]
19. George SR, O'Dowd BF, Lee SP (2002) G-Protein-coupled receptor oligomerization and its potential for drug discovery. *Nat Rev Drug Discov* 1:808–820. 10.1038/nrd913 [PubMed: 12360258]
20. Milligan G (2004) G protein-coupled receptor dimerization: function and ligand pharmacology. *Mol Pharmacol* 66:1–7. 10.1124/mol.104.000497 [PubMed: 15213289]
21. Milligan G (2007) G protein-coupled receptor dimerisation: molecular basis and relevance to function. *Biochim Biophys Acta* 1768:825–35. 10.1016/j.bbamem.2006.09.021 [PubMed: 17069751]
22. Milligan G (2008) A day in the life of a G protein-coupled receptor: the contribution to function of G protein-coupled receptor dimerization. *Br J Pharmacol* 153 Suppl 1:S216–29. 10.1038/sj.bjp.0707490 [PubMed: 17965750]
23. Ferré S, Baler R, Bouvier M, et al. (2009) Building a new conceptual framework for receptor heteromers. *Nat Chem Biol* 5:131–4. 10.1038/nchembio0309-131 [PubMed: 19219011]
24. Ferré S, Casadó V, Devi LA, et al. (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol Rev* 66:413–34. 10.1124/pr.113.008052 [PubMed: 24515647]
25. Casadó V, Cortés A, Mallol J, et al. (2009) GPCR homomers and heteromers: a better choice as targets for drug development than GPCR monomers? *Pharmacol Ther* 124:248–57. 10.1016/j.pharmthera.2009.07.005 [PubMed: 19664655]
26. Fuxe K, Marcellino D, Borroto-Escuela DO, et al. (2010) The changing world of G protein-coupled receptors: from monomers to dimers and receptor mosaics with allosteric receptor–receptor interactions. *J Recept Signal Transduct* 30:272–283. 10.3109/10799893.2010.506191
27. Milligan G, Canals M, Pediani JD, et al. (2006) The role of GPCR dimerisation/oligomerisation in receptor signalling. *Ernst Schering Found Symp Proc* 145–61 [PubMed: 17703581]
28. Lopez-Gimenez JF, Canals M, Pediani JD, Milligan G (2007) The $\alpha 1b$ -adrenoceptor exists as a higher-order oligomer: effective oligomerization is required for receptor maturation, surface delivery, and function. *Mol Pharmacol* 71:1015–29. 10.1124/mol.106.033035 [PubMed: 17220353]
29. Milligan G (2013) The prevalence, maintenance, and relevance of G protein-coupled receptor oligomerization. *Mol Pharmacol* 84:158–69. 10.1124/mol.113.084780 [PubMed: 23632086]
30. Ciruela F, Gómez-Soler M, Guidolin D, et al. (2011) Adenosine receptor containing oligomers: their role in the control of dopamine and glutamate neurotransmission in the brain. *Biochim Biophys Acta* 1808:1245–55. 10.1016/j.bbamem.2011.02.007 [PubMed: 21316336]
31. Ulloa-Aguirre A, Zariñán T, Dias JA, Conn PM (2014) Mutations in G protein-coupled receptors that impact receptor trafficking and reproductive function. *Mol Cell Endocrinol* 382:411–423. 10.1016/j.mce.2013.06.024 [PubMed: 23806559]
32. Rossi M, Fasciani I, Marampon F, et al. (2017) The First Negative Allosteric Modulator for Dopamine D 2 and D 3 Receptors, SB269652 May Lead to a New Generation of Antipsychotic Drugs. *Mol Pharmacol* 91:586–594. 10.1124/mol.116.107607
33. Lane JR, Donthamsetti P, Shonberg J, et al. (2014) A new mechanism of allostery in a G protein-coupled receptor dimer. *Nat Chem Biol* 10:745–752. 10.1038/nchembio.1593 [PubMed: 25108820]

34. Pulido D, Casadó-Anguera V, Pérez-Benito L, et al. (2018) Design of a True Bivalent Ligand with Picomolar Binding Affinity for a G Protein-Coupled Receptor Homodimer. *J Med Chem* 61:9335–9346. 10.1021/acs.jmedchem.8b01249 [PubMed: 30257092]
35. Berque-Bestel I, Lezoualc'h F, Jockers R (2008) Bivalent ligands as specific pharmacological tools for G protein-coupled receptor dimers. *Curr Drug Discov Technol* 5:312–8 [PubMed: 19075611]
36. Tabor A, Weisenburger S, Banerjee A, et al. (2016) Visualization and ligand-induced modulation of dopamine receptor dimerization at the single molecule level. *Sci Rep* 6:33233 10.1038/srep33233 [PubMed: 27615810]
37. Kasai RS, Suzuki KGN, Prossnitz ER, et al. (2011) Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J Cell Biol* 192:463–80. 10.1083/jcb.201009128 [PubMed: 21300851]
38. Kasai RS, Ito SV, Awane RM, et al. (2018) The Class-A GPCR Dopamine D2 Receptor Forms Transient Dimers Stabilized by Agonists: Detection by Single-Molecule Tracking. *Cell Biochem Biophys* 76:29–37. 10.1007/s12013-017-0829-y [PubMed: 29116599]
39. Carli M, Kolachalam S, Aringhieri S, et al. (2018) Dopamine D2 Receptors Dimers: How can we Pharmacologically Target Them? *Curr Neuropharmacol* 16:222–230. 10.2174/1570159X15666170518151127 [PubMed: 28521704]
40. Scarselli M, Annibale P, McCormick PJ, et al. (2016) Revealing G-protein-coupled receptor oligomerization at the single-molecule level through a nanoscopic lens: methods, dynamics and biological function. *FEBS J* 283:1197–217. 10.1111/febs.13577 [PubMed: 26509747]
41. Casadó-Anguera V, Bonaventura J, Moreno E, et al. (2016) Evidence for the heterotetrameric structure of the adenosine A2A-dopamine D2 receptor complex. *Biochem Soc Trans* 44:595–600. 10.1042/BST20150276 [PubMed: 27068975]
42. Bonaventura J, Navarro G, Casadó-Anguera V, et al. (2015) Allosteric interactions between agonists and antagonists within the adenosine A2A receptor-dopamine D2 receptor heterotetramer. *Proc Natl Acad Sci U S A* 112:. 10.1073/pnas.1507704112
43. Navarro G, Cordoní A, Casadó-Anguera V, et al. (2018) Evidence for functional pre-coupled complexes of receptor heteromers and adenylyl cyclase. *Nat Commun* 9:1242 10.1038/s41467-018-03522-3 [PubMed: 29593213]
44. Maguire JJ, Kuc RE, Davenport AP (2012) Radioligand binding assays and their analysis. *Methods Mol Biol* 897:31–77. 10.1007/978-1-61779-909-9_3 [PubMed: 22674160]
45. Maggio R, Rocchi C, Scarselli M (2013) Experimental strategies for studying G protein-coupled receptor homo- and heteromerization with radioligand binding and signal transduction methods. *Methods Enzymol* 521:295–310. 10.1016/B978-0-12-391862-8.00016-8 [PubMed: 23351746]
46. De Lean A, Stadel JM, Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylyl cyclase-coupled beta-adrenergic receptor. *J Biol Chem* 255:7108–17 [PubMed: 6248546]
47. Strange PG (2005) Oligomers of D2 dopamine receptors: evidence from ligand binding. *J Mol Neurosci* 26:155–60. 10.1385/JMN:26:2-3:155 [PubMed: 16012188]
48. Gracia E, Moreno E, Cortés A, et al. (2013) Homodimerization of adenosine A1 receptors in brain cortex explains the biphasic effects of caffeine. *Neuropharmacology* 71:56–69. 10.1016/j.neuropharm.2013.03.005 [PubMed: 23523559]
49. Navarro G, Cordoní A, Zelman-Femiak M, et al. (2016) Quaternary structure of a G-protein-coupled receptor heterotetramer in complex with Gi and Gs. *BMC Biol* 14:26 10.1186/s12915-016-0247-4 [PubMed: 27048449]
50. Łukasiewicz S, Błasiak E, Faron-Górecka A, et al. Fluorescence studies of homooligomerization of adenosine A2A and serotonin 5-HT1A receptors reveal the specificity of receptor interactions in the plasma membrane. *Pharmacol Rep* 59:379–92 [PubMed: 17901566]
51. Gracia, Pérez-Capote K, Moreno E, et al. (2011) A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. *Biochem J* 435:701–9. 10.1042/BJ20101749 [PubMed: 21306300]
52. May LT, Bridge LJ, Stoddart LA, et al. (2011) Allosteric interactions across native adenosine-A3 receptor homodimers: quantification using single-cell ligand-binding kinetics. *FASEB J* 25:3465–76. 10.1096/fj.11-186296 [PubMed: 21715680]

53. Herrick-Davis K, Grinde E, Cowan A, Mazurkiewicz JE (2013) Fluorescence correlation spectroscopy analysis of serotonin, adrenergic, muscarinic, and dopamine receptor dimerization: the oligomer number puzzle. *Mol Pharmacol* 84:630–42. 10.1124/mol.113.087072 [PubMed: 23907214]
54. Guitart X, Navarro G, Moreno E, et al. (2014) Functional Selectivity of Allosteric Interactions within G Protein-Coupled Receptor Oligomers: The Dopamine D1-D3 Receptor Heterotetramer. *Mol Pharmacol* 86:417–429. 10.1124/mol.114.093096 [PubMed: 25097189]
55. Guo W, Shi L, Javitch JA (2003) The fourth transmembrane segment forms the interface of the dopamine D2 receptor homodimer. *J Biol Chem* 278:4385–8. 10.1074/jbc.C200679200 [PubMed: 12496294]
56. Pou C, Mannoury la Cour C, Stoddart LA, et al. (2012) Functional homomers and heteromers of dopamine D2L and D3 receptors co-exist at the cell surface. *J Biol Chem* 287:8864–78. 10.1074/jbc.M111.326678 [PubMed: 22291025]
57. Herrick-Davis K, Grinde E, Mazurkiewicz JE (2004) Biochemical and biophysical characterization of serotonin 5-HT_{2C} receptor homodimers on the plasma membrane of living cells. *Biochemistry* 43:13963–71. 10.1021/bi048398p [PubMed: 15518545]
58. Herrick-Davis K, Grinde E, Lindsley T, et al. (2012) Oligomer size of the serotonin 5-hydroxytryptamine 2C (5-HT_{2C}) receptor revealed by fluorescence correlation spectroscopy with photon counting histogram analysis: evidence for homodimers without monomers or tetramers. *J Biol Chem* 287:23604–14. 10.1074/jbc.M112.350249 [PubMed: 22593582]
59. Mancia F, Assur Z, Herman AG, et al. (2008) Ligand sensitivity in dimeric associations of the serotonin 5HT_{2c} receptor. *EMBO Rep* 9:363–9. 10.1038/embor.2008.27 [PubMed: 18344975]
60. Teitler M, Toohey N, Knight JA, et al. (2010) Clozapine and other competitive antagonists reactivate risperidone-inactivated h5-HT₇ receptors: radioligand binding and functional evidence for GPCR homodimer protomer interactions. *Psychopharmacology (Berl)* 212:687–97. 10.1007/s00213-010-2001-x [PubMed: 20827463]
61. Mercier J-F, Salahpour A, Angers S, et al. (2002) Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem* 277:44925–31. 10.1074/jbc.M205767200 [PubMed: 12244098]
62. Gherbi K, May LT, Baker JG, et al. (2015) Negative cooperativity across β 1-adrenoceptor homodimers provides insights into the nature of the secondary low-affinity CGP 12177 β 1-adrenoceptor binding conformation. *FASEB J* 29:2859–71. 10.1096/fj.14-265199 [PubMed: 25837585]
63. Angers S, Salahpour A, Joly E, et al. (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* 97:3684–9. 10.1073/pnas.060590697 [PubMed: 10725388]
64. Parmar VK, Grinde E, Mazurkiewicz JE, Herrick-Davis K (2017) Beta2-adrenergic receptor homodimers: Role of transmembrane domain 1 and helix 8 in dimerization and cell surface expression. *Biochim Biophys Acta* 1859:1445–1455. 10.1016/j.bbamem.2016.12.007
65. Bagher AM, Laprairie RB, Toguri JT, et al. (2017) Bidirectional allosteric interactions between cannabinoid receptor 1 (CB1) and dopamine receptor 2 long (D2L) heterotetramers. *Eur J Pharmacol* 813:66–83. 10.1016/j.ejphar.2017.07.034 [PubMed: 28734930]
66. Szalai B, Barkai L, Turu G, et al. (2012) Allosteric interactions within the AT₁ angiotensin receptor homodimer: role of the conserved DRY motif. *Biochem Pharmacol* 84:477–85. 10.1016/j.bcp.2012.04.014 [PubMed: 22579851]
67. Levitz J, Habrian C, Bharill S, et al. (2016) Mechanism of Assembly and Cooperativity of Homomeric and Heteromeric Metabotropic Glutamate Receptors. *Neuron* 92:143–159. 10.1016/j.neuron.2016.08.036 [PubMed: 27641494]
68. Goin J, Nathanson NM (2006) Quantitative analysis of muscarinic acetylcholine receptor homo- and heterodimerization in live cells: regulation of receptor down-regulation by heterodimerization. *J Biol Chem* 281:5416–25. 10.1074/jbc.M507476200 [PubMed: 16368694]
69. Park PS-H, Wells JW (2003) Monomers and Oligomers of the M₂ Muscarinic Cholinergic Receptor Purified from Sf 9 Cells[†]. *Biochemistry* 42:12960–12971. 10.1021/bi034491m [PubMed: 14596611]

70. McMillin SM, Heusel M, Liu T, et al. (2011) Structural basis of M3 muscarinic receptor dimer/oligomer formation. *J Biol Chem* 286:28584–98. 10.1074/jbc.M111.259788 [PubMed: 21685385]
71. Cvejic S, Devi LA (1997) Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J Biol Chem* 272:26959–64 [PubMed: 9341132]
72. McVey M, Ramsay D, Kellett E, et al. (2001) Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta -opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. *J Biol Chem* 276:14092–9. 10.1074/jbc.M008902200 [PubMed: 11278447]
73. Johnston JM, Aburi M, Provasi D, et al. (2011) Making structural sense of dimerization interfaces of delta opioid receptor homodimers. *Biochemistry* 50:1682–90. 10.1021/bi101474v [PubMed: 21261298]
74. Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399:697–700. 10.1038/21441 [PubMed: 10385123]
75. He L, Fong J, von Zastrow M, Whistler JL (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 108:271–82 [PubMed: 11832216]
76. White JF, Grodnitzky J, Louis JM, et al. (2007) Dimerization of the class A G protein-coupled neurotensin receptor NTS1 alters G protein interaction. *Proc Natl Acad Sci U S A* 104:12199–204. 10.1073/pnas.0705312104 [PubMed: 17620610]
77. Ayoub MA, Levoe A, Delagrange P, Jockers R (2004) Preferential formation of MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction properties compared with MT2 homodimers. *Mol Pharmacol* 66:312–21. 10.1124/mol.104.000398 [PubMed: 15266022]
78. Mandrika I, Petrovska R, Klovins J (2010) Evidence for constitutive dimerization of niacin receptor subtypes. *Biochem Biophys Res Commun* 395:281–7. 10.1016/j.bbrc.2010.04.011 [PubMed: 20380810]
79. Babcock GJ, Farzan M, Sodroski J (2003) Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *J Biol Chem* 278:3378–85. 10.1074/jbc.M210140200 [PubMed: 12433920]
80. Chidiac P, Green MA, Pawagi AB, Wells JW (1997) Cardiac muscarinic receptors. Cooperativity as the basis for multiple states of affinity. *Biochemistry* 36:7361–79. 10.1021/bi961939t [PubMed: 9200684]
81. Durroux T (2005) Principles: a model for the allosteric interactions between ligand binding sites within a dimeric GPCR. *Trends Pharmacol Sci* 26:376–84. 10.1016/j.tips.2005.05.006 [PubMed: 15946747]
82. Franco R, Casadó V, Mallol J, et al. (2005) Dimer-based model for heptaspanning membrane receptors. *Trends Biochem Sci* 30:360–6. 10.1016/j.tibs.2005.05.010 [PubMed: 15951182]
83. Casadó V, Cortés A, Ciruela F, et al. (2007) Old and new ways to calculate the affinity of agonists and antagonists interacting with G-protein-coupled monomeric and dimeric receptors: the receptor-dimer cooperativity index. *Pharmacol Ther* 116:343–54. 10.1016/j.pharmthera.2007.05.010 [PubMed: 17935788]
84. Rovira X, Roche D, Serra J, et al. (2008) Modeling the Binding and Function of Metabotropic Glutamate Receptors. *J Pharmacol Exp Ther* 325:443–456. 10.1124/jpet.107.133967 [PubMed: 18287211]
85. Rovira X, Vivó M, Serra J, et al. (2009) Modelling the interdependence between the stoichiometry of receptor oligomerization and ligand binding for a coexisting dimer/tetramer receptor system. *Br J Pharmacol* 156:28–35. 10.1111/j.1476-5381.2008.00031.x [PubMed: 19133989]
86. Casadó V, Mallol J, Canela EI, et al. (1991) The binding of [3H]R-PIA to A1 adenosine receptors produces a conversion of the high- to the low-affinity state. *FEBS Lett* 286:221–4 [PubMed: 1864372]
87. Franco R, Casadó V, Ciruela F, et al. (1996) The cluster-arranged cooperative model: a model that accounts for the kinetics of binding to A1 adenosine receptors. *Biochemistry* 35:3007–15. 10.1021/bi952415g [PubMed: 8608139]
88. Albizu L, Balestre M-N, Breton C, et al. (2006) Probing the existence of G protein-coupled receptor dimers by positive and negative ligand-dependent cooperative binding. *Mol Pharmacol* 70:1783–91. 10.1124/mol.106.025684 [PubMed: 16926282]

89. de Meyts P, Roth J, Neville DM, et al. (1973) Insulin interactions with its receptors: experimental evidence for negative cooperativity. *Biochem Biophys Res Commun* 55:154–61 [PubMed: 4361269]
90. Urizar E, Montanelli L, Loy T, et al. (2005) Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J* 24:1954–64. 10.1038/sj.emboj.7600686 [PubMed: 15889138]
91. Kara E, Lin H, Strange PG (2010) Co-operativity in agonist binding at the D2 dopamine receptor: evidence from agonist dissociation kinetics. *J Neurochem* 112:1442–53. 10.1111/j.1471-4159.2009.06554.x [PubMed: 20050980]
92. Wreggett KA, Wells JW (1995) Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J Biol Chem* 270:22488–99 [PubMed: 7673239]
93. Casadó V, Ferrada C, Bonaventura J, et al. (2009) Useful pharmacological parameters for G-protein-coupled receptor homodimers obtained from competition experiments. Agonist-antagonist binding modulation. *Biochem Pharmacol* 78:1456–63. 10.1016/j.bcp.2009.07.012 [PubMed: 19643089]
94. Casadó V, Cantí C, Mallol J, et al. (1990) Solubilization of A1 adenosine receptor from pig brain: characterization and evidence of the role of the cell membrane on the coexistence of high- and low-affinity states. *J Neurosci Res* 26:461–73. 10.1002/jnr.490260409 [PubMed: 2122003]
95. Sarrió S, Casadó V, Escriche M, et al. (2000) The heat shock cognate protein hsc73 assembles with A(1) adenosine receptors to form functional modules in the cell membrane. *Mol Cell Biol* 20:5164–74 [PubMed: 10866672]
96. heng Y, Prusoff WH (1973) Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–108 [PubMed: 4202581]
97. Shinkre BA, Kumar TS, Gao Z-G, et al. (2010) Synthesis and evaluation of 1,2,4-triazolo[1,5-c]pyrimidine derivatives as A2A receptor-selective antagonists. *Bioorg Med Chem Lett* 20:5690–5694. 10.1016/j.bmcl.2010.08.021 [PubMed: 20801028]
98. Orru M, Bakešová J, Brugarolas M, et al. (2011) Striatal pre- and postsynaptic profile of adenosine A(2A) receptor antagonists. *PLoS One* 6:e16088 10.1371/journal.pone.0016088 [PubMed: 21264319]
99. Berde B, Boissonnas RA, Huguenin RL, Stürmer E (1964) Vasopressin analogues with selective pressor activity. *Experientia* 20:42–3 [PubMed: 5892275]
100. May LT, Leach K, Sexton PM, Christopoulos A (2007) Allosteric Modulation of G Protein–Coupled Receptors. *Annu Rev Pharmacol Toxicol* 47:1–51. 10.1146/annurev.pharmtox.47.120505.105159 [PubMed: 17009927]

Fig. 1. Competition experiments using 0.009 nM of the A_1R antagonist $[^3\text{H}]\text{DPCPX}$ vs. increasing concentrations of the A_1R R-PIA using sheep brain striatum membranes (0.2 mg protein/ml) were performed as indicated in Methods. Binding data of the same experiment were fitted to the two-independent-site model (Eq. (8)) (a) or to the dimer receptor model (Eq. (3) using $K_{\text{DA}1}=0.038$ nM) (b). Mean $\pm$ SEM values from a representative experiment performed in triplicates are shown. Parameter values appear in Table 1

Fig. 2.

Competition experiments using 24 nM of the $\text{A}_{2\text{A}}\text{R}$ agonist $[^3\text{H}]\text{CGS21680}$ vs. increasing concentrations of the $\text{A}_{2\text{A}}\text{R}$ antagonist SCH442416 using sheep brain striatum membranes (0.2 mg protein/ml) were performed as indicated in Methods. In (a), binding data were fitted to the one-site model (Eq. (7), dashed line) or to the dimer receptor model with B non-cooperative (Eq. (5)) and non-considering K_{DAB} , using $K_{\text{DA1}}=41$ nM (solid line). In (b), the same binding data were fitted to the dimer receptor model with B non-cooperative (Eq. (5)) and considering K_{DAB} . Mean $\pm$ SEM values from a representative experiment performed in triplicates are shown. Parameter values appear in Table 2

Fig. 3. Binding assays of D_1R in sheep brain striatum membranes (0.2 mg protein/ml) were performed as indicated in Methods. In (a), saturation experiment performed with increasing concentrations of the D_1R antagonist $[^3\text{H}]\text{SCH23390}$. Binding data were fitted to the Eq. (1), and $K_{\text{DA}1}$ was 0.27 nM. In (b), competition experiments using several D_1R antagonist $[^3\text{H}]\text{SCH23390}$ concentrations (0.15 nM in blue; 0.4 nM in black and 1.4 nM in red) vs. increasing concentrations of the D_1R agonist SKF81297. Binding data were fitted to the dimer receptor model with non-cooperative competitor (Eq. (5)). In (c), magnification of competition assay of 0.15 nM $[^3\text{H}]\text{SCH23390}$ vs. increasing concentrations of SKF81297 fitted to the dimer receptor model (Eq. (5)) (solid line) or fitted to the one-site model (Eq. (7)) (dotted line). Mean $\pm$ SEM values from a representative experiment performed in triplicates are shown. Parameter values appear in Table 3

Fig. 4.

Competition curve simulations considering the Eq. (5). In (a), simulation was performed following the parameter values: $R_T=0.5$ pmol/mg protein, $K_{DA1}=1$ nM ($D_{CA}=0$, $K_{DA2}=4$ nM), $K_{DB1}=10$ nM ($D_{CB}=0$, $K_{DB2}=40$ nM) and $K_{DAB}=2$ nM ($D_{AB}=1$), varying the radioligand concentration (A in Eq. (5); 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10 nM, bottom to top) and with increasing concentrations of the competitor (B in Eq. (5)). In (b), (c) and (d), simulation was performed following the parameter values: $R_T=0.5$ pmol/mg protein, $[A] = 5$ nM (b), 1 nM (c) or 2 nM (d), $K_{DA1}=1$ nM, $K_{DA2}=4$ nM ($D_{CA}=0$), $K_{DB1}=10$ nM, $K_{DB2}=40$ nM ($D_{CB}=0$), varying the K_{DAB} value (1, 2, 5, 10, 20, 50, 100, 200, 500) and with increasing concentrations of the competitor (B in Eq. (5))

Fig. 5. Graphical representation of a receptor homodimer showing cooperativity (D_{CB} , white arrow), in the binding of a competitor B (green ellipse), and allosteric interactions (D_{AB} and D_{BA} , black arrows) between B and the radioligand A (red rectangle)

Table 1

Parameter values obtained by fitting data from competition experiments of the antagonist [^3H]DPCPX binding to adenosine A_1 receptors vs. the agonist R-PIA to different models

Model	Parameter	0.009 nM [^3H]DPCPX
Two-independent-site model	B_{maxH} (pmol/mg protein)	0.7 ± 0.1
	K_{DH} (nM)	5 ± 2
	B_{maxL} (pmol/mg protein)	0.18 ± 0.09
	K_{DL} (nM)	120 ± 60
Dimer receptor model	R_{T} (pmol/mg protein)	0.38 ± 0.01
	K_{DB1} (nM)	0.02 ± 0.01
	K_{DB2} (nM)	5.3 ± 0.5
	K_{DAB} (nM)	0.018 ± 0.007
	D_{CB}	-1.8
	D_{AB}	$+0.35$

Values are mean $\pm$ SEM from three experiments performed with different striatum membranes of 3–4 animals. The radioligand affinity was $K_{\text{DA1}} = 0.038$ nM. B_{maxH} and B_{maxL} are, respectively, the maximum specific binding corresponding to, respectively, high- and low-affinity sites, and K_{DH} and K_{DL} are the equilibrium dissociation constants of the competing ligand B (R-PIA) for, respectively, high- and low-affinity sites. R_{T} is the total amount of receptor dimers, K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B to the dimer. K_{DAB} is the hybrid equilibrium dissociation constant of B binding to a receptor dimer semioccupied by the A ([^3H]DPCPX). D_{CB} is the dimer cooperativity index for the binding of ligand B and D_{AB} is the dimer radioligand-competitor modulation index (see Methods)

Table 2

Parameter values obtained by fitting data from competition experiments of the agonist [^3H]CGS21680 binding to adenosine $\text{A}_{2\text{A}}$ receptors vs. the antagonist SCH442416 to different models

Model	Parameter	24 nM [^3H]CGS21680
One-site model	B_{max} (pmol/mg protein)	1.7 ± 0.2
	K_{D} (nM)	180 ± 50
Dimer receptor model (without radioligand /competitor modulation)	R_{T} (pmol/mg protein)	0.8 ± 0.1
	K_{DB1} (nM)	90 ± 30
	K_{DB2} (nM)	-
	K_{DAB} (nM)	-
	D_{CB}	0
	D_{AB}	0
Dimer receptor model	R_{T} (pmol/mg protein)	0.56 ± 0.07
	K_{DB1} (nM)	$1.2 \pm 0.3^*$
	K_{DB2} (nM)	-
	K_{DAB} (nM)	0.03 ± 0.02
	D_{CB}	0
	D_{AB}	+1.9

Values are mean $\pm$ SEM from three experiments performed with different striatum membranes of 3–4 animals. The radioligand affinity was $K_{\text{DA1}}=41$ nM. B_{max} is the maximum specific binding and K_{D} is the equilibrium dissociation constant for the competing ligand B (SCH442416). R_{T} is the total amount of receptor dimers, K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B to the dimer. K_{DAB} is the hybrid equilibrium dissociation constant of B binding to a receptor dimer semioccupied by A ([^3H]CGS21680). D_{CB} is the dimer cooperativity index for the binding of ligand B and D_{AB} is the dimer radioligand-competitor modulation index (see Methods). Statistical differences between parameters obtained with the dimer receptor model were calculated by Student-t test

*
p<0.05 between K_{DB1} values

Table 3

Parameter values obtained by fitting data from competition experiments of the antagonist [³H]SCH23390 binding to dopamine D₁ receptors vs. the agonist SKF81297 to different models

Model	Parameter	0.15 nM [³ H]SCH	0.40 nM [³ H]SCH	1.4 nM [³ H]SCH
One-site model	B _{max} (pmol/mg protein)	0.9±0.1	0.541±0.005 [*]	nd
	K _D (nM)	28±5	20.6±0.8	nd
Two-independent-site model	B _{maxH} (pmol/mg protein)	Nd	nd	0.21±0.02
	K _{DH} (nM)	Nd	nd	0.8±0.3 ^{***#}
	B _{maxL} (pmol/mg protein)	Nd	nd	0.55±0.02
	K _{DL} (nM)	Nd	nd	55±6 ^{***#}
Dimer receptor model	R _T (pmol/mg protein)	0.41±0.02	0.39±0.01	0.44±0.01
	K _{DB1} (nM)	3±1	7±3	9±1
	K _{DB2} (nM)	-	-	-
	K _{DAB} (nM)	0.9±0.5	5±4	4±1
	D _{CB}	0	0	0
	D _{AB}	+0.7	+0.4	+0.7

Values are mean±SEM from three experiments performed with different striatum membranes of 3–4 animals. The radioligand affinity was K_{DA1}=0.27 nM. B_{max} is the maximum specific binding and K_D is the equilibrium dissociation constant for the competing ligand B (SKF81297). B_{maxH} and B_{maxL} are, respectively, the maximum specific binding corresponding to, respectively, high- and low-affinity sites, and K_{DH} and K_{DL} are the equilibrium dissociation constants of the competing ligand B for, respectively, high- and low-affinity sites. R_T is the total amount of receptor dimers, K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B to the dimer.

K_{DAB} is the hybrid equilibrium dissociation constant of B binding to a receptor dimer semioccupied by the A ([³H]SCH23390). D_{CB} is the dimer cooperativity index for the binding of ligand B and D_{AB} is the dimer radioligand-competitor modulation index (see Methods). nd: not determined.

Statistical differences between parameters obtained with the monomeric receptor models were calculated by one-way ANOVA followed by Bonferroni's post-hoc test

*
p<0.05 and

**
p<0.01 vs. value at 0.15 nM;

p<0.05 and

p<0.01 vs. value at 0.40 nM

Table 4

Effect of modulators/ligands on dissociation kinetic parameters of dopamine D₁ and adenosine A_{2A} receptor radiolabelled antagonists

Receptor	Radioligand	Modulator	K _{off1} (min ⁻¹)	K _{off2} (min ⁻¹)	RT ₁ (min)	RT ₂ (min)
A _{2A}	[³ H]ZM 241385	-	0.025±0.002	-	40	-
		CGS21680	0.19±0.03 ^{**}	0.004±0.004	5	250
		Caffeine	0.028±0.004	-	36	-
D1	[³ H]SCH23390	-	0.0246 ± 0,0008	-	41	-
		SKF81297	0.0325 ± 0,0004 ^{***}	-	31	-
		SKF83566	0.0256 ± 0,0007	-	39	-

Dissociation kinetic assays of D1R were performed at RT on sheep striatum membranes with 0.17 nM [³H]SCH23390 in the absence or presence of 5 nM SKF81297 or 0.5 nM SKF83566. Dissociations were initiated after 90 min of radioligand association by the addition of 50 volumes of the binding buffer (the infinite dilution method) in the presence or the absence of the modulator at the same concentration. Dissociation kinetic parameters of A_{2A}R data are adapted from [42]. RT: residence time. K_{off} values are mean±SEM from three experiments performed with different striatum homogenates of 3–4 animals. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's post-hoc test

^{**}
p<0.01 and

^{***}
p<0.001 vs. each respective control