Current Neuropharmacology, 2022, *20*, 1035-1045

REVIEW ARTICLE

Overcoming the Challenges of Detecting GPCR Oligomerization in the Brain

Víctor Fernández-Dueñas[1,2,*], Jordi Bonaventura[1,2], Ester Aso[1,2], Rafael Luján[3], Sergi Ferré[4] and Francisco Ciruela[1,2,*]

[1]*Pharmacology Unit, Department of Pathology and Experimental Therapeutics, School of Medicine and Health Sciences, Institute of Neurosciences, University of Barcelona, 08907 L'Hospitalet de Llobregat, Spain;* [2]*Neuropharmacology & Pain Group, Neuroscience Program, Bellvitge Institute for Biomedical Research, 08907 L'Hospitalet de Llobregat, Spain;* [3]*Synaptic Structure Laboratory, Instituto de Investigación en Discapacidades Neurológicas (IDINE), Departamento de Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Albacete, Spain;* [4]*Integrative Neurobiology Section, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD, USA*

ARTICLE HISTORY

Received: July 31, 2021
Revised: September 14, 2021
Accepted: October 14, 2021

DOI:
10.2174/1570159X19666211104145727

Abstract: G protein-coupled receptors (GPCRs) constitute the largest group of membrane receptor proteins controlling brain activity. Accordingly, GPCRs are the main target of commercial drugs for most neurological and neuropsychiatric disorders. One of the mechanisms by which GPCRs regulate neuronal function is by homo- and heteromerization, with the establishment of direct protein-protein interactions between the same and different GPCRs. The occurrence of GPCR homo- and heteromers in artificial systems is generally well accepted, but more specific methods are necessary to address GPCR oligomerization in the brain. Here, we revise some of the techniques that have mostly contributed to reveal GPCR oligomers in native tissue, which include immunogold electron microscopy, proximity ligation assay (PLA), resonance energy transfer (RET) between fluorescent ligands and the Amplified Luminescent Proximity Homogeneous Assay (ALPHA). Of note, we use the archetypical GPCR oligomer, the adenosine A_{2A} receptor (A_{2A}R)-dopamine D_2 receptor (D_2R) heteromer as an example to illustrate the implementation of these techniques, which can allow visualizing GPCR oligomers in the human brain under normal and pathological conditions. Indeed, GPCR oligomerization may be involved in the pathophysiology of neurological and neuropsychiatric disorders.

Keywords: GPCR oligomerization, immunoelectron microscopy, proximity ligation assay, TR-FRET, ALPHA assay, RET.

1. INTRODUCTION

G protein-coupled receptors (GPCRs), also known as seven transmembrane (7TM) domain receptors or heptahelical receptors, constitute the largest group of proteins (~800) in our genome [1]. There are different classification systems for GPCRs. The classical A-F system distinguishes six classes of GPCRs based on their amino acid sequences and functional similarities [2], while the more recent GRAFS system [Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S)] is based on the phylogenetic origin [3, 4]. From these classes, the largest group (~80 %) of GPCRs is class A (Rhodopsin family), which includes receptors for light, hormones or neuromodulators [3, 4]. Class A has been the most studied group, but most of the uncovered general principles are applied to the other GPCR classes [5]. GPCRs have 7TM helices and are associated with the heterotrimeric guanine nucleotide-binding proteins (G proteins) [5]. When a ligand binds and activates a GPCR expressed at the cell plasma membrane, the receptor interacts and promotes the activation of a specific G protein, leading to activation of different cellular signaling pathways [6, 7]. GPCRs can also activate G-protein independent signaling systems, such as the arrestin pathway [8]. Arrestin-mediated cellular actions have gained interest in the past few years, due to the emergence of pharmacological concepts such as functional selectivity or biased signaling [9-12]. A functionally selective ligand can preferentially trigger the activation of a G protein-dependent or a G protein-independent and arrestin-dependent signaling pathway. As a result, this biased activation could lead to different responses generated by from the same receptor, depending on the tissue where receptors are located. Moreover, protein-protein interactions may lead to the selective activation of different subpopulations of receptors, lead to the selective activation of a subpopulation of receptors [9-12]. One of the molecular mechanisms proposed can explain functional selectivity and the complexity of GPCR pharmacology

*Address correspondence to these authors at the Pharmacology Unit, Department of Pathology and Experimental Therapeutics, School of Medicine and Health Sciences, Institute of Neurosciences, University of Barcelona, 08907 L'Hospitalet de Llobregat, Spain;
E-mails: vfernandez@ub.edu, fciruela@ub.edu

is the formation of receptor complexes, in which a direct protein-protein interaction between two receptors is established [10-12]. This phenomenon is known as GPCR oligomerization. Although some controversy still exists regarding the existence of GPCR oligomerization [13], this phenomenon is becoming an important focus in drug discovery, especially when developing drugs targeting the central nervous system (CNS), where multiple receptors coexist in small subcellular spaces (*i.e.*, synaptic cleft). Hence, the integration of different signals by complexes of different GPCRs may allow the fine-tuning modulation of cellular responses to neurochemicals [14]. Accordingly, drugs acting at GPCR oligomers may offer therapeutic advantages for the treatment of diseases where traditional drugs have failed or exhibit poor efficacy [14, 15].

The occurrence of GPCR oligomerization was first postulated in the 1980s, when seminal work by Fuxe, Agnati and collaborators led to the hypothesis that clusters of receptors worked as integrative units in the CNS [16, 17]. This hypothesis was first supported by indirect evidence, such as the modulation of neuropeptides on the binding of monoamines to their receptors or the detection of receptor species showing twice the expected molecular size in western-blot experiments [18-21]. Based on these data, the study of GPCR oligomerization gained increasing attention, and several new tools were developed to detect GPCR oligomerization in living cells. Resonance energy transfer (RET), which was first described by Theodor Förster in the late 1940s [22], has been the most popular and probably most reliable of them. However, RET-based experiments in living cells require the fusion of fluorescent/bioluminescent proteins to the receptors of interest [23]. Accordingly, most of the results obtained with RET experiments indicating GPCR oligomerization have been accomplished in heterologous expression systems where receptors are fused with other proteins and expressed out of their physiological context. Indeed, the study of GPCR oligomerization in native receptors has remained a challenge; hence, the most valuable demonstrations of GPCR oligomerization are represented by the artificial crystallization of class A GPCR dimers [24-26].

One of the main examples illustrating the progress in the GPCR oligomerization field is the study of the adenosine A_{2A} receptor ($A_{2A}R$)-dopamine D_2 receptor (D_2R) heteromer. The oligomerization between $A_{2A}R$ and D_2R within GABAergic, enkephalinergic, striato-pallidal neurons in the striatum was postulated at the beginning of the 1990s [27, 28]. As recently reviewed [29], the crosstalk and allosteric interactions between these two class A GPCRs in the striatopallidal neurons may allow the fine tuning of some striatal functions (*i.e.*, control of voluntary movements). As initially hypothesized [30], $A_{2A}R$ antagonists have been introduced as an adjuvant treatment to L-DOPA in Parkinson's disease based on an allosteric modulation in the $A_{2A}R$-D_2R heteromer [27-29, 31, 32]. Prior to the investigation of $A_{2A}R$ antagonists as potential drugs for Parkinson's disease treatment, the existence of $A_{2A}R$-D_2R heteromers was extensively studied in heterologous expression systems by using RET-based approaches[31, 33-37]. The oligomeric interface was further explored and some critical residues within intracellular and transmembrane domains were identified as responsible for the interaction [37, 38]. In addition, the first attempts to prove direct receptor oligomerization in native tissues were made. Both receptors co-immunoprecipitated in rat striatum homogenates and, more importantly, they were closely co-localized in striatal neurons, as shown with immunogold electron microscopy (immunogold EM) [34]. Nonetheless, classical biochemical techniques, including co-immunoprecipitation, *in situ hybridization* and immunohistochemistry, may suggest colocalization but do not demonstrate the existence of GPCR oligomers in native tissue. In co-immunoprecipitation, one of the receptors present in a cell lysate is recognized with an antibody, immobilized, purified and then detected by western blotting [39]. However, it is difficult to detect low affinity or transient interactions and it is not possible to conclude that a direct interaction occurs. In addition, membrane proteins are often solubilized with detergents, fostering the artificial aggregation or disaggregation of putatively interacting proteins [39]. Another disadvantage is that immunoprecipitation does not provide information on the neuron population and neuronal compartment where the molecular interaction is taking place. In addition, the inaccuracy inherent to the dissection of a specific brain region due to its anatomical shape or location, added to the softness of the fresh tissue, as well as the proteolytic degradation produced when samples are not processed immediately after brain extraction (*i.e.*, human *post-mortem* tissue), are also important drawbacks [40]. On the other hand, immunohistochemical approaches have anatomical resolution and permit visualizing receptors colocalizing in the same neuron, but, due to their restricted spatial resolution, they cannot demonstrate the establishment of direct protein-protein interactions [40]. For instance, the formation of macromolecular complexes for GIRK channels and associated proteins like RGS7, Gβ5, and $GABA_B$ receptors was inferred after detection of their overlapping expression profiles in the hippocampus and cerebellum [41-44].

Overall, these classical biochemical techniques such as co-immunoprecipitation are not sufficient to demonstrate oligomerization in native tissue. Accordingly, novel techniques have been developed to detect GPCR oligomers between unmodified receptors in their native environment. This is exemplified by studies performed to detect $A_{2A}R$-D_2R heteromers, which include immunogold EM, the proximity ligation assay (PLA) and RET using fluorescent ligands [45]. These studies were performed in rodents and non-human primates, but the demonstration of $A_{2A}R$-D_2R oligomerization in post-mortem human brains was still missing. Recently, by means of an optimized PLA method and the novel Amplified Luminescent Proximity Homogeneous Assay (ALPHA), it has finally been possible to detect $A_{2A}R$-D_2R heteromers in human necropsies [46, 47]. Here, we illustrate and revise the strengths and weaknesses of all these techniques (immunogold EM, PLA, RET between fluorescent ligands and ALPHA), as techniques that have been instrumental in demonstrating the occurrence of GPCR oligomerization (*i.e.*, $A_{2A}R$-D_2R) in native tissue, and that have permitted to overcome the challenge of detecting GPCR oligomers in the human brain.

2. IMMUNOGOLD ELECTRON MICROSCOPY

Immunogold electron microscopy (immunogold EM), in which detection is performed with secondary antibodies conjugated to gold particles, is a technique that allows the exam-

ination and analysis of the subcellular localization of neurotransmitter receptors, ion channels and their associated proteins in different brain regions and neuron populations. Compared to optical microscopy, it provides higher spatial resolution, higher sensitivity and renders good ultrastructural and antigenicity preservation [48, 49]. There are two main methodologies used in immunogold EM: pre-embedding and post-embedding immunogold. In pre-embedding, immunolabelling steps are performed before samples are embedded in resins required to facilitate ultrathin sectioning and perform EM, while in post-embedding immunolabelling steps take place on embedded and ultrathin sections [48, 49]. Pre-embedding is considered to be more sensitive than post-embedding immunogold. In post-embedding, antigen preservation may be hampered by cell fixation and by dehydration and resin embedding. In addition, in post-embedding, antibodies cannot penetrate into resins and, therefore, labelling is restricted to the thin section surface [48, 49].

On the other hand, while pre-embedding is the most reliable manner to localize receptors at extrasynaptic and perisynaptic sites, post-embedding immunogold is considered a better approach to localize receptors at post-synaptic densities (PSDs) of excitatory synapses [48, 49]. Another approach that can be used in immunogold EM is the sodium dodecyl sulfate-digested freeze-fracture replica labelling (SDS-FRL). There are three steps in freeze-fracture replica of brain tissues, which are: i, fixation with paraformaldehyde; ii, freezing under high-pressure; and iii, replication by deposition of carbon and platinum following fracturing, followed by digestion of biological tissue [50]. Of note, sensitivity and labelling efficiency in the replica are very high, also because of the use of SDS. Thus, it is capturing and stabilizing integral membrane proteins and preserving antigenicity [50].

The use of secondary antibodies conjugated with gold particles of different sizes enables the simultaneous localization of different proteins in the same neuronal compartments. For this reason, these techniques have been successfully used to assess GPCR oligomerization by measuring the distance between gold particles corresponding to different proteins [41, 42]. It is important to note that the distance is measured between the gold particles, which have a small size (*i.e.*, 5 and 10 nm), but the real distance between the proteins forming a putative complex cannot be directly assessed. Indeed, the maximum projected distance between epitope and gold particle (two IgG diameters plus gold particle radius) will be in the range of 20-30 nm [51]. Nevertheless, given that in immunogold EM the spatial resolution is maintained, measuring the distance between gold particles bound to the different protomers allows a good estimation of their proximity with high sensitivity. On the other hand, it is important to note that the detection and quantification process could be affected when receptors' relative density is high, since some particle overlapping could occur.

As mentioned above, the existence of $A_{2A}R$-D_2R heteromers in striatal neurons was firstly supported by means of immunogold EM [34]. In the study by Cabello *et al.*, triple labelling was performed using three gold particles of different sizes and showed the colocalization and proximity of $mGlu_5R$, D_2R and $A_{2A}R$ along the extra-synaptic and perisynaptic plasma membrane of spines (Figs. **1A** and **B**) [34]. Later on, it was possible to obtain further information by measuring and quantifying the distance between immunoparticles for D_2R and $A_{2A}R$ in dendrites and spines (Fig. **1C**), demonstrating that both proteins were close to each other and that those distances were significantly increased in an animal model (6-OHDA striatal injection) of Parkinson's disease [52].

3. PROXIMITY LIGATION ASSAY

Proximity ligation assay (PLA) [53] is a technology that overcomes classical immunohistochemistry and complements immunogold EM to reveal GPCR oligomerization [54-58]. Thus, it allows to both visualize subcellular localization and detect transient or weak receptor-receptor interactions. Like fluorescent immunohistochemistry and immunogold EM, PLA is based on the detection of the pair of receptors by primary antibodies, which are raised in different species and then recognized by secondary antibodies. However, in this case, the secondary antibodies are conjugated to single strand complementary short DNAs. These DNA strands, when in close proximity (around 16 nm), can interact and generate an amplifiable circular DNA strand in the presence of linear connector oligonucleotides. Then, the circular strand serves as a template for a local rolling-circle amplification reaction that generates a product that can be detected by hybridization with fluorescently labeled complementary oligonucleotides [56]. The result of this process is the visualization of each oligomer as distinct puncta (fluorescent dots) by means of confocal microscopy. Of note, similar to immunogold EM, the use of primary and secondary antibodies (which can measure around 20-30 nm) leads to certain imprecision to determine the real distance between proteins forming a complex. On the other hand, a single signal (coming from the interaction of the DNA strands) is obtained in PLA. This may be an advantage on the detection and quantification process but depends on the relative receptors' density. Accordingly, a single signal is easy-to-count but, when receptors' density is high, secondary antibodies may hybridize with more than one partner, and several interactions can be omitted.

The PLA technique critically depends on two major factors: the quality of the tissue and the primary antibodies [54]. Fixation avoids the mobility of receptors and possible random collisions within the cell; thus, similar to EM, may allow to spatially localize where the GPCR oligomerization takes place [54]. However, proper fixation is a critical step for successful results. Poor or improper fixation dramatically increases background and non-specific signals and makes it hard to identify positive and specific signals. Indeed, it is extremely recommended to include as many negative controls (*i.e.*, knock-out animals, different brain areas) as possible to discriminate the specific signal [56]. On the other hand, over-fixation may blunt epitope recognition by antibodies, again facilitating unspecific signals [56]. The recognition of epitopes by antibodies depends on the preservation of the conformation of the receptor. Accordingly, polyclonal antibodies, which may be less affected by conformational changes than monoclonal ones, are preferable in PLA [56].

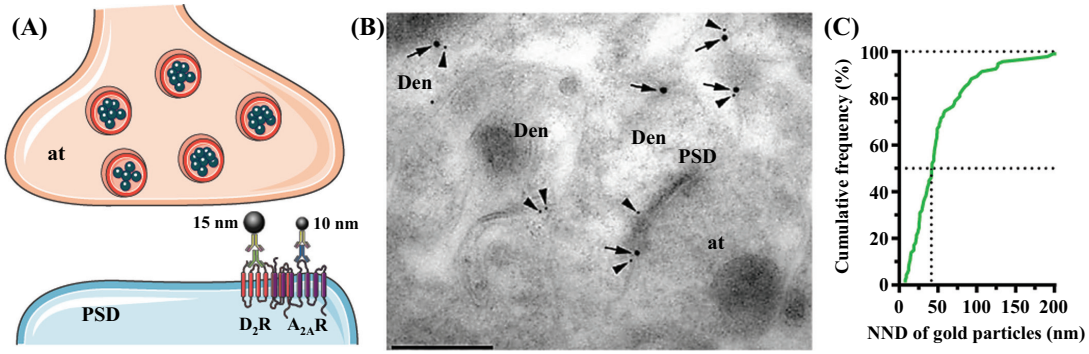

Fig. (1). Immunogold EM. (**A**) Schematic diagram showing the immunogold/EM-based approach for the detection of D_2R and $A_{2A}R$ within a synapse. Axon terminal (at), postsynaptic density (PSD). (**B**) Electron micrographs revealing the subcellular distribution of D_2R and $A_{2A}R$ in rat striatum, revealed using double-labelling post-embedding immunogold detection. Immunoparticles for D_2R (15 nm size, arrows) and $A_{2A}R$ (10 nm size, arrowheads) were detected along the extrasynaptic and perisynaptic plasma membrane of the same dendritic shafts (Den) establishing excitatory synaptic contact with at Scale bar: 0.2 µm. (**C**) Quantitative analysis of the spatial distance between D_2R and $A_{2A}R$ immunoparticles. NND, nearest neighbour distance [52]. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

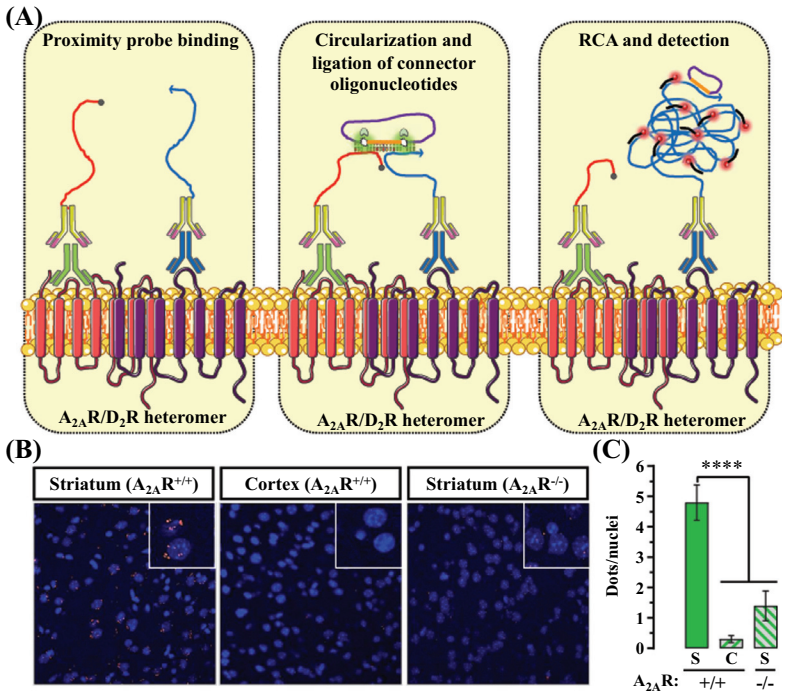

Fig. (2). Proximity ligation assay. (**A**) Schematic representation of *in situ* PLA. Primary antibodies are used to detect D_2R and $A_{2A}R$ (left image). Next, the dual binding of a pair of proximity probes (secondary species-antibodies with attached DNA strands, illustrated in red and blue) targeting the primary antibodies serves to template the hybridization of circularization oligonucleotides, which are then joined by ligation by T4 DNA ligase (illustrated as green oval) into a circular ssDNA molecule (purple curvy circle) (middle image). The closed circular ssDNA may serve as a template for the phi29 DNA polymerase which extends the 3′-OH ends of one of the PLA probes (blue) acting as a primer for rolling circle amplification (RCA). Finally, the generated concatemeric product is hybridized with fluorescent oligonucleotide probes (red spot) (right image). Extracted from [56]. **(B)** Representative images of dual recognition of D_2R and $A_{2A}R$ by *in situ* PLA in mouse striatum (S). Both cortex (C) from wild-type mice and striatum (S) from $A_{2A}R^{-/-}$ were used as negative controls. **(C)** Quantification of PLA signal for D_2R and $A_{2A}R$ proximity in mouse striatum. Values correspond to the mean ± SEM (PLA dots/nuclei) of at least 6 animals for condition. ****: significantly different ($P < 0.0001$), as compared to the striatum of $A_{2A}R^{+/+}$ mice (one-way ANOVA with Dunnett's multiple comparison test). Adapted from [52]. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

PLA was successfully used to reveal $A_{2A}R$-D_2R heteromers in native tissue from animal models (Fig. **2**) [45, 52, 54]. However, it initially failed to detect these kinds of complexes in postmortem human brains. Recently, Javitch, Trifilieff and collaborators were able to use PLA to detect $A_{2A}R$-D_2R heteromers in the human brain [46]. Basically, they achieved it by optimizing the fixation protocol. Thus, differently from rodent brains, which are typically fixed by rapid intracardial perfusion with paraformaldehyde, post-mortem human brains are classically fixed by immersion and embedding in paraformaldehyde and subsequent embedding in paraffin. Here, the authors first optimized different immersion proto-

cols in mice. Interestingly, they realized that nuclear background was further reduced upon immersion, thus providing a novel approach to obtain specific PLA signals. On the other hand, they introduced another change, which consisted of using a chromogenic substrate based on horseradish peroxidase enzymatic activity instead of fluorescently labeled probes. The use of horseradish peroxidase to reveal the PLA signal dramatically reduced background [46]. This fact could indicate that the high background observed in PLA using fluorescent probes could be related with autofluorescent components of tissues (*i.e.*, lipofuscins), which may be taken into account to avoid misinterpretation of the results [59]. Overall, together with the development of a superior automated quantification of PLA puncta, it was possible to finally demonstrate the existence of $A_{2A}R$-D_2R heteromers in human striatal (caudate nucleus) samples [46].

4. RET BETWEEN FLUORESCENT LIGANDS

Both immunogold EM and PLA may be considered outstanding tools to reveal GPCR oligomerization in native tissue. The main advantage of these techniques relies on their ability to determine the possible spatial localization of the oligomers. On the other hand, the need for antibodies and the uncertainty about the actual distance between the two GPCRs forming the complex are the main drawbacks. It is important to note that antibodies are molecules larger than 150 kDa (10-15 nm), making it difficult to fully ensure that the antibody-targeted receptors are in contact. Conversely, RET-based approaches require real close proximity (<10 nm) between the two fluorophores that are virtually in contact with the receptor, hence, they are indicative of GPCR oligomerization [23].

RET is a nonradiative transfer of energy from an excited (donor) to a relaxed chromophore (acceptor), which results in light emission from the acceptor and an apparent decrease of the emission of the donor [22]. The efficiency of the energy transfer depends on the distance between donor and acceptor molecules, which should be less than 10 nm. Apart from the distance between chromophores, RET also depends on the relative spatial localization of the molecules (dipole orientation), their spectral overlap, quantum yields and extinction coefficients [22, 60-65]. One of the most common pairs of chromophores used in RET experiments are variants from the Green Fluorescent Protein (GFP), namely Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP). Thus, a fluorescence RET (FRET) process can occur when CFP and YFP are close together. If CFP is excited at 430 nm, it emits light at 480 nm, which may excite YFP and lead to an emission at 530 nm. However, the use of fluorescent proteins as RET pairs is also one of the main drawbacks of classical RET techniques. First, CFP and YFP excitation and emission spectra partially overlap (phenomena known as crosstalk and bleed-through). In addition, they may exhibit some auto-fluorescence (emission in the absence of an exciting beam), and they may be photobleached upon continuous irradiation [23]. The use of lanthanide elements (*i.e.*, europium, terbium) as donor molecules can help overcome some of these issues. These rare-earth elements exhibit a long-lived emission that results in emission after the excitation source is interrupted, and hence they allow a time-resolved (usually about 50 μs) FRET (TR-FRET) [66]. In addition, their excitation peak is at 340 nm (UV-shifted compared to GFP analogs), and thus they are compatible with acceptor molecules (*i.e.*, fluorescein, allophycocyanin) without overlap. Similarly, the bleed-through is almost negligible, since lanthanides emit a series of sharp emission peaks that may not contaminate the acceptor emission spectra [67-69]. Overall, TR-FRET approaches may allow high temporal resolution and spectral compatibility, which finally results in a high signal-to-noise ratio required to detect GPCR oligomerization.

Both FRET and TR-FRET approaches, together with bioluminescent RET (BRET), in which the donor molecule is a bioluminescent protein (*i.e.*, *Renilla* luciferase, *R*luc), have been extensively used to reveal GPCR oligomerization [23] in heterologous expression systems. For instance, $A_{2A}R$-D_2R heteromers have been extensively detected in HEK293 cells transiently expressing constructs fused to compatible donor and acceptor molecules [33, 37]. By transfecting a constant amount of $A_{2A}R$ tagged with the Rluc protein as a donor and increasing amounts of D_2R tagged with a YFP protein as an acceptor, it was possible to obtain a saturable BRET signal, indicative of a specific GPCR oligomerization [33, 37]. Upon these conditions, different mutations to the receptors were introduced, in order to reveal the molecular determinants of the $A_{2A}R$-D_2R interface. For instance, the mutation to alanine of a rich arginine domain at the third intracellular loop of the D_2R led to a reduction in the BRET signal [37]. Similarly, by using Bimolecular Fluorescence Complementation (BiFC) and transmembrane-disruptive peptides it was possible to selectively disrupt $A_{2A}R$-D_2R heteromers [70]. Thus, the formation of the complexes, which was assessed by measuring the reconstitution of the two halves of a fluorescent protein was blocked when incubating cells with peptides targeting the heteromeric interface [70].

Nevertheless, fusing receptors with fluorescent proteins to engage in RET represents a major drawback (*i.e.*, the need for genetically modified receptors). Thus, this approach is incompatible with performing RET in native tissue and physiologically expressed unaltered receptors. Other strategies were envisioned to allow performing RET on native tissues, such as the use of two fluorescently labelled selective ligands, which, once bound to their respective receptors, could engage in RET. The first use of this strategy to detect GPCR oligomers was performed in rat mammary glands, where oxytocin receptor homodimers were found [71]. Interestingly, the ligands were based on a lanthanide (*i.e.* europium) as a donor and a red dye (*i.e.* alexa647) as an acceptor [71]. Oxitocyn receptors are very dense in mammary glands [71], and this particularly high expression of oxytocin receptors drove a very high signal-to-noise ratio, which allowed the detection of such oligomers in native tissue for the first time [71].

The oxytocin receptor study led to the development of an adapted protocol to reveal $A_{2A}R$-D_2R heteromers in the striatum, where $A_{2A}R$ and D_2R are also enriched [52]. A D_2R antagonist (N-(paminophenethyl)spiperone) labelled with terbium was used as a donor and an $A_{2A}R$ antagonist (SCH442-416) conjugated to DY-647 was used as an acceptor (Fig. **3A**). A specific TR-FRET signal was obtained after incubating striatal membranes with fluorescent ligands (Fig. **3B**) [52]. Nevertheless, $A_{2A}R$-D_2R heteromer expression in the brain is lower (at least 10 times) than that observed for

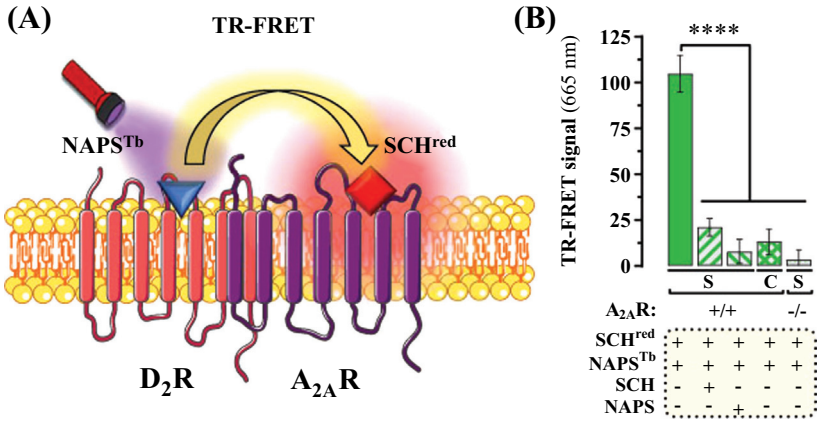

Fig. (3). TR-FRET. (A) Diagram illustrating the principle of TR-FRET between fluorescent ligands bound to D_2R and $A_{2A}R$. $NAPS^{Tb}$ and SCH^{red} are selective D_2R and $A_{2A}R$ antagonists, respectively. These antagonists are labelled with compatible fluorophores (*i.e.*, Terbium and Alexa649, respectively) for a TR-FRET process. **(B)** Membrane preparations from rat striatum (S) were labelled with $NAPS^{Tb}$ (1 nM) plus SCH^{red} (10 nM), which resulted in a TR-FRET signal (1st column). Incubation with cold ligands (1 µM of non-labelled NAPS or SCH) lead to a significantly lower TR-FRET signal (2nd and 3rd columns). Membranes from the cortex (C) of the same rats (4th column) and striatal membranes from $A_{2A}R^{-/-}$ mouse (5th column) were used as negative controls of the TR-FRET signal. Data are expressed as means ± SEM of three independent experiments performed in triplicate. ****: significantly different ($P < 0.0001$) as compared to the striatum of $A_{2A}R^{+/+}$ mice (one-way ANOVA followed by Bonferroni's test) [52]. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

oxytocin receptors at mammary glands; thus the signal-to-noise ratio obtained was much lower [71, 72]. This decrease in sensitivity made it difficult to detect a positive TR-FRET signal and, along with some adaptations of the original protocol, several controls and corrections were necessary [73]. In general, to obtain specific TR-FRET signal in these conditions it is important to subtract: 1) the background signal coming from the device and the autofluorescence of the sample; 2) the signal resulting from non-specific TR-FRET due to non-specific binding; 3) the signal due to direct excitation of the acceptor; and 4) the signal caused by the bleed-through of donor fluorescence into the acceptor emission filters [73]. On the other hand, it is important to note that it was not possible to use TR-FRET in fresh or fixed striatal slices, but instead, striatal membranes were used [52]. Also, it is important to note that the same strategy failed to detect the same oligomer in human brain preparations. We hypothesize that the nature of the necropsies, together with the variable time and conservation protocols of tissue extraction, could explain why this approach was unsuccessful in post-mortem human brains.

5. ALPHA

Revealing GPCR oligomerization in human post-mortem tissue may be the final goal of studies aimed at studying this phenomenon. To our best of knowledge, to date, only two techniques have succeeded. The first, as mentioned above, is the PLA. The second is the Amplified Luminescent Proximity Homogeneous Assay (ALPHA), which is an assay based on Luminescent Oxygen Channeling Immunoassay (LOCI™) [24]. In brief, the ALPHA consists of the use of two types of latex nanobeads that, like RET, are donor and acceptor beads. Donor beads are loaded with a photosensitizer (phthalocyanine), while acceptor hydrogel-coated beads contain a chromophore (*i.e.*, rubrene, europium). The donor bead is excited at ~680 nm, which leads to the production of singlet oxygen. This singlet of oxygen has a halftime life of 4 µsec before reducing its energy to the ground state. Within this time, the oxygen singlet can diffuse 200 nm in solution. Therefore, if the acceptor bead is located in close proximity, it can be excited and produce a luminescent/fluorescent signal [15]. It is important to note that the composition of the acceptor bead determines the two ALPHA techniques, which are ALPHAScreen and ALPHALisa. In ALPHAScreen, the acceptor is coupled to thioxene, anthracene or, most commonly, to a rubrene molecule and emits a detectable light at 520-620 nm. In ALPHALisa, the acceptor bead is coupled to europium, which has a sharp emission peak at ~615 nm. Since the emission bandwidth is narrower, ALPHALisa displays less susceptibility to artificial or natural interferences than ALPHAScreen. In addition, its light emission is more intense leading to higher signal-to-noise ratios.

A major advantage regarding the use of latex nanobeads as donor and acceptor molecules is the high signal-to-noise ratio. Accordingly, ALPHA may allow detecting GPCR oligomers with superior sensitivity, either when the expression is relatively low, as it happens in the brain as compared with transfected mammalian cells, or when the interaction is weak and difficult to detect. However, although these features help in overcoming some of the problems observed when using fluorescent ligands in RET, the ALPHA presents some drawbacks common to immunohistochemistry, immunogold EM or the PLA approach. Accordingly, the receptors forming a putative complex need to be first detected by means of primary antibodies, which can be readily recognized by secondary antibodies labelled with the latex nanobeads (with a diameter of around 200 nm) subsequently. Accordingly, the maximum projected distance between epitope and beads will be around 200 nm, which is a distance much higher than EM, PLA and, especially RET. In addition, it is also important to consider that the APLHA is performed in membranes, thus spatial information, like in RET, is lost. Indeed, ALPHA

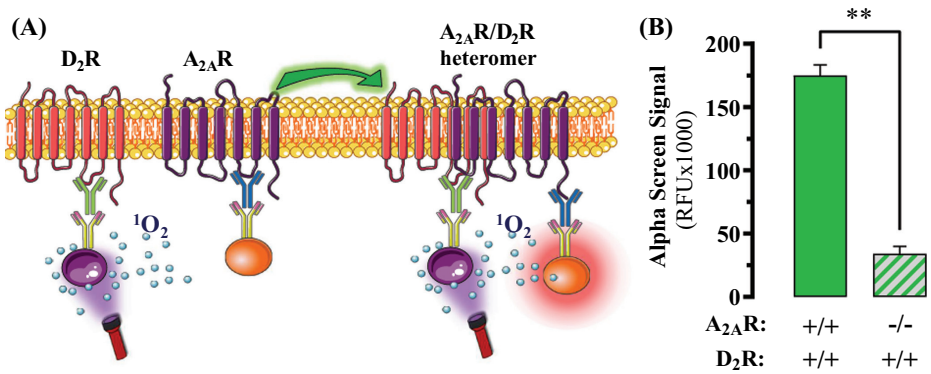

Fig. (4). ALPHA assay. **(A)** Diagram illustrating the principle of the ALPHA assay, in which the putative receptors forming an oligomer are first recognized by primary antibodies, and these by secondary antibodies labelled with latex nanobeads compatible with eliciting an energy transfer if they are in close proximity. (**B)** ALPHA signal obtained from striatal extracts of wild-type ($A_{2A}R^{+/+}$) and $A_{2A}R$ deficient mice ($A_{2A}R^{-/-}$). Data are expressed as means ± SEM of three independent experiments performed in triplicate. $^{**}P < 0.01$ Student's *t*-test. Adapted from [47]. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

Table 1. Techniques used to reveal GPCR oligomers in the brain.

-	Immunogold EM	PLA	RET	ALPHA
Fixation	YES	YES	NO	NO
Spatial resolution	YES	YES	NO	NO
Sensitivity	↑↑	↑	↑	↑↑
Use of antibodies	YES	YES	NO	YES
Proximity	~25 nm	~25-40 nm	<10 nm	~200 nm

Abbreviations: EM, electron microscopy; PLA, proximity ligation assay; RET, resonance energy transfer; ALPHA, amplified luminescent proximity homogeneous assay.

does not allow assessing whether the interactions are occurring at the plasma membrane or during the trafficking/recycling of receptors.

As discussed previously, the ALPHA approach is the second assay, together with the PLA, that has permitted to reveal the existence of $A_{2A}R$-D_2R heteromers in human post-mortem brains [47, 74]. In the first study using ALPHA [47], purified caudate membranes from control and Parkinson's disease patients were evaluated. The samples were first incubated with primary antibodies, and then, nanobead-labelled secondary antibodies labelled were added. The assay was also validated in striatal membranes of mice lacking the $A_{2A}R$, and in an animal model of Parkinson's disease (Fig. **4**). The results were comparable to those obtained by PLA and TR-FRET. In addition, the signal-to-noise ratio obtained led to the conclusion that the ALPHA could be applied for revealing GPCR oligomers in native tissue [47]. Indeed, the assay was next implemented in human brain membrane preparations and a robust signal was obtained, as assessed by the Z-value (statistical parameter that allows evaluation of the assay's robustness [17]). In the second study [74], purified caudate membranes from control and schizophrenic patients were used. Interestingly, ALPHALisa acceptor beads, instead of ALPHAScreen [47] were used, in order to increase the sensitivity (see above). Interestingly, a specific ALPHA signal was observed, thus validating that different combinations of antibodies and latex nanobeads can be used to perform the ALPHA. Importantly, a significant and pronounced reduction of $A_{2A}R$-D_2R heteromers was observed in schizophrenic subjects, which may indicate that $A_{2A}R$-D_2R heteromerization is involved both in the pathogenesis of schizophrenia and, hence, involved in the therapeutic effects of antipsychotic drugs [74]. Of note, the reduction in $A_{2A}R$-D_2R heteromerization was opposite to the increase observed in Parkinson's disease patients [47]. Overall, the ALPHA is a quantitative assay that allows detecting differences within pathophysiological conditions in human tissue. Such differences could be considered a pathological fingerprint and, eventually, could also lead to new therapeutic strategies aimed at restoring $A_{2A}R$-D_2R heteromer balance. Altogether, the ALPHA should be regarded as a novel complementary approach to reveal GPCR oligomerization in native tissue.

CONCLUSION

GPCRs are the most important targets for commercial drugs; thus characterizing the biology of GPCRs is key to improving the management of diseases in which GPCRs are involved. GPCR oligomerization is a phenomenon that can lead to the development of novel drug types. Accordingly, reliable, and robust techniques to study GPCR oligomerization in native tissues are desirable. Here, we have presented the most reliable techniques available (immunogold EM, PLA, TR-FRET between ligands and ALPHA). We have showed the strengths and weakness of these techniques (Table **1**) and how these tools have allowed revealing $A_{2A}R$-D_2R heteromerization, not only in animal models but also in human post-mortem brains.

It is important to note that, by themselves, these biophysical techniques can only demonstrate a very close proximity between the putative directly interacting GPCRs, and we cannot exclude that a positive result is due to a close co-localization, due to a high co-expression into the same membrane compartment or their presence in the same macromolecular complex. Accordingly, further work is required to validate true intermolecular interactions of the components of a signaling complex. One possibility is the use of nanobodies, which are smaller in size than regular antibodies, and hence, would allow to reduce the distance between actuators in immunochemistry-based methods (*i.e.*, spatial resolution) and of RET tools (*i.e.*, lower distances). On the other hand, it is also important to note that the presented tools aim to assess the establishment of direct receptor-receptor interactions, but except RET, they do not allow to assess dynamic changes. Thus, although many studies have suggested that GPCR oligomers dimers are stable entities, it has been shown that these complexes are indeed dynamic structures on a milli-second timescale, where changes in the quaternary structure can be driven by agonists or allosteric modulators [75, 76]. Accordingly, detecting the dynamics of GPCR oligomerization in unaltered receptors remain a challenge for the field. Interestingly, this tool would be valuable to prove the ability to restore/modulate a putative imbalance of GPCR oligomers by targeting these complexes.

Overall, the recent progress and development of novel technologies have allowed the identification of GPCR oligomers in the brain. The further study of the molecular determinants and functional properties of GPCR oligomers may help in designing novel strategies to better manage a number of pathologies in which GPCR oligomerization plays a major role.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This work was supported by grants from FEDER-EU/Ministerio de Ciencia, Innovación y Universidades-Agencia Estatal de Investigación (PID2020-118511RB-I00) and Generalitat de Catalunya (2017SGR1604) to FC, the intramural funds of the National Institute on Drug Abuse to SF, and Junta de Comunidades de Castilla-La Mancha (SBPLY/17/180501/000229) to RL.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

We thank Centres de Recerca de Catalunya (CERCA) Programme/Generalitat de Catalunya for IDIBELL institutional support, Esther Castaño, Benjamín Torrejón and Álvaro Gimeno from the CCiT-Bellvitge Campus of the University of Barcelona.

REFERENCES

[1] Fredriksson, R.; Lagerström, M.C.; Lundin, L.G.; Schiöth, H.B. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.,* **2003**, *63*(6), 1256-1272. http://dx.doi.org/10.1124/mol.63.6.1256 PMID: 12761335

[2] Attwood, T.K.; Findlay, J.B.C. Fingerprinting G-protein-coupled receptors. *Protein Eng.,* **1994**, *7*(2), 195-203. http://dx.doi.org/10.1093/protein/7.2.195 PMID: 8170923

[3] Schiöth, H.B.; Fredriksson, R. The GRAFS classification system of G-protein coupled receptors in comparative perspective. *Gen. Comp. Endocrinol.,* **2005**, *142*(1-2), 94-101. http://dx.doi.org/10.1016/j.ygcen.2004.12.018 PMID: 15862553

[4] Lagerström, M.C.; Schiöth, H.B. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat. Rev. Drug Discov.,* **2008**, *7*(4), 339-357. http://dx.doi.org/10.1038/nrd2518 PMID: 18382464

[5] Hu, G.M.; Mai, T.L.; Chen, C.M. Visualizing the GPCR network: classification and evolution. *Sci. Rep.,* **2017**, *7*(1), 15495-15510. http://dx.doi.org/10.1038/s41598-017-15707-9 PMID: 29138525

[6] Erlandson, S.C.; McMahon, C.; Kruse, A.C. Structural basis for G protein-coupled receptor signaling. *Annu. Rev. Biophys.,* **2018**, *47*, 1-18. http://dx.doi.org/10.1146/annurev-biophys-070317-032931 PMID: 29498889

[7] Weis, W.I.; Kobilka, B.K. The molecular basis of G protein-coupled receptor activation. *Annu. Rev. Biochem.,* **2018**, *87*, 897-919. http://dx.doi.org/10.1146/annurev-biochem-060614-033910 PMID: 29925258

[8] Gurevich, V.V.; Gurevich, E.V. The structural basis of the arrestin binding to GPCRs. *Mol. Cell. Endocrinol.,* **2019**, *484*, 34-41. http://dx.doi.org/10.1016/j.mce.2019.01.019 PMID: 30703488

[9] Urban, J.D.; Clarke, W.P.; von Zastrow, M.; Nichols, D.E.; Kobilka, B.; Weinstein, H.; Javitch, J.A.; Roth, B.L.; Christopoulos, A.; Sexton, P.M.; Miller, K.J.; Spedding, M.; Mailman, R.B. Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.,* **2007**, *320*(1), 1-13. http://dx.doi.org/10.1124/jpet.106.104463 PMID: 16803859

[10] Rozenfeld, R.; Devi, L.A. Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB J.,* **2007**, *21*(10), 2455-2465. http://dx.doi.org/10.1096/fj.06-7793com PMID: 17384143

[11] Guitart, X.; Navarro, G.; Moreno, E.; Yano, H.; Cai, N-S.; Sánchez-Soto, M.; Kumar-Barodia, S.; Naidu, Y.T.; Mallol, J.; Cortés, A.; Lluís, C.; Canela, E.I.; Casadó, V.; McCormick, P.J.; Ferré, S. Functional selectivity of allosteric interactions within G protein-coupled receptor oligomers: the dopamine D1-D3 receptor heterotetramer. *Mol. Pharmacol.,* **2014**, *86*(4), 417-429. http://dx.doi.org/10.1124/mol.114.093096 PMID: 25097189

[12] Kenakin, T.; Miller, L.J. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol. Rev.,* **2010**, *62*(2), 265-304. http://dx.doi.org/10.1124/pr.108.000992 PMID: 20392808

[13] Milligan, G.; Ward, R.J.; Marsango, S. GPCR homo-oligomerization. *Curr. Opin. Cell Biol.,* **2019**, *57*, 40-47. http://dx.doi.org/10.1016/j.ceb.2018.10.007 PMID: 30453145

[14] Ferré, S.; Casadó, V.; Devi, L.A.; Filizola, M.; Jockers, R.; Lohse, M.J.; Milligan, G.; Pin, J.P.; Guitart, X. G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. *Pharmacol. Rev.,* **2014**, *66*(2), 413-434. http://dx.doi.org/10.1124/pr.113.008052 PMID: 24515647

[15] Guidolin, D.; Marcoli, M.; Tortorella, C.; Maura, G.; Agnati, L.F. G protein-coupled receptor-receptor interactions give integrative dynamics to intercellular communication. *Rev. Neurosci.,* **2018**, *29*(7), 703-726. http://dx.doi.org/10.1515/revneuro-2017-0087 PMID: 29466243

[16] Agnati, L.F.; Fuxe, K.; Zoli, M.; Rondanini, C.; Ogren, S.O. New vistas on synaptic plasticity: the receptor mosaic hypothesis of the engram. *Med. Biol.,* **1982**, *60*(4), 183-190. PMID: 6128444

[17] Fuxe, K.; Agnati, L.F.; Benfenati, F.; Celani, M.; Zini, I.; Zoli, M.; Mutt, V. Evidence for the existence of receptor-receptor interactions in the central nervous system. Studies on the regulation of

monoamine receptors by neuropeptides. *J. Neural Transm. Suppl.,* **1983**, *18*, 165-179.
PMID: 6192208

[18] Fuxe, K.; Canals, M.; Torvinen, M.; Marcellino, D.; Terasmaa, A.; Genedani, S.; Leo, G.; Guidolin, D.; Diaz-Cabiale, Z.; Rivera, A.; Lundstrom, L.; Langel, U.; Narvaez, J.; Tanganelli, S.; Lluis, C.; Ferré, S.; Woods, A.; Franco, R.; Agnati, L.F. Intramembrane receptor-receptor interactions: a novel principle in molecular medicine. *J. Neural Transm. (Vienna),* **2007**, *114*(1), 49-75.
http://dx.doi.org/10.1007/s00702-006-0589-0 PMID: 17066251

[19] Ng, G.Y.K.; O'Dowd, B.F.; Lee, S.P.; Chung, H.T.; Brann, M.R.; Seeman, P.; George, S.R. Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem. Biophys. Res. Commun.,* **1996**, *227*(1), 200-204.
http://dx.doi.org/10.1006/bbrc.1996.1489 PMID: 8858125

[20] Zawarynski, P.; Tallerico, T.; Seeman, P.; Lee, S.P.; O'Dowd, B.F.; George, S.R. Dopamine D2 receptor dimers in human and rat brain. *FEBS Lett.,* **1998**, *441*(3), 383-386.
http://dx.doi.org/10.1016/S0014-5793(98)01588-9 PMID: 9891976

[21] Ciruela, F.; Casadó, V.; Mallol, J.; Canela, E.I.; Lluis, C.; Franco, R. Immunological identification of A1 adenosine receptors in brain cortex. *J. Neurosci. Res.,* **1995**, *42*(6), 818-828.
http://dx.doi.org/10.1002/jnr.490420610 PMID: 8847743

[22] Förster, T. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Phys.,* **1948**, *2*, 55-75.
http://dx.doi.org/10.1002/andp.19484370105

[23] Ciruela, F.; Vilardaga, J.P.; Fernández-Dueñas, V. Lighting up multiprotein complexes: lessons from GPCR oligomerization. *Trends Biotechnol.,* **2010**, *28*(8), 407-415.
http://dx.doi.org/10.1016/j.tibtech.2010.05.002 PMID: 20542584

[24] Wu, B.; Chien, E.Y.T.; Mol, C.D.; Fenalti, G.; Liu, W.; Katritch, V.; Abagyan, R.; Brooun, A.; Wells, P.; Bi, F.C.; Hamel, D.J.; Kuhn, P.; Handel, T.M.; Cherezov, V.; Stevens, R.C. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science,* **2010**, *330*(6007), 1066-1071.
http://dx.doi.org/10.1126/science.1194396 PMID: 20929726

[25] Manglik, A.; Kruse, A.C.; Kobilka, T.S.; Thian, F.S.; Mathiesen, J.M.; Sunahara, R.K.; Pardo, L.; Weis, W.I.; Kobilka, B.K.; Granier, S. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature,* **2012**, *485*(7398), 321-326.
http://dx.doi.org/10.1038/nature10954 PMID: 22437502

[26] Huang, J.; Chen, S.; Zhang, J.J.; Huang, X.Y. Crystal structure of oligomeric β1-adrenergic G protein-coupled receptors in ligand-free basal state. *Nat. Struct. Mol. Biol.,* **2013**, *20*(4), 419-425.
http://dx.doi.org/10.1038/nsmb.2504 PMID: 23435379

[27] Ferré, S.; Fuxe, K.; von Euler, G.; Johansson, B.; Fredholm, B.B. Adenosine-dopamine interactions in the brain. *Neuroscience,* **1992**, *51*(3), 501-512.
http://dx.doi.org/10.1016/0306-4522(92)90291-9 PMID: 1488111

[28] Ferré, S.; von Euler, G.; Johansson, B.; Fredholm, B.B.; Fuxe, K. Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. USA,* **1991**, *88*(16), 7238-7241.
http://dx.doi.org/10.1073/pnas.88.16.7238 PMID: 1678519

[29] Ferré, S.; Bonaventura, J.; Zhu, W.; Hatcher-Solis, C.; Taura, J.; Quiroz, C.; Cai, N-S.; Moreno, E.; Casadó-Anguera, V.; Kravitz, A.V.; Thompson, K.R.; Tomasi, D.G.; Navarro, G.; Cordomí, A.; Pardo, L.; Lluís, C.; Dessauer, C.W.; Volkow, N.D.; Casadó, V.; Ciruela, F.; Logothetis, D.E.; Zwilling, D. Essential control of the function of the striatopallidal neuron by pre-coupled complexes of adenosine A_{2A}-Dopamine D_2 receptor heterotetramers and adenylyl cyclase. *Front. Pharmacol.,* **2018**, *9*, 243.
http://dx.doi.org/10.3389/fphar.2018.00243 PMID: 29686613

[30] Fuxe, K.; Ferré, S.; Zoli, M.; Agnati, L.F. Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. *Brain Res.,* **1998**, *26*(2-3), 258-273.
http://dx.doi.org/10.1016/S0165-0173(97)00049-0 PMID: 9651540

[31] Canals, M.; Marcellino, D.; Fanelli, F.; Ciruela, F.; de Benedetti, P.; Goldberg, S.R.; Neve, K.; Fuxe, K.; Agnati, L.F.; Woods, A.S.; Ferré, S.; Lluis, C.; Bouvier, M.; Franco, R. Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.,* **2003**, *278*(47), 46741-46749.
http://dx.doi.org/10.1074/jbc.M306451200 PMID: 12933819

[32] Beggiato, S.; Antonelli, T.; Tomasini, M.C.; Borelli, A.C.; Agnati, L.F.; Tanganelli, S.; Fuxe, K.; Ferraro, L. Adenosine A2A-D2 receptor-receptor interactions in putative heteromers in the regulation of the striato-pallidal gaba pathway: possible relevance for parkinson's disease and its treatment. *Curr. Protein Pept. Sci.,* **2014**, *15*(7), 673-680.
http://dx.doi.org/10.2174/1389203715666140901103205 PMID: 25175458

[33] Bonaventura, J.; Navarro, G.; Casadó-Anguera, V.; Azdad, K.; Rea, W.; Moreno, E.; Brugarolas, M.; Mallol, J.; Canela, E.I.; Lluís, C.; Cortés, A.; Volkow, N.D.; Schiffmann, S.N.; Ferré, S.; Casadó, V. Allosteric interactions between agonists and antagonists within the adenosine A2A receptor-dopamine D2 receptor heterotetramer. *Proc. Natl. Acad. Sci. USA,* **2015**, *112*(27), E3609-E3618.
http://dx.doi.org/10.1073/pnas.1507704112 PMID: 26100888

[34] Cabello, N.; Gandía, J.; Bertarelli, D.C.; Watanabe, M.; Lluís, C.; Franco, R.; Ferré, S.; Luján, R.; Ciruela, F. Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. *J. Neurochem.,* **2009**, *109*(5), 1497-1507.
http://dx.doi.org/10.1111/j.1471-4159.2009.06078.x PMID: 19344374

[35] Kamiya, T.; Saitoh, O.; Yoshioka, K.; Nakata, H. Oligomerization of adenosine A2A and dopamine D2 receptors in living cells. *Biochem. Biophys. Res. Commun.,* **2003**, *306*(2), 544-549.
http://dx.doi.org/10.1016/S0006-291X(03)00991-4 PMID: 12804599

[36] Ciruela, F.; Burgueño, J.; Casadó, V.; Canals, M.; Marcellino, D.; Goldberg, S.R.; Bader, M.; Fuxe, K.; Agnati, L.F.; Lluis, C.; Franco, R.; Ferré, S.; Woods, A.S. Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization. Direct epitope-epitope electrostatic interactions between adenosine A2A and dopamine D2 receptors. *Anal. Chem.,* **2004**, *76*(18), 5354-5363.
http://dx.doi.org/10.1021/ac049295f PMID: 15362892

[37] Fernández-Dueñas, V.; Gómez-Soler, M.; Jacobson, K.A.; Kumar, S.T.; Fuxe, K.; Borroto-Escuela, D.O.; Ciruela, F. Molecular determinants of A2AR-D2R allosterism: role of the intracellular loop 3 of the D2R. *J. Neurochem.,* **2012**, *123*(3), 373-384.
http://dx.doi.org/10.1111/j.1471-4159.2012.07956.x PMID: 22924752

[38] Borroto-Escuela, D.O.; Marcellino, D.; Narvaez, M.; Flajolet, M.; Heintz, N.; Agnati, L.; Ciruela, F.; Fuxe, K. A serine point mutation in the adenosine A2AR C-terminal tail reduces receptor heteromerization and allosteric modulation of the dopamine D2R. *Biochem. Biophys. Res. Commun.,* **2010**, *394*(1), 222-227.
http://dx.doi.org/10.1016/j.bbrc.2010.02.168 PMID: 20197060

[39] Morató, X.; Borroto-Escuela, D.O.; Fuxe, K.; Fernández-Dueñas, V.; Ciruela, F. Co-immunoprecipitation from brain. *Neuromethods,* **2016**, *110*, 19-30.
http://dx.doi.org/10.1007/978-1-4939-3064-7_2

[40] Aguado, C.; Luján, R. The histoblot technique: a reliable approach to analyse expression profile of proteins and to predict their molecular association. *Neuromethods,* **2019**, *144*, 65-88.
http://dx.doi.org/10.1007/978-1-4939-8985-0_6

[41] Aguado, C.; Orlandi, C.; Fajardo-Serrano, A.; Gil-Minguez, M.; Martemyanov, K.A.; Luján, R. Cellular and subcellular localization of the RGS7/Gβ5/R7BP complex in the cerebellar cortex. *Front. Neuroanat.,* **2016**, *10*, 114-130.
http://dx.doi.org/10.3389/fnana.2016.00114 PMID: 27965545

[42] Ciruela, F.; Fernández-Dueñas, V.; Sahlholm, K.; Fernández-Alacid, L.; Nicolau, J.C.; Watanabe, M.; Luján, R. Evidence for oligomerization between GABAB receptors and GIRK channels containing the GIRK1 and GIRK3 subunits. *Eur. J. Neurosci.,* **2010**, *32*(8), 1265-1277.
http://dx.doi.org/10.1111/j.1460-9568.2010.07356.x PMID: 20846323

[43] Fernández-Alacid, L.; Watanabe, M.; Molnár, E.; Wickman, K.; Luján, R. Developmental regulation of G protein-gated inwardly-rectifying K+ (GIRK/Kir3) channel subunits in the brain. *Eur. J. Neurosci.,* **2011**, *34*(11), 1724-1736.

http://dx.doi.org/10.1111/j.1460-9568.2011.07886.x PMID: 22098295

[44] Luján, R. *International Review of Neurobiology*; Academic Press Inc., **2015**, Vol. 123, pp. 161-200.

[45] Bonaventura, J.; Rico, A.J.; Moreno, E.; Sierra, S.; Sánchez, M.; Luquin, N.; Farré, D.; Müller, C.E.; Martínez-Pinilla, E.; Cortés, A.; Mallol, J.; Armentero, M-T.; Pinna, A.; Canela, E.I.; Lluís, C.; McCormick, P.J.; Lanciego, J.L.; Casadó, V.; Franco, R. L-DOPA-treatment in primates disrupts the expression of A(2A) adenosine-CB(1) cannabinoid-D(2) dopamine receptor heteromers in the caudate nucleus. *Neuropharmacology,* **2014**, *79*, 90-100. http://dx.doi.org/10.1016/j.neuropharm.2013.10.036 PMID: 24230991

[46] Zhu, Y.; Mészáros, J.; Walle, R.; Fan, R.; Sun, Z.; Dwork, A.J.; Trifilieff, P.; Javitch, J.A. Detecting G protein-coupled receptor complexes in postmortem human brain with proximity ligation assay and a Bayesian classifier. *Biotechniques,* **2020**, *68*(3), 122-129. http://dx.doi.org/10.2144/btn-2019-0083 PMID: 31859535

[47] Fernández-Dueñas, V.; Gómez-Soler, M.; Valle-León, M.; Watanabe, M.; Ferrer, I.; Ciruela, F. Revealing adenosine A_{2A}-dopamine D_2 receptor heteromers in Parkinson's disease post-mortem brain through a new alphascreen-based assay. *Int. J. Mol. Sci.,* **2019**, *20*(14), 3600-3611. http://dx.doi.org/10.3390/ijms20143600 PMID: 31340557

[48] Luján, R.; Watanabe, M. Post-embedding immunohistochemistry in the localisation of receptors and ion channels. *Neuromethods,* **2016**, *110*, 211-232. http://dx.doi.org/10.1007/978-1-4939-3064-7_16

[49] Luján, R. Pre-embedding methods for the localization of receptors and ion channels. *Neuromethods,* **2016**, *110*, 191-210. http://dx.doi.org/10.1007/978-1-4939-3064-7_15

[50] Harada, H.; Shigemoto, R. High-resolution localization of membrane proteins by SDS-digested freeze-fracture replica labeling (SDS-FRL). In: *Neuromethods,* **2016**, vol *110*, pp. 233-245.

[51] Ries, J.; Kaplan, C.; Platonova, E.; Eghlidi, H.; Ewers, H. A simple, versatile method for GFP-based super-resolution microscopy *via* nanobodies. *Nat. Methods,* **2012**, *9*(6), 582-584. http://dx.doi.org/10.1038/nmeth.1991 PMID: 22543348

[52] Fernández-Dueñas, V.; Taura, J.J.; Cottet, M.; Gómez-Soler, M.; López-Cano, M.; Ledent, C.; Watanabe, M.; Trinquet, E.; Pin, J-P.; Luján, R.; Durroux, T.; Ciruela, F. Untangling dopamine-adenosine receptor-receptor assembly in experimental parkinsonism in rats. *Dis. Model. Mech.,* **2015**, *8*(1), 57-63. http://dx.doi.org/10.1242/dmm.018143 PMID: 25398851

[53] Söderberg, O.; Gullberg, M.; Jarvius, M.; Ridderstråle, K.; Leuchowius, K-J.; Jarvius, J.; Wester, K.; Hydbring, P.; Bahram, F.; Larsson, L-G.; Landegren, U. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods,* **2006**, *3*(12), 995-1000. http://dx.doi.org/10.1038/nmeth947 PMID: 17072308

[54] Trifilieff, P.; Rives, M-L.; Urizar, E.; Piskorowski, R.A.; Vishwasrao, H.D.; Castrillon, J.; Schmauss, C.; Slättman, M.; Gullberg, M.; Javitch, J.A. Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques,* **2011**, *51*(2), 111-118. http://dx.doi.org/10.2144/000113719 PMID: 21806555

[55] Narváez, M.; Crespo-Ramírez, M.; Fores-Pons, R.; Pita-Rodríguez, M.; Ciruela, F.; Filip, M.; Beggiato, S.; Ferraro, L.; Tanganelli, S.; Ambrogini, P.; de la Mora, M.P.; Fuxe, K.; Borroto-Escuela, D.O. Study of GPCR homo- and heteroreceptor complexes in specific neuronal cell populations using the *in situ* proximity ligation assay. *Neuromethods,* **2021**, *169*, 117-134. http://dx.doi.org/10.1007/978-1-0716-1522-5_9

[56] Taura, J.; Fernández-Dueñas, V.; Ciruela, F. Visualizing G protein-coupled receptor-receptor interactions in brain using proximity ligation *in situ* assay. *Curr. Protoc. Cell Biol.,* **2015**, *67*, 17.1-17.17, 16. http://dx.doi.org/10.1002/0471143030.cb1717s67 PMID: 26061241

[57] Borroto-Escuela, D.O.; Narvaez, M.; Valladolid-Acebes, I.; Shumilov, K.; Di Palma, M.; Wydra, K.; Schaefer, T.; Reyes-Resina, I.; Navarro, G.; Mudó, G.; Filip, M.; Sartini, S.; Friedland, K.; Schellekens, H.; Beggiato, S.; Ferraro, L.; Tanganelli, S.; Franco, R.; Belluardo, N.; Ambrogini, P.; de la Mora, M.; Fuxe, K. Detection, analysis, and quantification of GPCR homo- and heteroreceptor complexes in specific neuronal cell populations using the *in situ* proximity ligation assay. *Neuromethods,* **2018**, *140*, 299-315. http://dx.doi.org/10.1007/978-1-4939-8576-0_19

[58] López-Cano, M.; Fernández-Dueñas, V.; Ciruela, F. Proximity ligation assay image analysis protocol: Addressing receptor-receptor interactions. *Methods Mol. Biol.,* **2019**, *2040*, 41-50. http://dx.doi.org/10.1007/978-1-4939-9686-5_3 PMID: 31432474

[59] Moreno-García, A.; Kun, A.; Calero, O.; Medina, M.; Calero, M. An overview of the role of lipofuscin in age-related neurodegeneration. *Front. Neurosci.,* **2018**, *12*, 464. http://dx.doi.org/10.3389/fnins.2018.00464 PMID: 30026686

[60] Cardullo, R.A. Theoretical principles and practical considerations for fluorescence resonance energy transfer microscopy. *Methods Cell Biol.,* **2007**, *81*, 479-494. http://dx.doi.org/10.1016/S0091-679X(06)81023-X PMID: 17519181

[61] Stryer, L.; Haugland, R.P. Energy transfer: a spectroscopic ruler. *Proc. Natl. Acad. Sci. USA,* **1967**, *58*(2), 719-726. http://dx.doi.org/10.1073/pnas.58.2.719 PMID: 5233469

[62] Stryer, L. Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.,* **1978**, *47*, 819-846. http://dx.doi.org/10.1146/annurev.bi.47.070178.004131 PMID: 354506

[63] Gandía, J.; Lluís, C.; Ferré, S.; Franco, R.; Ciruela, F.; Gandia, J.; Lluis, C.; Ferre, S.; Franco, R.; Ciruela, F. Light resonance energy transfer-based methods in the study of G protein-coupled receptor oligomerization. *BioEssays,* **2008**, *30*(1), 82-89. http://dx.doi.org/10.1002/bies.20682 PMID: 18081019

[64] Sheng, M.; Hoogenraad, C.C. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu. Rev. Biochem.,* **2007**, *76*, 823-847. http://dx.doi.org/10.1146/annurev.biochem.76.060805.160029 PMID: 17243894

[65] Ferrandon, S.; Feinstein, T.N.; Castro, M.; Wang, B.; Bouley, R.; Potts, J.T.; Gardella, T.J.; Vilardaga, J.P. Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat. Chem. Biol.,* **2009**, *5*(10), 734-742. http://dx.doi.org/10.1038/nchembio.206 PMID: 19701185

[66] Cottet, M.; Faklaris, O.; Maurel, D.; Scholler, P.; Doumazane, E.; Trinquet, E.; Pin, J.P.; Durroux, T. BRET and Time-resolved FRET strategy to study GPCR oligomerization: from cell lines toward native tissues. *Front. Endocrinol. (Lausanne),* **2012**, *3*, 92.

[67] Mathis, G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin. Chem.,* **1995**, *41*(9), 1391-1397. http://dx.doi.org/10.1093/clinchem/41.9.1391 PMID: 7656455

[68] Bazin, H.; Trinquet, E.; Mathis, G. Time resolved amplification of cryptate emission: a versatile technology to trace biomolecular interactions. *J. Biotechnol.,* **2002**, *82*(3), 233-250. PMID: 11999692

[69] Terrillon, S.; Durroux, T.; Mouillac, B.; Breit, A.; Ayoub, M.A.; Taulan, M.; Jockers, R.; Barberis, C.; Bouvier, M. Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. *Mol. Endocrinol.,* **2003**, *17*(4), 677-691. http://dx.doi.org/10.1210/me.2002-0222 PMID: 12554793

[70] Navarro, G.; Cordomí, A.; Casadó-Anguera, V.; Moreno, E.; Cai, N-S.; Cortés, A.; Canela, E.I.; Dessauer, C.W.; Casadó, V.; Pardo, L.; Lluís, C.; Ferré, S. Evidence for functional pre-coupled complexes of receptor heteromers and adenylyl cyclase. *Nat. Commun.,* **2018**, *9*(1), 1242. http://dx.doi.org/10.1038/s41467-018-03522-3 PMID: 29593213

[71] Albizu, L.; Cottet, M.; Kralikova, M.; Stoev, S.; Seyer, R.; Brabet, I.; Roux, T.; Bazin, H.; Bourrier, E.; Lamarque, L.; Breton, C.; Rives, M.L.; Newman, A.; Javitch, J.; Trinquet, E.; Manning, M.; Pin, J.P.; Mouillac, B.; Durroux, T. Time-resolved FRET between GPCR ligands reveals oligomers in native tissues. *Nat. Chem. Biol.,* **2010**, *6*(8), 587-594. http://dx.doi.org/10.1038/nchembio.396 PMID: 20622858

[72] Hide, I.; Padgett, W.L.; Jacobson, K.A.; Daly, J.W.A. A2A adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: characterization with radioligand binding and by activation of adenylate cyclase. *Mol. Pharmacol.,* **1992**, *41*(2), 352-359.

PMID: 1311411

[73] Fernández-Dueñas, V.; Durroux, T.; Ciruela, F. Receptor–Receptor Interactions in the Brain. *Neuromethods,* **2016**, *110*, 99-107. http://dx.doi.org/10.1007/978-1-4939-3064-7_8

[74] Valle-León, M.; Callado, L.F.; Aso, E.; Cajiao-Manrique, M.M.; Sahlholm, K.; López-Cano, M.; Soler, C.; Altafaj, X.; Watanabe, M.; Ferré, S.; Fernández-Dueñas, V.; Menchón, J.M.; Ciruela, F. Decreased striatal adenosine A_{2A}-dopamine D_2 receptor heteromerization in schizophrenia. *Neuropsychopharmacology,* **2021**, *46*(3), 665-672. http://dx.doi.org/10.1038/s41386-020-00872-9 PMID: 33010795

[75] Kawano, K.; Yano, Y.; Omae, K.; Matsuzaki, S.; Matsuzaki, K. Stoichiometric analysis of oligomerization of membrane proteins on living cells using coiled-coil labeling and spectral imaging. *Anal. Chem.,* **2013**, *85*(6), 3454-3461. http://dx.doi.org/10.1021/ac400177a PMID: 23427815

[76] Felce, J.H.; Davis, S.J.; Klenerman, D. Single-molecule analysis of G protein-coupled receptor stoichiometry: Approaches and limitations. *Trends Pharmacol. Sci.,* **2018**, *39*(2), 96-108.