Chapter 9

In Situ Proximity Ligation Assay to Study and Understand the Distribution and Balance of GPCR Homo- and Heteroreceptor Complexes in the Brain

Dasiel O. Borroto-Escuela, Beth Hagman, Miles Woolfenden, Luca Pinton, Antonio Jiménez-Beristain, Julia Oflijan, Manuel Narvaez, Michael Di Palma, Kristin Feltmann, Stfano Sartini, Patrizia Ambrogini, Francisco Ciruela, Riccardo Cuppini, and Kjell Fuxe

Abstract

The existence of homo- and heteroreceptor complexes with allosteric receptor-receptor interactions increases the diversity of receptor function including recognition, trafficking, and signaling. This phenomenon increases our understanding of how brain function is altered through molecular integration of receptor signals. An alteration in specific heteroreceptor complexes or their balance/equilibrium with the corresponding homoreceptors is considered to have a role in the pathogenic mechanisms that lead to mental and neurological diseases, including drug addiction, depression, Parkinson's disease, and schizophrenia. However, despite extensive experimental work supporting the formation of these receptor complexes in cellular models, their detection and visualization in the brain remained largely unknown until recent years, when a well-characterized in situ proximity ligation assay (in situ PLA) was adapted to validate the existence of GPCR homo- and heteroreceptor complexes in their native environment. In this chapter we will describe the in situ PLA procedure as a high selectivity and sensitivity assay to detect and characterize GPCR homo- and heteroreceptor complexes and their balance and distribution ex vivo in the brain by confocal laser microscopy. Herein, we outlined in detail the in situ PLA assay and how to use it in an optimal way on work with formalin-fixed free-floating rat brain sections.

Key words G protein-coupled receptors, Immunohistochemistry, In situ proximity ligation assay, Heteroreceptor complexes, Dimerization, Receptor-receptor interaction, Stoichiometry

1 Introduction

Nervous system-related diseases are highly complex in their etiology. It is not surprising that the underlying pathological processes are poorly understood and treatment possibilities are inadequate. One emerging concept is that direct physical interactions of different receptors named homo-/heteroreceptor complexes may be

Rafael Luján and Francisco Ciruela (eds.), *Receptor and Ion Channel Detection in the Brain: Methods and Protocols*, Neuromethods, vol. 110, DOI 10.1007/978-1-4939-3064-7_9, © Springer Science+Business Media New York 2016

involved with disease onset and progression. Thus, these receptor complexes could potentially serve as a biomarker and/or drug target of the disease [2–4]. Accordingly, the orthosteric targeting of heteroreceptor complexes with pharmacological agents (i.e., bivalent ligands) and the disruption of the receptor-receptor interface by means of interfering peptides may become new therapeutical strategies [5]. Several groups have contributed to the development of the concept of heteroreceptor complexes, in which receptors physically interact to produce either an integrated regulation of receptor tyrosine kinases [2, 5], ion channels [6], and/or an activation of intracellular signaling cascades generating changes in gene expression [7].

Recently, GPCR heteroreceptor complexes were rigorously collected, and their magnitude is represented in a global GPCR heterodimer network (GPCR-HetNet; www.gpcr-hetnet.com) [8]. The results from the GPCR-HetNet indicate a scale-free model in which some of the protomers dominate the connectivity and hold the network together. Using two different hub criteria revealed the following hubs in the network: the dopamine D2 receptor, the beta-2 adrenergic receptor, the growth hormone secretagogue receptor type 1, the mu-type opioid receptor, the secretin receptor, the delta-type opioid receptor, the adenosine A1 receptor, the adenosine A2A receptor, and the cannabinoid receptor 1. Other highly connected protomers were also identified. The emergence of potential allosteric mechanism avenues and higher-order heteroreceptor complexes are also described. Although, in this work, the homoreceptor complex information was excluded, the data highlighted that more than 87 % of the total numbers of protomers identified exist as homoreceptor complexes too [8].

These direct interactions involving GPCRs were demonstrated through diverse biophysical (FRET, BRET, BiFC) and biochemical or microscopy-based procedures (e.g., co-immunolocalization, co-immunoprecipitation, radioligand binding, co-internalization analysis) that assess receptor-receptor interactions in heteroreceptor complexes [1, 9–11]. Each methodology used has provided precise and valuable information which was considered with caution in view of the limitations of the techniques. Some controversy regarding some approaches also emerged [12, 13]. Nevertheless, when these methods are properly assessed, it is possible to safely demonstrate the direct interactions between GPCRs as well as their dynamics [14, 15]. Novel technologies have also been developed, such as real-time FRET experiments in living cells [16] and dual-color fluorescence cross-correlation spectroscopy [17]. However, despite the extensive experimental results obtained with these biophysical/biochemical techniques, supporting the formation of GPCR homo- and heteroreceptor complexes in heterologous systems, the existence of such receptor complexes in their native environment (brain tissue) was not confirmed until recent

years. Thus, a well-characterized in situ proximity ligation assay (in situ PLA) was adapted to validate the existence of GPCR complexes in cellular models first [18] and then in brain sections ex vivo [2, 5, 7, 19–23].

It should be highlighted that until now, most experimental work was focused on the study of receptor-receptor interactions in GPCRs as a phenomenon of binary interactions. In other words, it was only focused on the understanding of face-to-face protomer-protomer interactions and their allosteric receptor-receptor modulation and functional specificities. However, the phenomenon of GPCR oligomerization is a more complex and dynamic process, which involves many more parameters than the classical analysis of the interactions of the two protomers involved [24]. One emerging concept is that direct physical receptor-receptor interactions in heteroreceptor complexes or altered balance with their homoreceptor complex populations can contribute to brain disease progression inter alia of Parkinson's disease [3, 4, 25], depression [5, 26], schizophrenia [21], Alzheimer's, and addiction. When discussing the role of GPCR heteroreceptor complexes, it is of substantial interest to understand the balance/equilibrium between the corresponding homo- and heteroreceptor complexes in the plasma membrane of the cell (Fig. 1). Consider a disruption or shift of this balance as a mechanism of disease development could be important in the discovery of pharmacological targets. We have suggested that the introduction of in situ PLA (Fig. 1) in combination with Western blot, radioligand-binding experiments, and co-immunoprecipitation could open a new window to the understanding of the importance of the balance of the corresponding homo- and heteroreceptor complexes [19].

In this chapter, we will describe the in situ PLA procedure as a high selectivity and sensitivity assay to study and characterize the balance and distribution of GPCR homo- and heteroreceptor complexes ex vivo in the brain using confocal laser microscopy, including a detailed description of how to perform the assay in an optimal way. At the end of the chapter, we will discuss the advantages and disadvantages of this method compared to other available techniques.

2 The In Situ PLA: Principle of the Assay

The in situ PLA is a method that combines the dual recognition of a probe-targeted assay with a split-reporter approach, creating a selective and sensitive method for specific detection of two receptors in close proximity forming a homo- or heteroreceptor complex [27]. In this method a pair of antibodies, in which complementary oligonucleotides have been attached, is used to target the receptor protomers in the receptor complex of interest.

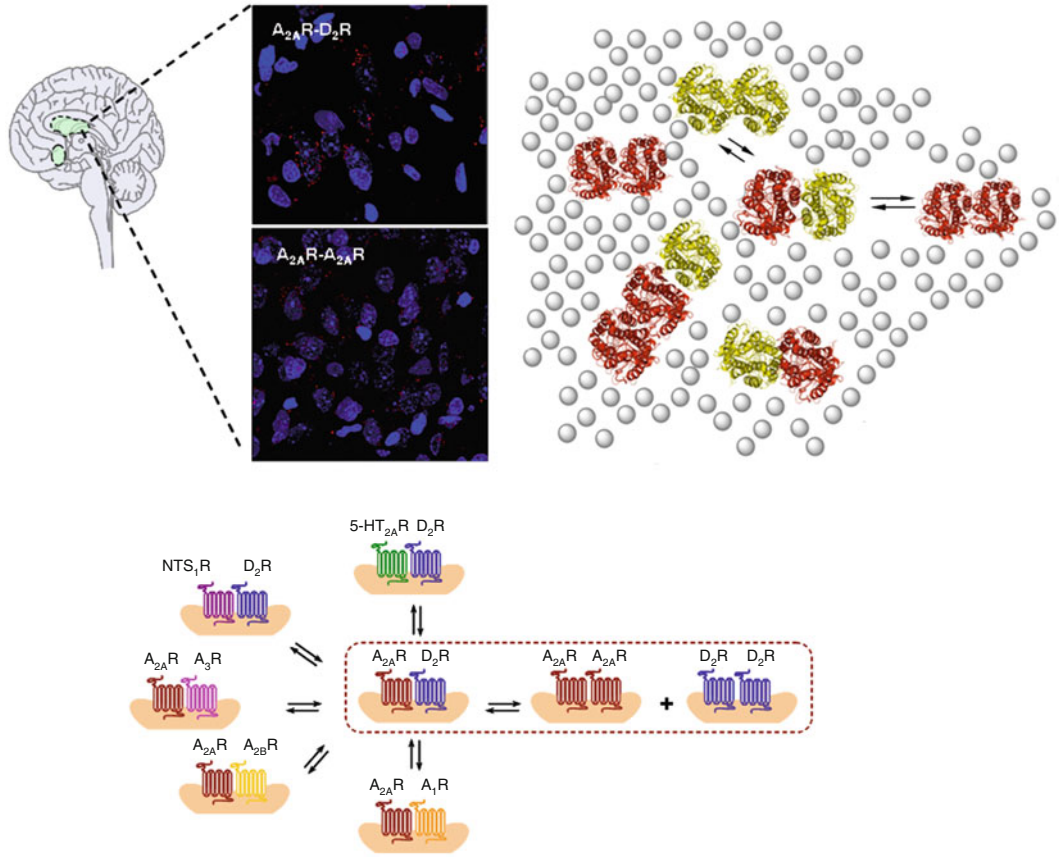

Fig. 1 On the understanding of the balance/equilibrium between GPCR homo- and heteroreceptor complexes in cell populations in the brain. (*Top right*) Schematic representation of the balance between different populations of A2AR-D2R heteroreceptor complexes and their corresponding homoreceptor complexes in a portion of a neural membrane (D2R and A2A homoreceptor complexes are indicated in *red* and *yellow* colour respectively). (*Top left*) The panel represents an example of in situ PLA experiment performed in striatal free-floating sections using primary antibodies of different species directed to adenosine A2A and dopamine D2 receptors. The detected A2A-A2A homoreceptor complexes and A2A-D2R heteroreceptor complexes are seen as *red* clusters (blobs, puncta) in each panel. (*Bottom*) Understanding the complexity of the balance between A2AR-D2R and its homoreceptor complexes, iso A2AR- and iso D2R-heteroreceptor complexes, and other A2A- and D2R-heteroreceptor complexes. Thus, it is clear that the stoichiometry and topology of the complexes is unknown (e.g., A2AR-D2R = A2AR-A2AR + D2R-D2R + A2AR-A2AR-D2R + A2AR-D2R-D2R + A2AR-A2AR-D2R-D2R + A2AR-A2AR-A2AR-D2R-D2R)

Only when the receptor protomers are in close proximity, i.e., interacting in a receptor complex, are the oligonucleotides attached to the antibody able to join together. After hybridization of both proximity probes, enzymatic ligation follows and a circular DNA molecule is formed. This DNA circle strand is a surrogate marker for the detected complexes and serves as a template for amplification (rolling circle amplification (RCA)) using a highly efficient polymerase (e.g., phi29 DNA polymerase). The long single-concatemeric-stranded rolling circle product (RCP), attached to one

of the proximity probes, is a result of the first round amplification, displacing the newly created strand. The RCP is linked to the proximity probe and, thereby, stays attached to one of the two protomer of the receptor complex, allowing to reveal the location of the receptor complex [28]. The RCPs are detected and visualized by hybridization with fluorescent-labeled oligonucleotides complementary to the repeated sequences encoded in the RCPs, which renders them visible for fluorescence microscopy (Fig. 1).

3 Materials

3.1 Buffers

1. Hoffman solution (cryoprotection for free-floating brain sections): 250 mL 0.4 M PBS, ethylene glycol 300 mL, 300 g sucrose, 10 g polyvinylpyrrolidone, and 9 g NaCl. Add high purity water to 1000 mL. Keep the resulting solution in a freezer (cold storage: –20 °C).
2. Phosphate-buffered saline (PBS). PBS is prepared by mixing 0.23 g NaH_2PO_4 (anhydrous; 1.90 mM), 1.15 g Na_2HPO_4 (anhydrous; 8.10 mM), and 9.00 g NaCl (154 mM). Then, add H_2O to 900 mL and, if needed, adjust to desired pH (7.2–7.4) with 1 M NaOH or 1 M HCl. Finally, add H_2O to 1 L, filter, sterilize, and store indefinitely at 4 °C. PBS could also be prepared at a 10× concentration (commercially available at Sigma-Aldrich (Cat. No: P5493-1L)) and stored until dilution into the working solution.
3. Glycine buffer (10 mM): dissolve 0.75 g glycine in 100 mL PBS. Store at 4 °C.
4. Permeabilization buffer: 0.1 % Triton X-100 in PBS (e.g., 0.1 mL Triton X-100 in 100 mL PBS). Store at 4 °C.
5. Blocking solution: Prepare the blocking solution by preparing 0.2 % BSA in PBS (e.g., 0.2 g in 100 mL PBS, stored at 4 °C). Adjust the amount of reagents accordingly so that the total volume is kept at 400 μL × well (12-well plate). Prepare this solution fresh. This blocking solution can be replaced by, e.g., the Duolink blocking solution (Sigma-Aldrich) or the Odyssey blocking buffer (Licor Biosciences). However, choose the blocking agent best suited for the antibodies used. If animal serum is used to replace the BSA, for example, the use of 5 % sterile-filtered goat serum, make sure that it is sterile filtered, as unfiltered serum may increase the amount of background signals.
6. Primary antibodies and proximity probes (oligonucleotide-labeled secondary antibodies), incubation buffer. We strongly recommend to dilute the antibodies in the blocking solution to be used (see above 5).

7. Ligation buffer: 10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, and pH 7.5 adjusted with HCl. Stored at −20 °C [29].
8. Hybridization-ligation solution: BSA (250 g/mL), T4 DNA ligase (final concentration of 0.05 U/μL), Tween-20 (0.05 %), NaCl 250 mM, ATP 1 mM, and the circularization or connector oligonucleotides (125–250 nM). Circularization or connector oligonucleotides can be designed and synthesized as described previously [29]. Before usage, vortex briefly to mix the ligase with the solution. Alternatively, the ligation buffer and the hybridization-ligation solution can be ordered from Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008).
9. Washing buffer A: 8.8 g NaCl, 1.2 g Tris base, and 0.5 mL Tween-20. Dissolve in 800 mL high purity water and adjust the pH to 7.4 using HCl. Adjust with high purity water to 1000 mL and filter through a 0.22 μm filter. Store at 4 °C.
10. Amplification solution: Instead of preparing the solutions described below [11–13], one amplification solution can be purchased (Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008)) and used.
11. Rolling circle amplification (RCA) buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and pH 7.5 adjusted with HCl. Stored at −20 °C.
12. RCA solution (final concentration: phi-29 polymerase 0.125–0.200 U/μL, BSA (250 g/mL), 1× RCA buffer, Tween-20 (0.05 %), and dNTP (250 M for each)).
13. Detection solution (final concentration: BSA (250 g/mL), 2 μL sodium citrate 20X (A 20X stock solution consists of 3 M sodium chloride and 300 mM trisodium citrate (adjusted to pH 7.0 with HCl)), 4 μL Tween-20 (0.5 %), and the fluorescence detection by fluorophores (e.g., Texas Red or Alexa 555)-oligonucleotide strand (5 μM) (*see* [29])
14. Washing buffer B: 5.84 g NaCl, 4.24 g Tris base, and 26.0 g Tris-HCl. Dissolve in 500 mL high purity water and adjust the pH to 7.5 using HCl. Then add again high purity water to 1000 mL. Filter through a 0.22 μm filter. Store at 4 °C.
15. Mounting medium (e.g., VectaShield, Vector Labs)

3.2 Brain Tissue Preparation

For the analysis of GPCR homo- and heteroreceptor complexes in the rat brain, we highly recommend the use of formaldehyde-fixed frozen free-floating sections, whose production is described in detail in the following (**Note 1**). First, animals are deeply anesthetized by an intraperitoneal (i.p.) injection of a high dose of pentobarbital (60 mg/mL, [0.1 mL/100 g]) and then perfused intracardially with 30–50 mL ice-cold 4 % paraformaldehyde (PFA)

in 0.1 M phosphate-buffered saline (PBS, pH 7.4) solution. After perfusion, brains are collected and transferred into well-labeled glass vials filled with 4 % PFA fixative solution for 6–12 h. Then, the brains are placed in 10 and 30 % sucrose (0.1 M PBS, pH 7.4) and incubated for 1 day (10 % sucrose) and a number of days (30 % sucrose) at 4 °C with several sucrose buffer changes, until freezing the brain (in a bowl with dry ice: put inside a beaker with isopentane; when the isopentane reaches −45 °C, enter the mold with tissue; once frozen store at −80 °C). Proceed to generate the tissue sections (10–30 μm thick) using a cryostat (stored tissue at −20 °C on the day before cutting). After cutting, store them in Hoffman solution (e.g., in a 24-well plate). Alternatively, to the use of fixed free-floating sections, you can use fixed frozen sections attached to microscopy slides. The mounted sections on slides must be kept at −20 °C until use.

3.3 Proximity Probes: Conjugation of Oligonucleotides to Antibodies

Proximity probes are created through the attachment of oligonucleotides to antibodies. The antibodies are functionalized by either direct covalent coupling of an oligonucleotide [29] or non-covalent coupling by incubating biotinylated antibodies with a streptavidin-modified oligonucleotide [30]. The oligonucleotide component of the proximity probes can be functionalized to either primary antibodies or secondary antibodies. The latter approach avoids the need to conjugate the oligonucleotide components to each primary antibody protomer pair, saving time and costs. Mainly three types of chemical methods can be used for the conjugation of oligonucleotides to antibodies: the maleimide/NHS-esther chemistry (SMCC) [31], the succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH) [32], or the commercially available Antibody-Oligonucleotide All-in-One Conjugation Kit from Solulink company (http://www.solulink.com/). It is based on two complementary hetero-bifunctional linkers (Sulfo-S-4FB (formylbenzamide) and S-HyNic (hydrazino-nicotinamide)). The act of conjugation can severely affect the ability of some antibodies to bind an antigen. For this reason, different antibodies, conjugation chemistries, and reaction conditions to obtain suitable proximity probes must be tested prior to any experiments. Alternatively, antigen-validated, proximity probes from specialized companies like Duolink (Uppsala, Sweden; http://www.olink.com/) or Sigma-Aldrich can be bought directly.

4 Assay Protocol

1. First, wash the fixed free-floating sections (storage at −20 °C in Hoffman solution) four times with PBS.
2. Quench your brain slices with 10 mM glycine buffer, for 20 min at room temperature (**Note 2**).

3. Wash twice, for 5 min each, with PBS at room temperature.
4. Then incubate with the permeabilization buffer (10 % fetal bovine serum (FBS) and 0.5 % Triton X-100 or Tween-20 in Tris buffer saline (TBS), pH 7.4) for 30 min at room temperature.
5. Wash twice, for 5 min each, with PBS at room temperature.
6. Then incubate with the blocking buffer (0.2 % BSA in PBS) for 30 min at room temperature (**Note 3**). It should be checked regularly that the reaction does not dry out since this will cause a high background signal.
7. Turn on the incubator and preheat the humidity chamber until usage.
8. Incubate the brain sections with the primary antibodies diluted in a suitable concentration in the blocking solution for 1–2 h at 37 °C or at 4 °C overnight (**Note 4**).
9. Wash twice, for 5 min each, with the blocking solution at room temperature under gentle agitation to remove the excess of primary antibodies.
10. In the mean time, if primary antibodies are used in combination with secondary proximity probes, dilute the secondary antibodies proximity probes to a suitable concentration in the blocking solution. It is important to use the same buffer as those for the primary antibody to avoid background staining. Apply the proximity probe mixture to the sample and incubate for 1 h at 37 °C in a humidity chamber. Do not allow the samples to dry as this will also cause artifacts.
11. To remove the unbound proximity probes, wash the slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation.
12. Prepare the hybridization-ligation solution. To ensure optimal conditions for the enzymatic reactions, the sections should be soaked for 1 min in ligation buffer (10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.5 [29]), prior to addition of the hybridization-ligation solution (final concentration: BSA (250 g/mL), T4 DNA ligase buffer, Tween-20 (0.05 %), NaCl 250 mM, ATP 1 mM, and the circularization or connector oligonucleotides 125–250 nM). Circularization or connector oligonucleotides can be designed and synthesized as described previously ([29]). Remove the soaking solution (ligation buffer), and add T4 DNA ligase at a final concentration of 0.05 U/μL to the hybridization-ligation solution. Vortex briefly to mix the ligase with the solution. Apply the mixture immediately to the sections and incubate slides in a humidity chamber at 37 °C for 30 min. Alternatively, the ligation buffer and the hybridization-ligation solution can

be ordered from Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008).

13. To remove the excess of connector oligonucleotides, wash twice, for 5 min each, with the washing buffer A at room temperature under gentle agitation. Tap off all excess washing solution.
14. Prepare the rolling circle amplification mixture. Soak the sections in RCA buffer for 1 min. Remove the soaking solution, and add the RCA solution (final concentration: phi-29 polymerase 0.125–0.200 U/μL, BSA (250 g/mL), RCA buffer, Tween-20 (0.05 %), and dNTP (250 M for each)). Vortex briefly the RCA solution and incubate in a humidity chamber for 100 min at 37 °C. Prepare the detection solution and incubate the sections in a humidity chamber for 30 min at 37 °C. Keep the detection solution in the dark to prevent fluorophore bleaching. From now on, all reactions and washing steps should be performed in the dark. Alternatively to preparing these buffers and solutions by yourself, the amplification mixture and the detection solution can be ordered from Olink Bioscience or Sigma-Aldrich (Cat No. DUO92008).
15. Wash the sections twice in the dark, for 10 min each, with the washing buffer B at room temperature under gentle agitation.
16. Dip the sections in a washing buffer B dilution of 1:10 and let sections dry at room temperature in the dark.
17. The free-floating sections are put on a microscope slide and a drop of appropriate mounting medium (e.g., VectaShield or Dako) is applied. The cover slip is placed on the section and sealed with nail polish. The sections should be protected against light and can be stored for several days at 4 °C or for several months/years at −20 °C (**Note 5**).

5 Quantitative PLA Image Analysis

1. Visualize the sections with fluorescence microscopy equipped with excitation/emission filters compatible with the fluorophores used. The in situ PLA signals have a very characteristic bright sub-micrometer-sized puncta appearance that is easily recognized and distinguishable from potential background fluorescence (*see* Fig. 2). While moving the focus through the sample tissue, appearance and disappearance of PLA signals should be noticeable. Up to a certain density of PLA signals, they appear as discrete dots (puncta, blobs) that can be easily counted/quantified using image analysis software.
2. Analyze the captured images using image techniques to quantify the number of dots. Many commercially image analysis

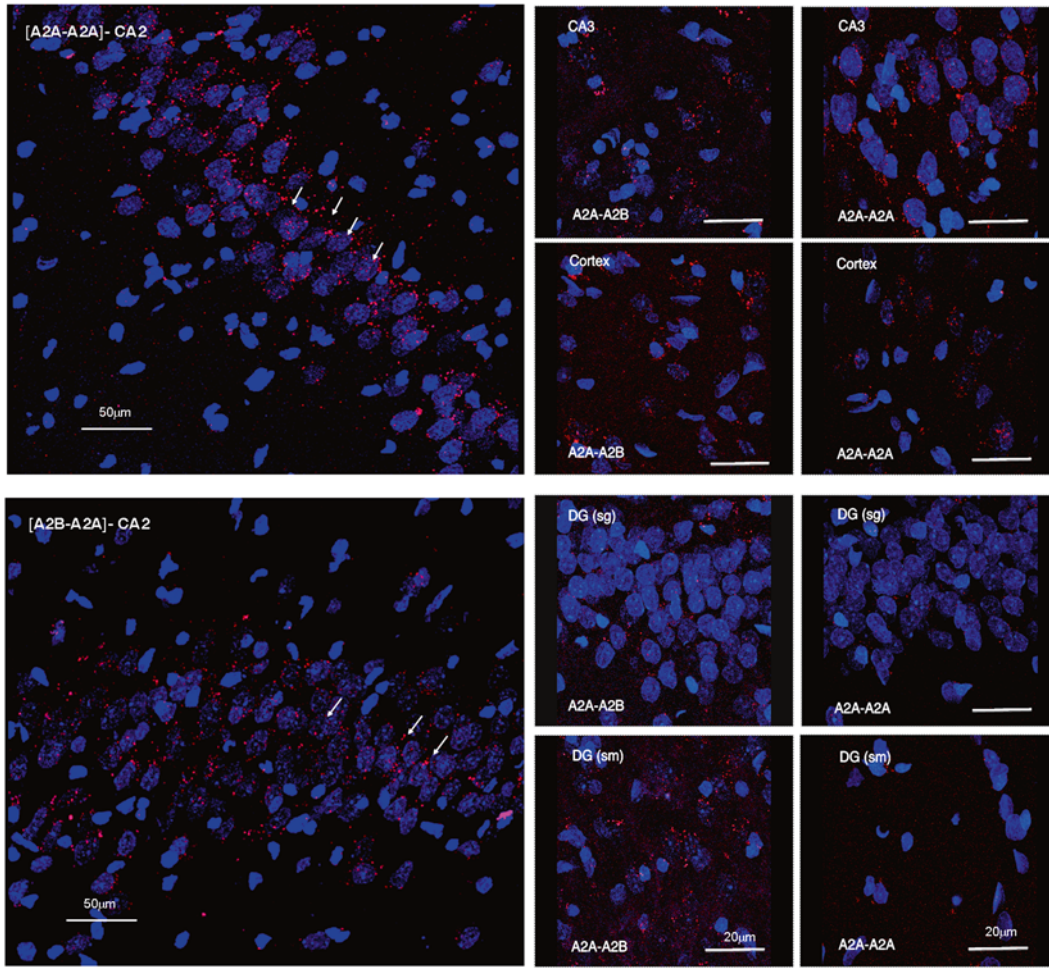

Fig. 2 Examples of in situ PLA assay in the hippocampus formation and cerebral cortex. (*Upper-left panel*) High densities of PLA-positive profiles (*red*) are shown in the pyramidal cell layer CA2 representing the A2A-A2A homoreceptor complexes. (*Lower-left panel*) Medium densities of the PLA-positive blobs are shown in the pyramidal cell layer of the CA2 representing the A2B-A2A isoreceptor complexes. In the two images, *arrows* point to examples of PLA-positive blobs. In the *right panels*, examples of PLA-positive blobs representing A2A-A2B isoreceptor complexes and A2A-A2A homoreceptor complexes are shown from the same regions of the hippocampus formation and the cerebral cortex. There presences in the CA3 regions and in the granular and molecular layers of the dentate gyros are illustrated. The scale bar are shown in the *lower-right panels* (20 µm)

software packages can be used (e.g., Duolink ImageTool (Sigma-Aldrich)), but also free software packages are available (e.g., BlobFinder, CellProfiler).

3. Usually four important parameters should be kept in mind for a proper analysis and result interpretation: (1) the number of DAPI nuclei in the sample field, (2) the number of positive PLA/dots per sample field, (3) the total number of positive

PLA cells/nuclei per sample field, and (4) the diameter sizes of the individual PLA blobs (the diameter may indicate if aggregates (higher order) of receptor complexes exists). Within these four values, it will be possible to get an overall view of the expression/enrichment of GPCR heteroreceptor complexes in the different brain areas analyzed and extract relevant conclusions from the comparisons between brain areas (Fig. 2). We have proposed the molecular phenomenon of receptor-receptor interactions as a fruitful way to understand how brain function can increase through molecular integration of signals. An alteration in specific receptor-receptor interactions or their balance/equilibrium (with the corresponding monomers-homomers) is indeed considered to have a role in the pathogenic mechanisms that lead to various brain diseases. Therefore, targeting protomer-protomer interactions in heteroreceptor complexes or changing the balance with their corresponding homoreceptor complexes in discrete brain regions may become an important field for developing novel drugs, including hetero-bivalent drugs and optimal types of combined treatments. The analysis of animal or human brain material with in situ PLA can reveal if the relative abundance of specific homo- and heteroreceptor complexes in discrete brain regions is altered in brain diseases or under certain drug treatments, for instance, chronic L-dopa treatment in Parkinson's disease [25]. In this analysis, it is important also to determine the ratio between heteroreceptor complex populations versus total number of the two participating protomer populations, using in addition to Western blots, receptor autoradiography, and biochemical binding methods. The two latter methods show the densities and affinities of the two functional receptor populations. The relationship between these parameters will help to normalize the heteroreceptor complexes values for comparison between groups in addition to evaluating the potential changes in the total number of the two protomer populations [19]. Certainly, we cannot compare or determine directly a balance between the homo- and heteroreceptor complexes populations in the same tissue using the in situ PLA approach, because of a technical limitation of the procedure itself. But the method could help us determine each population independently and compare their relative expression levels after an appropriate numerical analysis. Furthermore, increasing importance will be to determine the agonist/antagonist regulation of these homo-/heteroreceptor complexes in order to understand their potential roles as drug targets or as markers of brain disease progression.

6 Advantages and Disadvantages of the PLA Method

In situ PLA can offer advantages by:

1. Giving the opportunity to study the existence of any potential homo- and heteroreceptor complexes, for which a pair of suitable antibodies is available without the need of employing a genetic constructs.
2. The method could be performed in both cells and tissue samples, including human specimens collected from biobanks.
3. The in situ PLA could be useful to monitor the effects of different compounds like agonists and antagonists or their combined treatment on the balance of homo- and heteroreceptor complexes in cells and tissue.
4. The information obtained by the in situ PLA is at the resolution of individual cells or even of subcellular compartments providing profound insights into cellular heterogeneity in tissues.
5. The method also provides an enhanced sensitivity and selectivity compared to many other methods since powerful rolling circle amplification and dual target recognition are used.

As with any method, there are limitations, for instance:

1. The in situ PLA cannot be used in live cells, as fixation it is a prerequisite for the cell/tissue material employed.
2. When studying receptor-receptor interactions, it is important to remember that the method, like many other methods for studying protein-protein interactions, can show that two proteins are in close proximity and, therefore, likely directly interact. Proteins can also interact indirectly through an adapter protein. The maximal distance between two epitopes to give rise to a signal with in situ PLA is 10–30 nm with direct-conjugated proximity probes and slightly longer when secondary proximity probes are used. This distance will be dependent on the size of the receptors/antibodies and the respective length of the attached oligonucleotides. By changing the length of the oligonucleotides, the maximal distance limits can be reduced or increased. However, in general, the functional distance is usually close to the one detected in a FRET assay [27].
3. Another critical parameters for achieving good results is the use of excellent antibodies. Importantly, the antibodies have to be used under optimal conditions taking into consideration parameters such as antibody concentration, epitopes targeted by the antibodies, fixation, antigen retrieval, blocking conditions, etc.

4. A range of controls, both positive and negative ones, should be used to guarantee the specificity of the PLA signal. Positive controls can include cells where the protein is known to be expressed, such as in certain cells or tissues or in cells transfected to express the protein. Negative controls include cells or tissues that do not express the protein or where the protein has been knocked out or downregulated by, e.g., siRNA.

7 Notes

1. As for all antibody-based staining methods, the samples should be sufficiently pretreated to fit the primary antibodies with respect to fixation, permeabilization, and antigen retrieval of the tissue to be investigated. Similar conditions as employed for immunohistochemistry can be used for in situ PLA procedures. For in situ PLA, the common options are fixed paraffin-embedded or cryostat sections. The choice of section is determined by a number of conditions, including the time and skill of the investigator. However, careful consideration of the fixation protocol is especially necessary to ensure the optimal preservation of the morphology of the specimen and target antigen (receptors).
2. Autofluorescence can be brought on by certain endogenous tissue constituents, e.g., fibronectin, lipofuscin, and elastin, and by fixation in aldehydes. Based on this phenomenon, after rehydrating your formalin-fixed frozen sections and before blocking the slices, it is important to incubate them in 10–100 mM glycine PBS, pH 7.4, for 20 min. Glycine solution has the ability to quench autofluorescence caused by reactive groups (free aldehydes) in your cell or tissue-fixed samples. Alternatively to glycine solution, we can employ as well a solution of 50 mM ammonium chloride in PBS for 10 min. or a solution of 1 % sodium borohydride in PBS for 10–20 min. Sodium borohydride is flammable on contact with water and harmful by ingestion or inhalation. Therefore, take adequate precautions. It varies from sample to sample which method works the best, but in formalin-fixed rat brain sections, we had most success with the glycine solution methods. Another measure to avoid or reduce background in your samples is reducing the section thickness, as the intensity of autofluorescence depends on this parameter.
3. To reduce the likelihood of unspecific binding of the antibodies to the tissue, the tissue needs to be blocked by a blocking agent, such as bovine serum albumin (BSA) (by adding 1 μL BSA (10 mg/mL) and 1 μL sonicated salmon sperm DNA (0.1 mg/mL) to 38 μL 0.5 Triton X-100 or Tween-20 in TBS, pH 7.4 [32]). An animal serum like 10 % FBS can be used, but

make sure that it is sterile filtered, as unfiltered serum may increase the amount of background signals. Use the blocking agent best suited for the antibodies used.

4. The conditions for incubation with the primary/secondary antibodies (probes) should be chosen according to the manufacturer's recommendations, or be identified by the users.
5. The intensity of the rolling circle product will go down quickly in some mounting media. It is necessary to use a mounting medium containing anti-fade reagents.

Acknowledgments

This work has been supported by the Karolinska Institutets Forskningsstiftelser 2014/2015 to D.O.B-E and by the Swedish Medical Research Council (62X-00715-50-3) and AFA Försäkring (130328) to KF and D.O.B-E. D.O.B-E belongs to Academia de Biólogos Cubanos.

References

1. Achour L, Kamal M, Jockers R, Marullo S (2011) Using quantitative BRET to assess G protein-coupled receptor homo- and heterodimerization. Methods Mol Biol 756:183–200
2. Borroto-Escuela DO, Narvaez M, Perez-Alea M, Tarakanov AO, Jimenez-Beristain A, Mudo G, Agnati LF, Ciruela F, Belluardo N, Fuxe K (2015) Evidence for the existence of FGFR1-5-HT1A heteroreceptor complexes in the midbrain raphe 5-HT system. Biochem Biophys Res Commun 456(1):489–493
3. Fuxe K, Guidolin D, Agnati LF, Borroto-Escuela DO (2015) Dopamine heteroreceptor complexes as therapeutic targets in Parkinson's disease. Expert Opin Ther Targets 19(3): 377–398
4. Fuxe K, Borroto-Escuela D, Fisone G, Agnati LF, Tanganelli S (2014) Understanding the role of heteroreceptor complexes in the central nervous system. Curr Protein Pept Sci 15(7):647
5. Borroto-Escuela DO, Romero-Fernandez W, Mudo G, Perez-Alea M, Ciruela F, Tarakanov AO, Narvaez M, Di Liberto V, Agnati LF, Belluardo N, Fuxe K (2012) Fibroblast growth factor receptor 1–5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity. Biol Psychiatry 71(1):84–91
6. Nai Q, Li S, Wang SH, Liu J, Lee FJ, Frankland PW, Liu F (2009) Uncoupling the D1-N-methyl-D-aspartate (NMDA) receptor complex promotes NMDA-dependent long-term potentiation and working memory. Biol Psychiatry 67(3):246–254
7. Narvaez M, Millon C, Borroto-Escuela D, Flores-Burgess A, Santin L, Parrado C, Gago B, Puigcerver A, Fuxe K, Narvaez JA, Diaz-Cabiale Z (2015) Galanin receptor 2-neuropeptide Y Y1 receptor interactions in the amygdala lead to increased anxiolytic actions. Brain Struct Funct 220(4):2289–2301
8. Borroto-Escuela DO, Brito I, Romero-Fernandez W, Di Palma M, Oflijan J, Skieterska K, Duchou J, Van Craenenbroeck K, Suarez-Boomgaard D, Rivera A, Guidolin D, Agnati LF, Fuxe K (2014) The G protein-coupled receptor heterodimer network (GPCR-HetNet) and its hub components. Int J Mol Sci 15(5):8570–8590
9. Borroto-Escuela DO, Flajolet M, Agnati LF, Greengard P, Fuxe K (2013) Bioluminescence resonance energy transfer methods to study G protein-coupled receptor-receptor tyrosine kinase heteroreceptor complexes. Methods Cell Biol 117:141–164
10. Fernandez-Duenas V, Llorente J, Gandia J, Borroto-Escuela DO, Agnati LF, Tasca CI, Fuxe K, Ciruela F (2012) Fluorescence resonance energy transfer-based technologies in the study of protein-protein interactions at the cell surface. Methods 57(4):467–472
11. Skieterska K, Duchou J, Lintermans B, Van Craenenbroeck K (2013) Detection of G protein-coupled receptor (GPCR) dimerization

by coimmunoprecipitation. Methods Cell Biol 117:323–340

12. James JR, Oliveira MI, Carmo AM, Iaboni A, Davis SJ (2006) A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. Nat Methods 3(12):1001–1006
13. Bouvier M, Heveker N, Jockers R, Marullo S, Milligan G (2007) BRET analysis of GPCR oligomerization: newer does not mean better. Nat Methods 4(1):3–4; author reply 4
14. Marullo S, Bouvier M (2007) Resonance energy transfer approaches in molecular pharmacology and beyond. Trends Pharmacol Sci 28(8):362–365
15. Audet M, Lagace M, Silversides DW, Bouvier M (2010) Protein-protein interactions monitored in cells from transgenic mice using bioluminescence resonance energy transfer. FASEB J Off Publ Fed Am Soc Exp Biol 24(8): 2829–2838
16. Fernandez-Duenas V, Gomez-Soler M, Jacobson KA, Santhosh Kumar T, Fuxe K, Borroto-Escuela DO, Ciruela F (2012) Molecular determinants of a(2a) r-d(2) r allosterism: role of the intracellular loop 3 of the d(2) r. J Neurochem 123(3):373–384
17. 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(4):630–642
18. Borroto-Escuela DO, Van Craenenbroeck K, Romero-Fernandez W, Guidolin D, Woods AS, Rivera A, Haegeman G, Agnati LF, Tarakanov AO, Fuxe K (2010) Dopamine D2 and D4 receptor heteromerization and its allosteric receptor-receptor interactions. Biochem Biophys Res Commun 404(4):928–934
19. Borroto-Escuela DO, Romero-Fernandez W, Garriga P, Ciruela F, Narvaez M, Tarakanov AO, Palkovits M, Agnati LF, Fuxe K (2013) G protein-coupled receptor heterodimerization in the brain. Methods Enzymol 521: 281–294
20. Trifilieff P, Rives ML, Urizar E, Piskorowski RA, Vishwasrao HD, Castrillon J, Schmauss C, Slattman M, Gullberg M, Javitch JA (2011) Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. Biotechniques 51(2):111–118
21. Borroto-Escuela DO, Romero-Fernandez W, Narvaez M, Oflijan J, Agnati LF, Fuxe K (2014) Hallucinogenic 5-HT2AR agonists LSD and DOI enhance dopamine D2R protomer recognition and signaling of D2-5-HT2A heteroreceptor complexes. Biochem Biophys Res Commun 443(1):278–284
22. Borroto-Escuela DO, Narvaez M, Di Palma M, Calvo F, Rodriguez D, Millon C, Carlsson J, Agnati LF, Garriga P, Diaz-Cabiale Z, Fuxe K (2014) Preferential activation by galanin 1-15 fragment of the GalR1 protomer of a GalR1-GalR2 heteroreceptor complex. Biochem Biophys Res Commun 452(3): 347–353
23. Romero-Fernandez W, Borroto-Escuela DO, Agnati LF, Fuxe K (2013) Evidence for the existence of dopamine D2-oxytocin receptor heteromers in the ventral and dorsal striatum with facilitatory receptor-receptor interactions. Mol Psychiatry 18(8):849–850
24. Borroto-Escuela DO, Corrales F, Narvaez M, Oflijan J, Agnati LF, Palkovits M, Fuxe K (2013) Dynamic modulation of FGFR1-5-HT1A heteroreceptor complexes. Agonist treatment enhances participation of FGFR1 and 5-HT1A homodimers and recruitment of beta-arrestin2. Biochem Biophys Res Commun 441(2):387–392
25. Antonelli T, Fuxe K, Agnati L, Mazzoni E, Tanganelli S, Tomasini MC, Ferraro L (2006) Experimental studies and theoretical aspects on A2A/D2 receptor interactions in a model of Parkinson's disease. Relevance for L-dopa induced dyskinesias. J Neurol Sci 248(1–2): 16–22
26. Millón C, Flores-Burgess A, Narváez M, Borroto-Escuela DO, Santín L, Parrado C, Narváez JA, Fuxe K, Díaz-Cabiale Z. A role for galanin N-terminal fragment (1-15) in anxiety- and depression-related behaviors in rats. Int J Neuropsychopharmacol. 2014 Oct 31;18(3). pii: pyu064. doi: 10.1093/ijnp/pyu064.
27. Weibrecht I, Leuchowius KJ, Clausson CM, Conze T, Jarvius M, Howell WM, Kamali-Moghaddam M, Soderberg O (2010) Proximity ligation assays: a recent addition to the proteomics toolbox. Expert Rev Proteomics 7(3):401–409
28. Soderberg O, Leuchowius KJ, Kamali-Moghaddam M, Jarvius M, Gustafsdottir S, Schallmeiner E, Gullberg M, Jarvius J, Landegren U (2007) Proximity ligation: a specific and versatile tool for the proteomic era. Genet Eng (N Y) 28:85–93
29. Soderberg O, Leuchowius KJ, Gullberg M, Jarvius M, Weibrecht I, Larsson LG, Landegren U (2008) Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. Methods 45(3): 227–232

30. Gullberg M, Gustafsdottir SM, Schallmeiner E, Jarvius J, Bjarnegard M, Betsholtz C, Landegren U, Fredriksson S (2004) Cytokine detection by antibody-based proximity ligation. Proc Natl Acad Sci U S A 101(22):8420–8424
31. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, Landegren U (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 3(12): 995–1000
32. Leuchowius KJ, Weibrecht I, Soderberg O (2011) In situ proximity ligation assay for microscopy and flow cytometry. Curr Protoc Cytom Chapter 9:Unit 9.36