> CHAPTER FIFTEEN

C0075

# G Protein–Coupled Receptor Heterodimerization in the Brain

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## Contents

## Abstract

G protein–coupled receptors (GPCRs) play critical roles in cellular processes and signaling and have been shown to form heteromers with diverge biochemical and/or pharmacological activities that are different from those of the corresponding monomers or homomers. However, despite extensive experimental results supporting the formation of GPCR heteromers in heterologous systems, the existence of such receptor heterocomplexes in the brain remains largely unknown, mostly because of the lack of appropriate methodology. Herein, we describe the *in situ* proximity ligation assay procedure underlining its high selectivity and sensitivity to image GPCR heteromers with confocal

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microscopy in brain sections. We describe here how the assay is performed and discuss advantages and disadvantages of this method compared with other available techniques.

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# 1. INTRODUCTION

p0005    G protein–coupled receptors (GPCRs) play critical roles in cellular processes and signaling and have been shown to form heteromers with diverge biochemical and/or pharmacological activities that are different from those of the corresponding monomers or homomers. The idea of the existence of direct interactions between two different GPCRs at the level of the plasma membrane has its origin in 1980/1981 on the basis of the discovery that peptides like cholecystokinin-8 (CCK-8) and substance P could modulate the density, and especially the affinity, of distinct monoamine receptors in membrane preparations from the CNS with *in vivo* functional correlates (Agnati, Fuxe, Zini, Lenzi, & Hokfelt, 1980; Fuxe et al., 1981). These initial findings were in line with the previous discovery of negative cooperativity between β adrenergic receptors in 1974/1975 by Lefkowitz and colleagues, indicating the possible existence of homodimers of β adrenergic receptors leading to site–site interactions in recognition (Limbird, Meyts, & Lefkowitz, 1975).

p0010    Nevertheless, despite extensive experimental results supporting the formation of GPCR heteromers in heterologous systems, the existence of such receptor heterocomplexes in their native environment remains largely unknown, mostly because of the lack of appropriate methodology. For instance, until recent years, the methods that have been developed to study receptor–receptor interactions in heteromers require that genetic constructs be expressed in the cells to enable detection of the receptor interactions, thus excluding the use of tissue samples (Borroto-Escuela, Garcia-Negredo, Garriga, Fuxe, & Ciruela, 2010; Ferre et al., 2009; Fuxe et al., 2012).

p0015    In order to demonstrate in native tissue the existence of GPCR heteromers, especially in a manner that can be generally applicable to different receptor pairs, a well-characterized *in situ* proximity ligation assay (*in situ* PLA) has been adapted to confirm the existence of GPCR heteromers in brain slices *ex vivo*.

p0020    *In situ* PLA is based on a pair of antibodies that can bind to target proteins and to which oligonucleotides have been attached. When the so-called proximity probes recognize a target, for example, if the two target receptors interact, the attached oligonucleotides are brought into a sufficiently close

spatial proximity to allow them to join followed by ligation of the two linear oligonucleotides into a circular DNA molecule. This newly formed DNA circle strand can serve as a template for rolling circle amplification (RCA), resulting in a long single-stranded rolling circle product (RCP) attached to one of the proximity probes. As the RCP is linked to the proximity probe, it is attached at the site where the proximity probe bound, which means that it can be used to reveal the location of the receptor complex (Soderberg et al., 2006, 2007). The RCPs can then be detected and quantified by hybridizing fluorescent oligonucleotides to the repeated sequences of the RCPs, rendering them visible by fluorescence microscopy (Fig. 15.1).

p0025    Herein, we describe the *in situ* PLA procedure as a high selectivity and sensitivity assay to image GPCR heteromers in brain sections by confocal microscopy. We describe how the assay is performed and discuss advantages and disadvantages of this method compared with other available techniques.

## s0010    2. *In Situ* PLA FOR DEMONSTRATING RECEPTOR HETEROMERS AND THEIR RECEPTOR–RECEPTOR INTERACTIONS IN BRAIN TISSUE

p0030    *In situ* PLA has previously been performed to confirm the existence of striatal $A_{2A}R$–$D_2R$ heteromers (Trifilieff et al., 2011). The PLA technique involved the use of two primary antibodies of different species directed to either $D_2R$ or to $A_{2A}R$ (Fig. 15.2). We recommend to use *in situ* PLA also to indicate the ratio between heteromers versus total number of the two participating receptor populations, using in addition to Western blots, receptor autoradiography, and biochemical binding methods, the two latter methods showing the densities and affinities of the two functional receptor populations. This will also help to normalize the heteromer values for comparison between groups in addition to evaluating the potential changes in the total number of the two receptor populations. The person doing the PLA measurements should be blind to the code of the experimental groups studied.

## s0015    3. BRAIN TISSUE PREPARATION

p0035    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. As the protocols provided in this section are general, it is highly suggested to apply the same protocol that has been working previously for your receptor
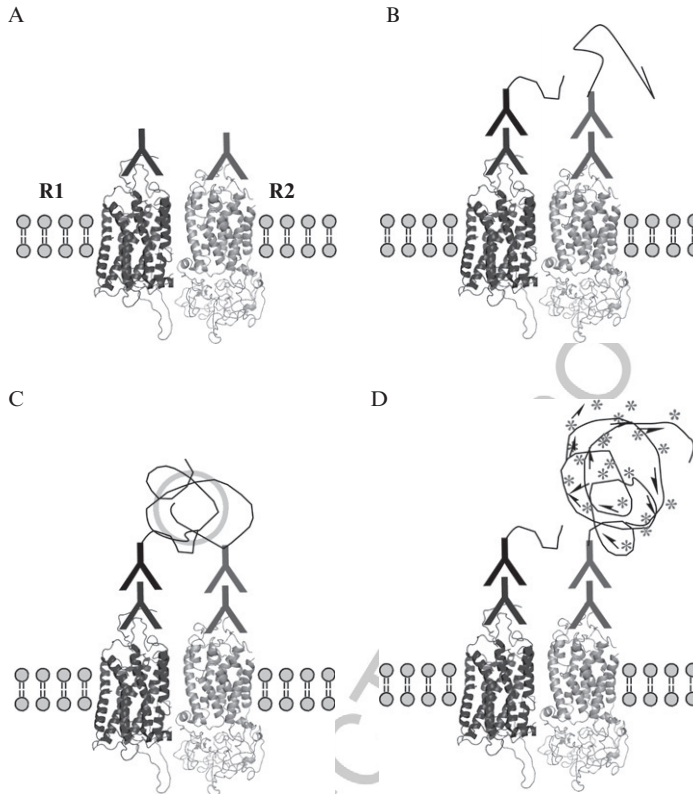
f0005 **Figure 15.1** Schematic presentation of *in situ* PLA for detection of GPCR heteromers in the brain. (A) Two primary antibodies from different species are used to detect each receptor heteromer protomers (R1 and R2). (B) Each species-specific secondary antibody with attached oligonucleotide DNA probes (proximity probes) is targeted to the corresponding primary antibody, and when they are in a close proximity (the distance between the two secondary antibodies is a maximum of 10–20 nm as calculated from a DNA arm of 25–35 bp), the proximity probes serves as a template for the hybridization of circularization oligonucleotides, which are then joined by ligation into a circular DNA molecule (C). The circular DNA molecule is then amplified by rolling circle amplification (RCA) primed by one of the proximity probes and using a polymerase to yield a long concatemeric copy of the rolling circle that remains covalently attached to the proximity probe (D). The RCA product can subsequently be identified by hybridization of fluorophore-labeled complementary oligonucleotide probes added to highlight the product. (For color version of this figure, the reader is referred to the online version of this chapter.)

pairs in immunohistochemistry. Thus, similar conditions as employed for immunohistochemistry can be used for *in situ* PLA reactions.

p0040     For *in situ* PLA, the common options are fixed (paraffin–embedded or cryostat sections and vibratome sections) or unfixed cryostat (frozen) sections. The choice of section is determined by a number of conditions,
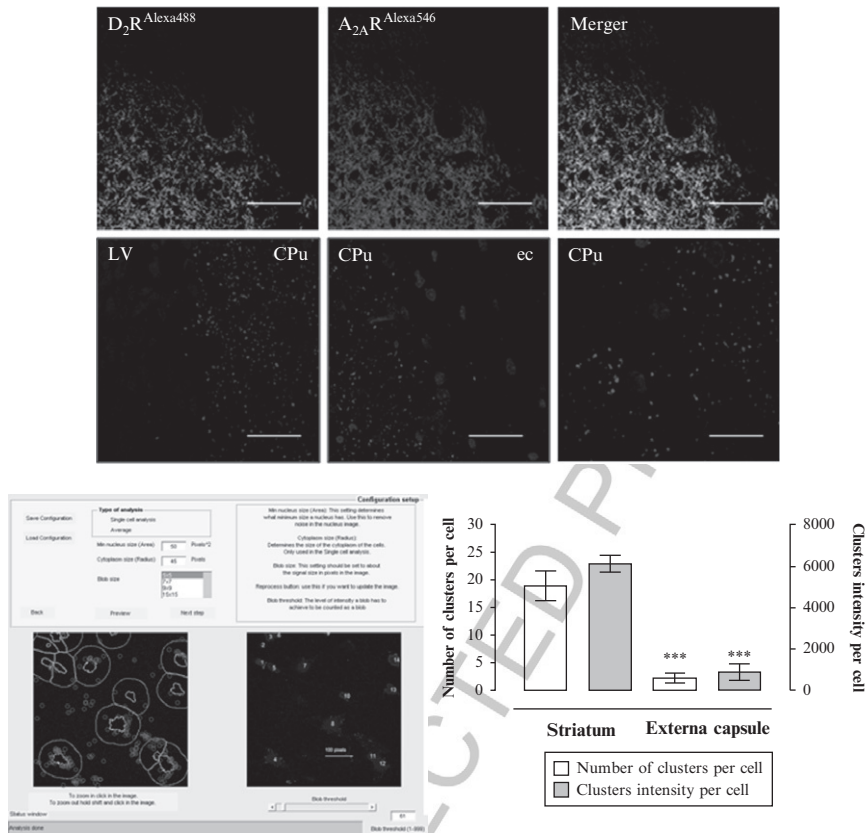
f0010 **Figure 15.2** Upper-upper panel: Specific $D_2R$ (green) and $A_{2A}R$ (red) immunoreactivities and colocalization (yellow) in striatal sections. $D_2R$ immunoreactivity was high in the striatum of rat surrounded by the external capsule (ec) using fluorescence immunohistochemistry (left). $A_{2A}R$ immunoreactivity (middle) showed a high level of colocalization with $D_2R$ (right) in the striatum of wild-type rat. Scale bars, 75 $\mu$m. Upper-down panel: PLA-positive $A_{2A}R$–$D_2R$ heteromers in striatal sections adjacent to the sections with immunoreactivity $A_{2A}R$–$D_2R$ heteromers were visualized as red clusters (blobs, dots) within the striatum which were almost absent within the lateral ventricle (LV, left) and the external capsule (ec, middle panel). Higher magnification image revealed a large number of PLA-positive red clusters within the caudate putamen (CPu, right). Each cluster represents a high concentration of fluorescence from the single-molecule amplification resulting from several hundred-fold replication of the DNA circle formed as a result of the probe proximity; the cluster/dot number can be quantified independently of the intensity. Nuclei are shown in blue (DAPI). Scale bars, 50 $\mu$m for left and middle panels; 20 $\mu$m for the right panels. Lower-left panel: Screendump from the corresponding BlobFinder analysis. The left pictures show how the software has identified the PLA signals, the nuclear limit, and the approximate limit of the cytoplasm based on a user-defined radius. The right picture shows the raw image based on 14 $Z$-planes with the nuclei enumerated. Lower-right panel: PLA-positive red clusters in striatum were quantified per cell using BlobFinder and the results are presented.

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). Incorrect specimen preparation can block or impede antigen labeling in the tissue. Unfortunately, the methods that are best for the preservation of tissue structure do so by modifying proteins, thereby reducing the efficiency of antigen detection. In cases of failure, it is important to try with multiple different conditions before you give up the *in situ* PLA.

o0005 **1.** Because of the ease of use, fixed frozen free-floating sections are often employed in most of the *in situ* PLA experiments. First, animals are anesthetized by an intraperitoneal injection of, for example, pentobarbital (60 mg/ml, [0.1 ml/100 g]) and perfused intracardially with 30–50 ml of 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 h. Then, the brain pieces are placed in sucrose 20% in PBS and incubated for 24 h until sections (10–30 μm thick) are generated and serially collected using a cryostat. Alternatively to the use of fixed free-floating sections, we can use tissue fixed frozen sections attached to microscopy slides. Mounted sections on slides must be kept at $-20\,^{\circ}C$ until use. Encircle the tissue section on the glass slide by creating a hydrophobic barrier using a grease pen or a silicon mask and proceed as follows.

o0010 **2.** Wash the fixed free-floating sections four times with PBS, then incubate with the blocking solution (10% fetal bovine serum [FBS] and 0.5% Triton X-100 or Tween-20 in Tris buffer saline [TBS], pH 7.4) for 2 h at room temperature or 1 h at 37 $^{\circ}C$ and then follow the Protocol step 5.

p0055 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 of 0.5% Triton X-100 or Tween-20 in TBS, pH 7.4; Leuchowius, Weibrecht, & Soderberg, 2011) or animal serum like 10% FBS (if animal serum is used, 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. Each time must be

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Quantification of $A_{2A}R$–$D_2R$ heteromers demonstrates highly significant differences in PLA clusters per cell between caudate putamen and external capsule (***$P < 0.001$ by Student's $t$-test). (See Color Insert.)

checked that the reaction should never become dry as this will cause high background.

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## 4. PROXIMITY PROBES: CONJUGATION OF OLIGONUCLEOTIDES TO ANTIBODIES

p0060 Proximity probes are created through the attachment of oligonucleotides to antibodies. The oligonucleotide component of the proximity probes can be covalently coupled to an antigen-binding component or attached to secondary antibodies specific for antibodies raised in different species. This approach avoids the need to conjugate the oligonucleotide components to each primary antibody pair.

p0065 Several different types of chemistry can be used for the conjugation of oligonucleotides to antibodies. Mainly three methods have been used extensively in recent years: the maleimide/NHS-esther chemistry (SMCC; Soderberg et al., 2006), the succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH; Leuchowius et al., 2011), or the commercially available Antibody-Oligonucleotide All-in-One Conjugation Kit from Solulink company (http://www.solulink.com/), based on two complementary heterobifunctional linkers (Sulfo-S-4FB (formylbenzamide) and S-HyNic (hydrazino–nicotinamide)). Because the act of conjugation can severely affect the ability of some antibodies to bind antigen, it may be necessary to analyze different antibodies, conjugation chemistries, and reaction conditions to obtain suitable proximity probes.

p0070 Another possibility, not less useful, is to buy directly proximity probes from specialized companies on antibody-oligonucleotide conjugation, for example, Duolink (Uppsala, Sweden; http://www.olink.com/).

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## 5. PLA REACTIONS, REAGENTS, AND SOLUTIONS

o0015 **1.** If primary antibodies directly labeled with oligonucleotides or primary antibodies are used in combination with secondary proximity probes (see Protocol step 4), the conditions for incubation with the primary antibodies should be chosen according to the manufacturer's recommendations or will have to be identified by the users. For instance, incubate the tissue with the primary antibodies diluted into a suitable concentration in the wash buffer (0.5% Triton X-100 or Tween-20 in TBS, pH 7.4) at 1–2 h at 37 °C or +4 °C overnight.

o0020  **2.** After incubation of the primary antibodies at conditions specified above or determined by the user, excess antibody should be removed. Wash the slides four times, 5 min each time, with wash buffer.

o0025  **3.** In the mean time, if primary antibodies are used in combination with secondary proximity probes, dilute the proximity probes to a suitable concentration in the wash buffer. 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 cause also artifacts. To remove unbound proximity probes, wash the slides four times, 5 min each time, with wash buffer.

o0030  **4.** Prepare the hybridization–ligation solution. To ensure optimal conditions for the enzymatic reactions, the sections should be soaked for 1 min in $1 \times$ ligation buffer (10 m$M$ Tris–acetate, 10 m$M$ magnesium acetate, 50 m$M$ potassium acetate, pH 7.5; Soderberg et al., 2008), prior to addition of the hybridization–ligation solution (final concentration: BSA (250 μg/ml), $1 \times$ T4 DNA ligase buffer, Tween-20 (0.05%), NaCl 250 m$M$, ATP 1 m$M$, and the circularization or connector oligonucleotides 125–250 n$M$). Circularization or connector oligonucleotides can be designed and synthesized as described previously (Soderberg et al., 2008). 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 slides in a humidity chamber for 30 min at 37 °C.

o0035  **5.** Wash the sections three times with wash buffer in a washing jar for 5 min to remove excess connector oligonucleotides.

o0040  **6.** Prepare the RCA mixture. Soak the sections in $1 \times$ RCA buffer (50 m$M$ Tris–HCl, 10 m$M$ MgCl$_2$, 10 m$M$ (NH$_4$)$_2$SO$_4$, pH 7.5 adjusted with HCl) 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 μg/ml), $1 \times$ RCA buffer, Tween-20 (0.05%), and dNTP (250 μ$M$ for each)). Vortex briefly the RCA solution and incubate in a humidity chamber for 100 min at 37 °C.

o0045  **7.** Wash the sections three times with wash buffer in a washing jar for 5 min. Prepare the detection solution (final concentration: BSA (250 μg/ml), $2 \times$ sodium citrate, sodium chloride buffer, and the fluorescence detection (e.g., Texas Red or Alexa 555)-oligonucleotide strand (6.25 n$M$)), see Soderberg et al., 2008) 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 wash steps should be performed in the dark.

o0050 **8.** Wash the sections twice, each time with wash buffer in a washing jar for 5 min in the dark.

o0055 **9.** Wash the sections once with TBS in a washing jar for 5 min in the dark.

o0060 **10.** Dry and mount sections with the appropriate mounting media (e.g., VectaShield or Dako). The sections should be protected against light and can be stored for several days at 4 °C or for several months at −20 °C.

s0030 ## 6. QUANTITATIVE PLA IMAGE ANALYSIS

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**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 appearance that is easily recognized once you know what to look for. The PLA detection reaction products are seen as bright fluorescent puncta of submicrometer size (see Fig. 15.2). By moving the focus up and down in your sample tissue, you should note appearance and disappearance of PLA signals. Up to a certain density of PLA signals, they appear as discrete dots (puncta, blobs) that can be easily enumerated using image analysis software.

o0070 **2.** Analyze the captured images by image techniques to quantify the number of dots (Fig. 15.2, lower panel). Many commercial image analysis software packages can also be used in addition to free software packages, such as BlobFinder (Allalou & Wahlby, 2009) or Cellprofiler (Carpenter et al., 2006). The BlobFinder is a free software tool developed by the Centre for Image Analysis-Uppsala University for such objectives (the freeware is available for download from http://www.cb.uu.se/∼amin/BlobFinder/; Fig. 15.2, lower panel). At higher densities per number of nuclei, the dots start to coalesce, thus making it more difficult to resolve and enumerate individual signals. It is important to use the same settings for image acquisition for all images in a series.

s0035 ## 7. ADVANTAGES AND DISADVANTAGES OF THE PLA METHOD

p0145    *Comparison with other methods to study receptor–receptor interactions in heteromers, such as FRET, BRET, and bimolecular fluorescence complementation: In situ* PLA can offer advantages by permitting analyses of interactions

among any receptors for which suitable antibodies are available without using genetic constructs. Such constructs can perturb cellular function and cannot always be used, for example, in cells and tissues from subjects. *In situ* PLA can be performed in all samples of cells and tissues, and the method is highly suited to investigate human specimens collected from biobanks, in order to investigate patterns of changes in heteromers that could provide insights on the role of basic heteromer mechanisms or have a diagnostic value (Nilsson et al., 2010). The method has also proven useful to monitor the effects of different compounds like agonists and antagonists or their combined treatment on the receptor heteromers in cells and tissue (Borroto-Escuela et al., 2011). The information is obtained at a resolution of individual cells or even of subcellular compartments, providing profound insights into cellular heterogeneity in tissues. The method also provides an enhanced sensitivity and selectivity compared with many other methods as powerful RCA and dual target recognition are used (Clausson et al., 2011).

p0150    As with any method there are limitations, for instance, *in situ* PLA cannot be used with live cells, as it requires cell fixation and, in some cases, permeabilized cells. 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 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. By changing the length of the oligonucleotides, the maximal distance limits can be reduced or increased.

p0155    Other critical parameters for achieving good results is the use of excellent antibodies. The antibodies must also be used under optimal conditions taking into consideration parameters such as antibody concentration, epitopes targeted by the antibodies, fixation, antigen retrieval, blocking conditions, etc. 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, for example, siRNA.

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## 8. APPLICATION

p0160    *In situ* PLA has been used to study proteins and protein–protein interactions in a range of applications (Leuchowius et al., 2010; Nilsson et al., 2010). In 2011, the method was employed to study GPCRs heteromers, mainly adenosine $A_{2A}$ and dopamine $D_2$ receptor heteromers in striatal sections (Trifilieff et al., 2011) and dopamine $D_2R$ and $D_4R$ in transiently transfected HEK293T cells (Borroto–Escuela et al., 2011). In addition, the methods were employed to demonstrate for the first time the existence of FGFR1 and 5-HT1A receptor heterocomplexes in the rat hippocampus and dorsal and median raphe in the midbrain (Fig. 15.3A; Borroto–Escuela et al., 2012).

p0165    In the analysis using *in situ* PLA, it is also important to determine the ratio between heteromers versus total number of the two participating receptor populations, using in addition to Western blots, receptor autoradiography, and biochemical binding methods, the two latter methods showing the densities and affinities of the two functional receptor populations. The relationship between these parameters will help to normalize the heteromer values for comparison between groups in addition to evaluating the potential
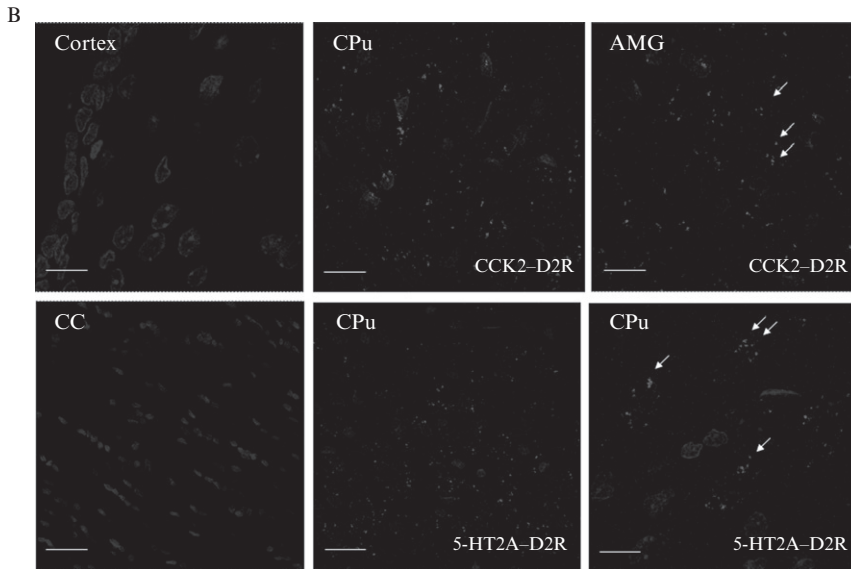
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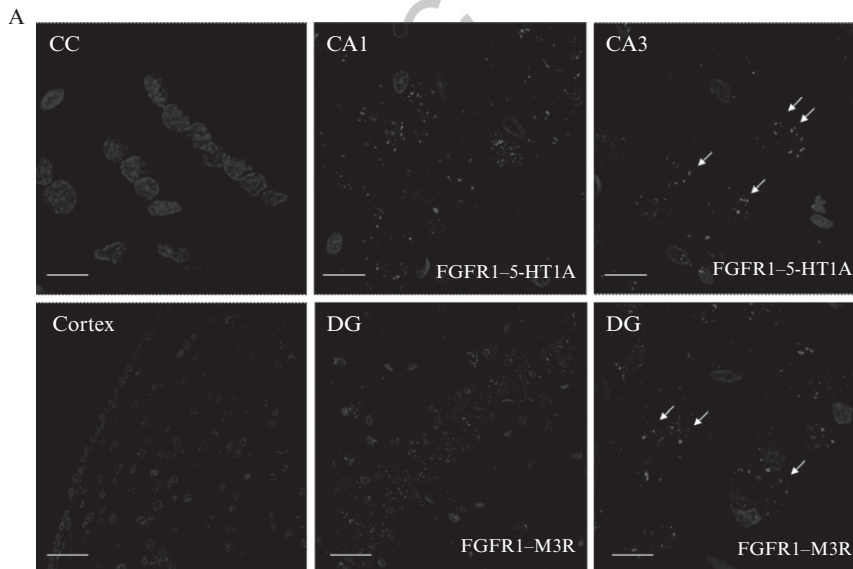


*(Continued)*

f0015 **Figure 15.3** Detection of different GPCR–GPCR heteromers and GPCR–RTK hetero-complexes in dorsal rat hippocampal and striatal sections by *in situ* PLA. (A-upper panel) Constitutive FGFR1–5-HT1A heteroreceptor complexes are detected by *in situ* PLA (red clusters) in dorsal rat hippocampus (Ammon's horn 1 and 3 (CA1, CA3) but not, as an example, in the corpus callosum (cc). Scale bars, 20 $\mu$m. (A-lower panel) Constitutive FGFR1–M3R heteroreceptor complexes are detected by *in situ* PLA (red clusters) in dorsal rat hippocampus (granular layer of the dentate gyrus [DG]) but not in the cortex cerebri. Scale bars from the left to the right, 50, 50, and 20 $\mu$m. (B-upper panel) Constitutive CCK2–$D_2$R heteromers are detected by *in situ* PLA (red clusters) in striatal sections (caudate putamen: CPu; amygdaloid cortex: AMG) but not, for example, in the cortex cerebri. Scale bars, 20 $\mu$m. (B-upper panel) Constitutive 5-HT2A–$D_2$R heteromers are detected by *in situ* PLA (red clusters) in striatal sections (caudate putamen: CPu; amygdaloid cortex: AMG) but not, for example, in the corpus callosum (cc). Scale bars from the left to the right, 75, 50, and 20 $\mu$m. Nuclei appear as a blue color in all panels and the white arrows indicate the red cluster formation (PLA signal). (See Color Insert.)

changes in the total number of the two receptor populations. Of increasing importance will be to determine the agonist/antagonist regulation of these receptor heteromers in order to understand their potential roles as targets for drugs used in neuropsychopharmacology for treatment of psychiatric and neurological diseases. Analysis of human brain material with *in situ* PLA can also reveal if the relative abundance of specific receptor heteromers in discrete brain regions is altered in brain diseases (Fig. 15.3A and B).

## ACKNOWLEDGMENTS

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# Non-Print Items