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G-Protein-Coupled Heteromers: Regulation in Disease

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Abstract

Over the past decade, an increasing number of studies have shown that G-protein-coupled receptors including opioid and cannabinoid receptors associate to form heteromers. Moreover, G-protein-coupled receptor heteromerization leads to the modulation of the binding, signaling, and trafficking properties of individual receptors. Although very little information is available about the physiological role of receptor heteromers, some studies have shown that the levels of some heteromers are upregulated in disease states such as preeclamptic pregnancy, schizophrenia, Parkinson's, ethanol-induced liver fibrosis, and development of tolerance to morphine. The recent generation of antibodies that selectively recognize distinct heteromers and, of peptides that selectively disrupt them, have started to elucidate the contribution of heteromers to the disease state. Here, we describe the methods for the generation of heteromer-selective antibodies and elucidation of their levels and localization under normal and pathological conditions.

1. INTRODUCTION

Drugs of abuse such as opioids and cannabinoids act through G-protein-coupled receptors (GPCRs), the opioid and cannabinoid receptors. Three opioid (μ , δ , and κ) and cannabinoid (CB₁R, CB₂R, and GPR55) receptor subtypes have been identified (Balenga, Henstridge, Kargl, & Waldhoer, 2011; Dietis, Rowbotham, & Lambert, 2011; Di Marzo, Piscitelli, & Mechoulam, 2011). Both receptors signal via G_{ai/o} proteins to activate similar signal transduction cascades leading to decreases in intracellular cyclic AMP levels, inhibition of neurotransmitter release, and to increases in mitogen-activated protein kinase phosphorylation (Bushlin, Rozenfeld, & Devi, 2010; Cichewicz, 2004; Howlett et al., 2002; Vigano, Rubino, & Parolaro, 2005). Moreover, activation of either receptor induces similar physiological responses such as antinociception, sedation, reward, and emotional responses (Maldonado, Valverde, & Berrendero, 2006; Manzanares et al., 1999). This similarity in mechanisms of action and physiological responses suggests the possibility of interactions between the opioid and cannabinoid systems.

Opioid receptor subtypes can associate to form higher-order structures, a process known as heteromerization. For example, μ (μ OR) and δ (δ OR) opioid receptors heteromerize and these modulate binding, signaling, and morphine-mediated analgesia (Gomes et al., 2004, 2000; Gomes, Ijzerman, Ye, Maillet, & Devi, 2011; Kabli et al., 2010; Levac, O'Dowd, & George, 2002; Rozenfeld & Devi, 2007). Heteromerization between δ OR and κ opioid receptors (κ OR) leads to novel pharmacology and alteration of individual receptor-traffic properties (Berg et al., 2012; Bhushan, Sharma, Xie, Daniels, & Portoghesi, 2004; Jordan & Devi, 1999). Furthermore, opioid receptors can heteromerize with other

family A GPCRs such as α 2A adrenergic (Jordan, Gomes, Rios, Filipovska, & Devi, 2003; Rios, Gomes, & Devi, 2004), β 2 adrenergic (Jordan, Trapaidze, Gomes, Nivarthi, & Devi, 2001), chemokine (Chen et al., 2004; Hereld & Jin, 2008; Pello et al., 2008), substance P (Pfeiffer et al., 2003), or somatostatin receptors (Pfeiffer et al., 2002). Interestingly, heteromerization between CB₁R and μ OR, δ OR, or angiotensin AT1 receptors (AT1Rs) leads to alterations in signaling and localization of CB₁R (Rios, Gomes, & Devi, 2006; Rozenfeld et al., 2012, 2011). However, little information is available about the physiological role of GPCR heteromers due to a lack of appropriate tools to study them in endogenous tissues and to distinguish from receptor homomers. Studies using mainly coimmuno-precipitation techniques suggest the involvement of some GPCR heteromers in disease. Heteromers between dopamine D1–D2 receptors have been implicated in major depression (Pei et al., 2010), between AT1R and adrenergic α 1D or AT1R and bradykinin B2 receptors with preeclamptic pregnancy (AbdAlla, Abdel-Baset, Lothar, el Massiery, & Quitterer, 2005; Gonzalez-Hernandez Mde, Godinez-Hernandez, Bobadilla-Lugo, & Lopez-Sanchez, 2010) and between dopamine receptor subtypes as well as dopamine D2 and adenosine 2A receptors in schizophrenia (Dziedzicka-Wasylewska, Faron-Gorecka, Gorecki, & Kusmider, 2008; Faron-Gorecka, Gorecki, Kusmider, Wasylewski, & Dziedzicka-Wasylewska, 2008; Fuxe et al., 2005; Maggio & Millan, 2010; Perreault, O'Dowd, & George, 2011). However, direct demonstration of heteromers *in vivo* has not been possible due to a lack of appropriate reagents.

We recently generated monoclonal antibodies (mAbs) that selectively recognize heteromers over individual receptor homomers using a subtractive immunization strategy. This enabled studies to directly explore the physiological role of GPCR heteromers. For example, these antibodies can be used to detect the presence of a heteromer in a specific tissue/region. A case in point is the detection of δ OR– κ OR heteromers in peripheral sensory neurons using δ OR– κ OR selective antibodies (Berg et al., 2012). Alternatively, the antibodies could implicate the heteromer in a disease state. μ OR– δ OR heteromer-selective antibodies detect increased heteromer levels in brain regions involved in pain processing following chronic morphine administration under conditions leading to the development of tolerance (Gupta et al., 2010), suggesting that they may play a role in tolerance. This is supported by studies showing that μ OR– δ OR heteromer disruption leads to enhanced morphine analgesia with a concomitant decrease in tolerance (He et al., 2011). CB₁R–AT1R heteromer-selective antibodies detect a significant heteromer upregulation in hepatic stellate cells of rats chronically treated with ethanol (Rozenfeld et al., 2011), suggesting its involvement in ethanol-induced liver fibrosis. Here, we describe the generation of heteromer-selective antibodies and their use with enzyme-linked immunosorbent assays (ELISAs), immunofluorescence, immunoprecipitation, and Western blotting to detect levels and localization of heteromers in native tissues under normal and pathological conditions.

2. GENERATION OF HETEROMER-SELECTIVE mAbs

The advantages of mAbs to probe for heteromer levels in normal and disease states are that they recognize a single epitope, are highly specific, and can be produced in large quantities. The challenge in the generation of heteromer-selective mAbs is that the “heteromer-specific” epitope may be present in a cell or tissue at relatively low levels thus limiting the chances of being detected by antibody-producing cells. This limitation can be overcome through the use of a subtractive immunization strategy (Salata et al., 1992; Sleister & Rao, 2001, 2002). The first step in subtractive immunization involves tolerizing mice with membrane preparations that either do not express the receptor of interest (usually HEK-293 membranes) or express one of the two receptors (e.g., Neuro2A neuroblastoma cell membranes used to generate CB₁R–AT1R heteromer-selective mAb (Rozenfeld et al., 2011) endogenously express CB₁R). Tolerization is achieved by killing activated antibody-

producing cells through the use of a chemical agent such as cyclophosphamide. Once a consistently low titer, as ascertained by ELISA, is obtained with the membranes used for the tolerization step, the mice are immunized with membranes from cells coexpressing the receptors forming the targeted heteromer. The immunizations are repeated until a high titer is obtained by ELISA at which time the mice are killed and spleens are removed for the generation of mAbs. Once the mAbs are generated, their heteromer selectivity is characterized by ELISA, immunofluorescence, or Western blot analysis using cells that express the individual or a combination of receptors or tissues from wild type and animals lacking one of the receptors. These steps are described below using the μ OR- δ OR heteromer as a model system.

2.1. Preparation of antigen for immunization

For the generation of μ OR- δ OR heteromer-selective mAbs (μ OR- δ OR mAb), we used membranes from human embryonic kidney 293 (HEK-293) cells (American Type Culture Collection, Manassas, VA) for the tolerization step and from cells coexpressing Flag- μ OR and myc- δ OR (HEK- $\mu\delta$) for the second immunogenic step.

1. Grow HEK-293 cells in 10-cm² dishes (Becton-Dickinson, Falcon) in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL).
2. When ~80–85% confluent, cotransfect cells with Flag- μ OR and myc- δ OR (6 μ g each) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).
3. Thirty-six to forty-eight hours after transfection, remove growth media and rinse cells twice with 5 ml phosphate-buffered saline (PBS).
4. Add 1.5 ml of prechilled buffer A (50 mM Tris-Cl, pH 7.4, containing protease inhibitor cocktail (Sigma-Aldrich)) to the dish, collect cells in an Eppendorf tube with the help of a rubber policeman, and centrifuge at 3000 $\times$ g for 3 min at 4 °C.
5. Resuspend the cell pellet in 0.2 ml of freshly prepared ice-cold buffer B (20 mM Tris-Cl buffer, pH 7.4, containing 2 mM EGTA, 1 mM MgCl₂, 250 mM sucrose, and protease inhibitor cocktail).
6. Homogenize cells on ice using a Teflon pestle for Eppendorf tubes (10–20 strokes), adjust the volume to 0.5 ml with ice-cold buffer B and pass through an insulin syringe three times.
7. Adjust the volume to 10 ml with ice-cold buffer B and centrifuge at 27,000 $\times$ g for 15 min at 4 °C.
8. Resuspend the pellet in ice-cold buffer B and repeat steps 6 and 7.
9. Resuspend the pellet in minimum volume of PBS containing 10% glycerol and pass through insulin syringe.
10. Determine membrane protein concentration using Pierce BCA protein assay reagent (Thermo Scientific) according to manufacturer's protocol.
11. Mix membranes from either HEK or HEK- $\mu\delta$ cells (50–100 μ g protein in 0.2 ml PBS) with 0.2 ml Freund's complete adjuvant (Gibco).
12. Pass the mixture through an insulin syringe 10 times.
13. Place the suspension in a rotating shaker at moderate speed for 20 min at 4 °C and repeat step 11.

14. Confirm the formation of an emulsion by placing a small drop in 10 ml of water in a petridish. If the drop disperses in water, the emulsification is incomplete in which case repeat steps 11 and 12. If the emulsification is complete, the drop does not disperse in water. For booster injections, prepare the emulsion in Freund's incomplete adjuvant.

2.2. Subtractive immunization

Subtractive immunization is carried out in Balb/c mice (6-week-old female). Use at least three mice for immunization. Prior to immunization, collect blood from the tail vein to prepare preimmune serum (described in Section 2.6).

1. Inject mice intraperitoneally with 50–100 μ g of HEK-293 membranes emulsified in complete Freund's adjuvant.
2. Administer cyclophosphamide (100 mg/kg body weight; Sigma-Aldrich) daily for the next 3 days.
3. Every 15 days, administer an intraperitoneal booster injection with membranes emulsified in incomplete Freund's adjuvant and repeat step 2.
4. Collect blood from the tail vein on the 4th day after each booster injection, prepare serum (described in Section 2.6), and monitor antibody production by ELISA (described in Section 3).
5. Continue booster injections until a consistent low titer is observed with HEK-293 membranes.
6. Inject the mice intraperitoneally with HEK- μ δ membranes emulsified in Freund's complete adjuvant.
7. Repeat steps 3 and 4 with HEK- μ δ membranes emulsified in incomplete Freund's adjuvant.
8. Continue booster injections until a consistent high titer is observed with HEK- μ δ membranes and not with HEK membranes alone or expressing individual receptors. Generate mAbs as described below.

2.3. Fusion protocol to generate hybridoma-secreting clones

To generate hybridoma-secreting clones, splenocytes from immunized mice are fused with mouse myeloma cells (Gupta et al., 2010; Kohler & Milstein, 1975). We use SP2/0-Ag-14 myeloma cells (American Type Culture Collection, Manassas, VA) that have the machinery necessary for antibody secretion although on their own they do not secrete antibodies (Shulman et al., 1978). SP2/0-Ag-14 cells have a mutation in one of the enzymes of the salvage pathway for purine nucleotide biosynthesis rendering the latter nonfunctional (Shulman et al., 1978). The fusion process is relatively inefficient, given that only 1 in 10^5 of starting cells form viable hybrids (Shulman et al., 1978). The unfused splenocytes do not grow in culture and eventually die. To kill unfused myeloma cells, compounds that block *de novo* purine biosynthesis such as aminopterin, methotrexate, or azaserine are used (Shulman et al., 1978). Fused myeloma cells do not die under these conditions since they have a functional salvage pathway derived from the splenocyte fusion partner (Shulman et al., 1978).

1. Thaw a vial of SP2/0-Ag-14 cells and grow them in DMEM containing 10% FBS and 1% penicillin–streptomycin. At the time of fusion, these cells should be under the exponential growth phase.

2. Three days before fusion, inject mice intravenously with HEK- $\mu\delta$ membranes (50–100 μg).
3. On the day of the fusion, kill an immunized mouse using CO_2 from compressed gas.
4. In a sterile hood, place the mouse on its right side and swab the body with 70% ethanol.
5. Cut open the mouse to expose the spleen taking care to not puncture the gut or surrounding splenic arteries and veins.
6. Remove the spleen and place it in a sterile Petridish containing 5 ml of sterile serum-free Iscove's modified Dulbecco's media (IMDM; Gibco-BRL).
7. Wash the spleen three to five times in serum-free IMDM.
8. Remove any adherent fat and transfer the spleen into a Petridish containing 5 ml of serum-free IMDM.
9. Tease apart the spleen with the help of a 21-G needle to release the splenocytes. The medium becomes cloudy as the cells are released from the spleen.
10. Transfer the cell suspension into a 50-ml centrifuge tube; let it stand for 2 min to allow the tissue clumps to settle down.
11. Transfer the supernatant containing the cell suspension minus the tissue clumps into a fresh 50-ml tube.
12. Make up the volume to 10 ml with serum-free IMDM and centrifuge at $400\times g$ for 10 min.
13. Resuspend cell pellet in 10 ml serum-free IMDM and count the number of live cells using 0.2% Trypan blue (Gibco-BRL) and an improved Neubauer counting chamber. A spleen from an immunized mouse yields $0.5\text{--}2\times 10^8$ splenocytes.
14. Wash the splenocytes twice in 10 ml of serum-free IMDM by centrifuging at $400\times g$ for 10 min.
15. Harvest the SP2/0-Ag-14 cells and wash them twice as described in step 14 for splenocytes.
16. Resuspend the SP2/0-Ag-14 cells in 10 ml serum-free IMDM and count the number of cells as described in step 13.
17. Mix the spleen and myeloma cells in a 50-ml centrifuge tube at a ratio of 10:1 (splenocytes:myeloma) and centrifuge at $800\times g$ for 5 min. Discard the supernatant.
18. Gently dislodge the cell pellet by tapping and place the tube in a beaker of water at 37°C .
19. Slowly add 1 ml of 50% PEG 4000 (Sigma-Aldrich) prewarmed to 37°C . This addition should be complete within a minute, continually stir the mixture with the pipette tip. The cells must not be in contact with the concentrated PEG solution for more than 2 min.
20. Slowly add (over a period of 1 min) 1 ml of serum-free IMDM prewarmed to 37°C . Continuously stir the cell suspension with the pipette tip while media is being added.
21. Add 4 ml of serum-free IMDM (prewarmed to 37°C) over a period of 3–4 min, without stirring, sliding it slowly down the side of the tube.

22. Add 20 ml of serum-free IMDM (prewarmed to 37 °C) followed by 20 ml of IMDM containing 15% FBS, again by sliding slowly down the side of the tube.
23. Cap the tube, invert it once, and leave it at 37 °C for 2 h.
24. Centrifuge the cells (400×g for 10 min), discard the supernatant, and resuspend the pellet in IMDM containing 15% FBS and 1× solution of hypoxanthine, aminopterin, and thymidine (HAT; Gibco-BRL).
25. Plate the cells in 24-well plates at 2 ml/well.
26. Put the plates in a 10% CO₂ incubator at 37 °C.
27. After a week, remove 1 ml of medium from each well and add 1 ml of complete media containing HAT supplement. Repeat this step once more. After 15 days in culture, screen the wells under a microscope for the presence of hybridoma-secreting clones.
28. Subclone wells containing clones into 96-well plates so as to obtain 1 cell/well.
29. Allow the clones to grow in 200 µl of complete media containing HAT supplement.
30. Change the media every 3 days by removing 100 µl of media from each well and adding 100 µl of complete media containing HAT supplement.
31. Check the wells after 1 week for the presence of hybridoma clones.
32. After 15 days in culture, test for the presence of hybridoma-secreting clones by ELISA (described under Section 3) using 50 µl of supernatant from each well and membranes from HEK-293, HEK-µδ, HEK-µ, or HEK-δ cells.
33. Clones that give a high titer with only HEK-µδ but not with HEK-µ or HEK-δ membranes are transferred into 25-cm² flasks along with 5 ml of complete growth media containing HAT supplement.
34. Determine the isotype of the antibody secreted by each hybridoma-secreting clone using the IsoStrip Mouse Monoclonal Antibody Iso-typing Kit (Roche) and 150 µl of hybridoma supernatant (1:10 diluted in PBS containing 1% BSA).
35. For large-scale preparation of mAbs, either grow hybridomas in large tissue culture flasks (Integra Celine 1000; Integra Biosciences AG, Switzerland) or generate ascites fluid as described by Hoogenraad and Wraight (1986). Either procedure will yield ~10 mg/ml of antibodies.

2.4. Purification of mAb from hybridoma supernatant

We routinely purify mAbs using a protocol described by Ey, Prowse, and Jenkin (1978) that takes advantage of protein A-Sepharose CL-4B beads.

1. Pass the hybridoma supernatant through a 0.2-µm filter to remove aggregates; adjust the pH to 8.0 by adding 1/10 volume of 1.0 M Tris.
2. Swell 1 g of protein A-Sepharose CL-4B beads (GE Healthcare) in 50 ml distilled water.
3. Transfer the slurry into a column (4–5 ml of gel); equilibrate with 1.0 M Tris, pH 8.0. Check that the pH of the eluate is 8.0.
4. Pass the antibody solution through the protein A column. About 10–20 mg of antibody/ml wet beads can be bound.

5. Wash the column with 10 volumes of 100 mM Tris (pH 8.0) followed by 10 volumes of 10 mM Tris (pH 8.0).
6. Elute bound antibodies by adding 500 μ l aliquots of 100 mM glycine (pH 3.0) and collecting the eluate in 1.5-ml Eppendorf tubes containing 50 μ l of 1 M Tris (pH 8.0). Mix each tube gently to bring the pH back to neutral. Do not shake vigorously to avoid frothing and bubbling. Collect ~25 fractions.
7. Identify immunoglobulin-containing fractions using a spectrophotometer at 280 nm; determine protein levels in positive fractions using Pierce BCA protein assay reagent (Thermo Scientific) according to manufacturer's protocol.
8. Store in aliquots at -20°C till use.

2.5. Serum preparation

Serum is prepared from blood collected from the tail vein of mice before (preimmune serum) and after (immune serum) immunization.

1. Place the mice in a small restraining cage.
2. Warm the tail with an infrared lamp or hot water (50 – 60°C) to increase the flow of blood to the tail.
3. Disinfect the tail with ethanol, make a small incision at the tip with a sterile blade, and collect blood (~ 75 – 100 μ l) into a sterile Eppendorf tube. Apply pressure or use a styptic pencil to stop the bleeding.
4. Allow blood to clot for 30–60 min at 37°C for 2 h.
5. Separate the clot from the sides of the tube using a gel-loading tip.
6. Keep the tube at 4°C overnight to allow the clot to contract.
7. Collect the serum by centrifugation at $10,000\times g$ for 10 min at 4°C .
8. Store the serum in aliquots at -20°C .
9. For working dilutions of preimmune and immune serum, add 50 μ l of 0.5 M EDTA (pH 7.4) and 2 μ l of 50% thimerosal to prevent microbial growth and store at 4°C .

2.6. Trouble-shooting and precautions

1. Instead of membranes, whole cells expressing the receptors of interest can be used to generate mAbs. In this case, cells should be washed free of phenol red with PBS since it is toxic and can lead to liver damage and jaundice in mice. Approximately, 10^6 – 10^8 cells should suffice for primary immunization and booster injections.
2. The most common problem during mAb production is that of bacterial, fungal, yeast, or mycoplasma contamination. Bacteria, seen under the light microscope as rods or cocci, can be treated with antibiotics. If the growth media contains penicillin and streptomycin, add 50 μ g/ml gentamycin. Alternatively, subclone cells into media containing antibiotics in an effort to dilute out the bacteria. However, it is best to discard the contaminated cells and sterilize the contaminated wells with 70% ethanol. Fungi or yeast can be detected under the microscope and this type of contamination prevented by adding fungizone (2.5 μ g/ml) to the growth media. However, once fungal or yeast contamination appears, it is best to discard the cells and sterilize the wells. Wells are sterilized by removing the media by suction, carefully avoiding touching adjacent wells, adding Chlorox (sodium hypochlorite) for 2–3 min, rinsing with sterile water, and leaving the wells empty. In addition, the

lid of the plate should be replaced to minimize the possibility of further contamination. Mycoplasma contamination is difficult to diagnose as the organism cannot be seen under the light microscope but can be the cause of slow cell growth. It is possible to rescue cells contaminated with mycoplasma by treating them with a mycoplasma removal agent (ICN Biomedicals, Inc.) according to manufacturer's protocol.

3. ELISA FOR DETECTION OF RECEPTOR HETEROMERS

ELISA, a technique commonly used to screen for the presence on an antigenic epitope on membrane preparations, in whole cells or tissue sections, can also detect GPCRs by using antibodies that recognize endogenous receptors or epitope tags (Flag, *myc*, or HA) present in the N-terminal region of the receptors. We use ELISA to screen for and determine the selectivity of heteromer-selective antibodies (Gupta et al., 2010; Rozenfeld et al., 2011). In the case of μ - δ mAb, we used membranes from (i) HEK cells, (ii) HEK cells expressing individual receptors, and (iii) HEK- $\mu\delta$ cells to detect hybridoma clones that gave a high titer with only HEK- $\mu\delta$ (Gupta et al., 2010). We also examined the selectivity of μ - δ mAb clones using membranes from the brain of wild type and mice lacking μ OR, δ OR, or both. We found that the μ - δ mAb recognized an epitope present only in membranes from wild type but not from knockout animals (Gupta et al., 2010). We examined whether the μ - δ mAb exhibited cross-reactivity with other heteromers involving μ OR or δ OR using membrane preparations from cells coexpressing μ OR- α 2A adrenergic receptor (α 2AR), μ OR-CB₁R, δ OR- α 2AR, or δ OR-CB₁R (Gupta et al., 2010). We found that the μ - δ mAb did not exhibit a significant degree of cross-reactivity with other heteromers involving μ OR or δ OR (Gupta et al., 2010). In addition, we used ELISA to determine the effect of a pathological condition such as development of tolerance to morphine (μ OR- δ OR heteromers) or chronic ethanol treatment (CB₁R-AT₁R heteromers) on heteromer levels (Gupta et al., 2010; Rozenfeld et al., 2011). Thus, ELISA is a versatile technique that can be used to determine heteromer levels under pathological techniques. Below, we describe a routinely used ELISA protocol.

1. Add 10–100 μ g of HEK, HEK- $\mu\delta$, HEK- μ , or HEK- δ membranes (in PBS) to each well of an ELISA plate (96-well, flat-bottom immuno plate; Nalgen Nunc International, USA) and air-dry the plates.
2. Incubate with 200 μ l of 3% BSA (Sigma) in PBS for 1 h at 37 °C.
3. Discard the BSA solution.
4. Add 100 μ l of μ OR- δ OR-selective hybridoma supernatant (or 1:500 to 1:1000 dilution of purified mAb) to the wells. Add 100 μ l of PBS to wells used to determine nonspecific binding.
5. Incubate for 16–18 h at 4 °C.
6. Wash wells four times (5 min each wash at RT) with 200 μ l of 1% BSA (Sigma) in PBS.
7. Incubate with 100 μ l of peroxidase-conjugated anti-mouse antibody (1:1000 in PBS containing 1% BSA) for 1 h at 37 °C.
8. Repeat step 6.
9. Incubate with 100 μ l of substrate (0.5 mg/10 ml of *ortho*-phenylenediamine in 0.15 M citrate-phosphate buffer, pH 5 containing 10 μ l of H₂O₂) for 10 min at RT; terminate the reaction by adding 50 μ l of 5N H₂SO₄.
10. Transfer the supernatant to another 96-well plate and read at 490 nm in an ELISA reader (Bio-Rad model 550).

This ELISA protocol can be adapted to monitor surface heteromer levels following chronic treatment with drugs. For example, cells ($\sim 2.5 \times 10^5$ cells/well) coexpressing μ OR and δ OR are seeded in growth media in a 24-well plate. After cells have attached, they are treated with morphine or any lipophilic opiate, preferably an antagonist ($1 \mu M$ final concentration in growth media) for different time periods (0–48 h). Controls are treated with same amount of growth media. At the end of the incubation period, media are removed, plates are placed on ice, and wells are rinsed with 200 μ l of cold PBS. Cells are fixed with ice-cold methanol for 5 min followed by two washes with 500 μ l PBS. Cells are then subjected to ELISA using primary and secondary antibodies as described above.

ELISA can also be carried out in tissue sections, to compare regional differences in antibody recognition in control and pathological conditions and to examine the effect of drugs/ligands on receptor levels. For this, 10- μ m tissue sections are placed on Fisher Brand Superfrost Plus slides (Fisher Scientific, PA) and circled immediately with ImmEdge PAP pen (Vector Laboratories, Inc., CA) to form a water-proof barrier; the resulting wells hold approximately 200 μ l solution. ELISA is then carried out as described above.

The most common problems encountered during the ELISAs are elevated background, poor, or absence of a signal. Elevated background signal can be due to inadequate washing and draining of the wells, contamination of the substrate solution with metal ions or oxidizing reagents. The latter can be avoided by using only distilled/deionized water in the preparation of the different solutions and by using clean plastic-ware. Prior substrate exposure to light leads to poor signal. The substrate should be prepared about 10 min before use and should be kept in the dark till use. Sometimes, the signal develops very rapidly (i.e., in less than a minute), giving very high titers that are outside the range of the plate reader. For this reason, pilot experiments using different dilutions of primary and secondary antibodies should be carried out to establish conditions where optimum signal is obtained after 10 min of incubation with substrate.

4. IMMUNOFLUORESCENCE FOR VISUALIZATION OF RECEPTOR HETEROMERS

Immunofluorescence uses fluorescently labeled secondary antibodies to visualize proteins in cells and tissues and can provide information about the tissue distribution of a given protein as well as its subcellular distribution. We used immunofluorescence to show that coexpression of μ OR with δ OR leads to intracellular retention of μ OR– δ OR heteromers in the Golgi apparatus, and this is rescued by the expression of RTP4 (Decaillot, Rozenfeld, Gupta, & Devi, 2008). We used this technique to visualize μ OR– δ OR heteromers in the brains of wild-type mice (but not μ OR or δ DOR knockout mice) treated chronically with morphine under a paradigm that leads to the development of tolerance. The increases in μ OR– δ OR heteromer levels were robust in the medial nucleus of the trapezoid body and in the rostral ventral medulla, brain regions involved in the processing of painful stimuli (Gupta et al., 2010). In the case of CB₁R–AT₁R heteromers, immunofluorescence studies showed that heteromerization leads to surface expression of CB₁R and colocalization of CB₁R and AT₁R at the cell surface of hepatic stellate cells that were chronically treated with ethanol (Rozenfeld et al., 2011). The protocol that we use for immunofluorescence is as follows:

1. If using cells, place poly-D-lysine-coated coverslips (Fisher brand, Fisher Scientific, USA) in 12-well plates (one/well). Seed the plates using complete growth media with cells (1×10^4) expressing the receptors of interest. Next day, remove media by suction and wash wells twice with sterile PBS prior to fixation and immunofluorescence.

2. If using tissue sections, collect brains, dissect them into different regions, snap freeze in liquid nitrogen, and store at -80°C until use. Cut cryostat sections ($5\text{--}8\text{ }\mu\text{m}$) and mount them in Superfrost Plus slides. Slides can be stored at -80°C until use. Before staining, warm the slides at RT for 30 min.
3. Fix cells/tissue sections with either acetone (5 min) or 4% paraformal-dehyde, pH 7.4 (15 min) at RT.
4. Wash wells/slides three to four times (5 min each) with Tris-buffered saline, pH 7.4 (TBS).
5. Block nonspecific binding sites with 1% goat serum in TBS for 30 min.
6. Incubate cells/tissue sections overnight at 4°C with the appropriate dilution of the primary antibody.
7. Wash wells/slides four times (5 min each) with gentle shaking in TBS.
8. Incubate wells/slides with the appropriate dilution of fluorescently labeled secondary antibody for 60–90 min in the dark with gentle shaking.
9. Repeat step 7.
10. Counterstain wells/slides with DAPI for nuclear visualization if desired.
11. Remove coverslips containing cells and mount them on slides using Vector shield (Vector Laboratories, Inc., Burlingame, CA); similarly coverslip tissue sections using Vector shield.
12. Examine cells/tissue sections under a fluorescence microscope (Leica DM 6000B).

Common problems encountered with immunofluorescence are high background and sensitivity particularly of heteromer-selective antibodies to fixatives. High background can be reduced by adding 0.1% Tween-20 to washes or increasing the concentration of normal serum (5–10%) used for blocking. If antibodies are sensitive to fixatives, then changing the fixation conditions (glutaraldehyde, methanol, or 10% formalin) followed by extensive washing to remove all traces of the fixative is recommended.

5. IMMUNOPRECIPITATION AND WESTERN BLOTTING

Immunoprecipitation is a useful technique to demonstrate heteromerization between differentially epitope-tagged GPCRs. After cell lysis, one of the receptors is immunoprecipitated using antibodies recognizing the epitope tag present on the receptor (*myc*-tagged receptors are immunoprecipitated using anti-*myc* antisera). The immunoprecipitates are then subjected to SDS-PAGE under nonreducing conditions, and Western blots are probed with antibodies to the epitope tag present on the other receptor. In order to avoid cross-reactivity, antibodies from two different species are used. A signal is detected in the blots only if there is an association between the two epitope-tagged receptors. We used this strategy to demonstrate heteromerization between μOR and δOR (Gomes et al., 2004, 2000), δOR and κOR (Jordan & Devi, 1999), μOR and $\alpha 2\text{AR}$ (Jordan et al., 2003), δOR and $\alpha 2\text{AR}$ (Rios et al., 2004), δOR and CB_1R (Rozenfeld et al., 2012), as well as CB_1R and AT_1R (Rozenfeld et al., 2011). When examining heteromerization in endogenous tissue, antibodies that selectively recognize each receptor or the heteromer are needed (Gomes et al., 2004; Gupta et al., 2010; Rozenfeld et al., 2012, 2011). In the case of the heteromer, the selective antibody could be used to immunoprecipitate the heteromer, and antibodies to individual receptors to probe the Western blots (Gupta et al., 2010). Coimmunoprecipitation and Western blot analysis can also be used to examine interactions between GPCR heteromers and signaling molecules such as β -arrestins, or chaperones such as RTP-4, AP-3,

or AP-2. Such studies showed that μ OR- δ OR heteromers are constitutively associated with β -arrestin (Rozenfeld & Devi, 2007), and that RTP-4 interacts with μ OR- δ OR heteromers (Decaillet et al., 2008). Thus, immunoprecipitation and Western blotting could be used to (i) probe heteromer levels under healthy and pathological conditions, (ii) identify differences in the levels of heteromer-associated proteins, or (iii) help identify novel proteins associated with the heteromer under pathological conditions. The following sections describe the different steps involved in coimmunoprecipitation and Western blotting.

5.1. Lysis of cells/tissues

Different buffers can be used to lyse transfected cells or tissues. Two important considerations in the choice of lysis buffer are the efficient solubilization of the desired receptor without affecting receptor associations and whether the lysis buffer interferes with receptor recognition by the antibody used for immunoprecipitation. Variables that can drastically affect the solubilization of proteins are salt concentration, pH, and type of detergent used. Lysis buffers commonly used to examine receptor interactions include

- Buffer G: 50 mM Tris-Cl, pH 7.4 containing 300 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, and 1 mM CaCl₂.
 - RIPA buffer: 50 mM Tris-Cl, pH 8 containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 1 mM CaCl₂.
 - NP-40 buffer: 10 mM Tris-Cl, pH 8 containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM CaCl₂.
 - CHAPS buffer: 1% in 50 mM Tris-Cl, pH 7.4.
 - Dodecyl maltoside buffer: 0.5% dodecyl maltoside in 50 mM Tris-Cl, pH 7.4.
1. Take a 10-cm dish containing cells expressing the receptors of interest. For tissues, use 20–50 mg of fresh or frozen tissue.
 2. Collect cells from plate by addition of 3 ml enzyme-free Hank's-based cell dissociation buffer (Invitrogen) followed by incubation for 3–5 min in a CO₂ incubator at 37 °C and up-and-down pipetting.
 3. Spin down the cells at 3000×g for 3 min.
 4. Resuspend the cell pellet in 1 ml PBS. Transfer to a 1.5-ml Eppendorf tube.
 5. Repeat steps 3–4. Discard the supernatant.
 6. Add 200 μ l of prechilled lysis buffer containing protease inhibitor cocktail and 10 mM iodoacetamide to the Eppendorf tube containing cells/tissue.
 7. Homogenize cells/tissue on ice with a Teflon pestle.
 8. Make the volume to 1.5 ml with prechilled lysis buffer.
 9. Pass the homogenate through an insulin syringe two to three times and transfer to a fresh Eppendorf tube.
 10. Incubate for 1 h at 4 °C using a rotating shaker.
 11. Centrifuge at 14,000 rpm for 20 min at 4 °C.
 12. Transfer supernatant to a fresh Eppendorf tube.
 13. Take an aliquot for protein estimation using BCA assay reagent.
 14. To an Eppendorf tube on ice, add 150 μ g of protein lysate, 5 μ g of antibody (polyclonal antibody to one receptor or to the epitope tag present on the receptor or

monoclonal heteromer-selective antibody), and 12 μ l of protease inhibitor cocktail. Make the volume to 1.2 ml with prechilled lysis buffer.

15. Incubate overnight on a rotating shaker at 4 °C.
16. Equilibrate protein A beads in prechilled lysis buffer and add 150 μ l to each tube.
17. Incubate for 2 h at 4 °C on a rotating shaker.
18. Centrifuge at 14,000 rpm for 1 min at 4 °C.
19. Wash the beads three times with 500 μ l of lysis buffer containing protease inhibitors.
20. Remove the lysis buffer completely with the help of an insulin syringe.
21. Add 70 μ l of 2 $\times$ sample buffer (120 mM Tris-Cl, pH 6.8 containing 4% SDS, 20% glycerol, and 0.002% bromophenol blue) to the pellet and incubate for 15 min at 60 °C.
22. Spin down the samples and run 10–15 μ l on 8% SDS-PAGE gels.
23. Transfer the separated proteins to nitrocellulose membranes overnight at 30 V.
24. Rinse membranes briefly in TBS-T (50 mM Tris-Cl, pH 7.4 containing 150 mM NaCl, 1 mM CaCl₂, and 0.1% Tween 20; prepared fresh).
25. Block the membranes for 1 h at RT with 10 ml of 5% nonfat dried milk in TBS-T.
26. Wash the membranes in TBS-T four times (15 min/wash) in a shaker.
27. Incubate with 1:1000 to 1:5000 primary antibody diluted in 30% Odyssey buffer (Li-cor) in TBS-T containing 0.01% sodium azide for 16 h at 4 °C.
28. Wash membranes four times (15 min each wash) with 15 ml TBS-T.
29. Incubate with 1:10,000 dilution of IRDye 680 or IRDye 800 secondary antibody (Li-cor) in 30% Odyssey buffer in TBS-T containing 0.01% sodium azide for 1–2 h at RT on a shaker.
30. Wash membranes four times (15 min each wash) with 15 ml TBS-T.
31. Visualize the signal and densitize it using the Odyssey Imaging system (Li-cor).

5.2. Problems and troubleshooting

Artifactual receptor aggregation during solubilization/immunoprecipitation due to the inherent hydrophobic nature of GPCRs is a major concern when examining receptor heteromerization. To rule this out, a variety of solubilization conditions including different combinations of detergents have to be used. In addition, controls where cells expressing individual receptors are mixed prior to solubilization and immunoprecipitation have to be used. If the heteromers are observed only in cells coexpressing both receptors and not in the mixed cells, this would suggest that they are not the result of artifactual aggregation.

Harsh solubilization procedures can disrupt receptor associations. Cross-linking reagents have been used to address this concern (Cvejic & Devi, 1997). The presence of receptor heteromers in the presence of cross-linking reagents, irrespective of the latter's functional properties, would then suggest that they stabilize the interactions and do not induce receptor heteromerization.

To prevent the masking of the antigenic epitope due to the presence of nonspecific proteins, it is advisable to preclear the cell lysate with normal serum (e.g., rabbit serum if using rabbit

polyclonal antibody for immunoprecipitation) followed by binding to protein A beads. This removes proteins that bind nonspecifically to the antibody or to the beads.

The presence of a high background or nonspecific bands in Western blots can be minimized through the use of a different blocking buffer, reducing the time of incubation with primary/secondary antibody, using harsher conditions for washing membranes after antibody incubation (e.g., 50 mM Tris-Cl, pH 7.5 containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate), or adding detergent to the primary/secondary antibody preparation up to a concentration of 1%.

6. SUMMARY AND PERSPECTIVES

Recent evidence indicates that some GPCR heteromers are upregulated in disease states. However, the physiological role of these heteromers in pathology is not clearly understood due to the lack of tools to distinguish between heteromer- and homomer-mediated effects. The development of heteromer-selective antibodies and of specific heteromer disrupting TAT peptides could help elucidate their role in pathological conditions. This is clearly shown in the case of μ OR- δ OR heteromers where selective antibodies detect increased heteromer levels in brain regions involved in pain perception following chronic treatment with morphine. Studies with TAT peptides that selectively disrupt μ OR- δ OR heteromers suggest that the latter may keep morphine-mediated signaling via μ OR in the desensitized state. Thus, GPCR heteromers could be novel targets for the development of therapeutics to treat diseases. This, however, would require an understanding of the functional role of GPCR heteromers during pathology. The different protocols described in this review used in combination with the heteromer-selective antibodies or with peptides that selectively disrupt the heteromers could help in elucidating these roles.

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