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Functional G-protein coupled receptors on nuclei from brain and primary cultured neurons

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Summary

A growing number of G-protein-coupled receptors (GPCRs) have been described on nuclear membranes. In many cases it is unknown how the intracellular GPCR is activated, how it is trafficked to the nuclear membranes, and what the long term signaling consequences are after nuclear receptor activation. Here we describe how to isolate nuclei that are free from plasma membrane and cytoplasmic contamination yet still exhibit physiological properties following receptor activation.

Keywords

GPCR; metabotropic glutamate receptor; mGluR5; nuclear preparation; calcium imaging; immunoblotting

1. Introduction

Signal transduction from G protein coupled receptors (GPCRs) has traditionally been thought to emanate from the cell surface where many signaling complexes are clustered and where extracellular stimuli can interact with GPCR ligand binding domains. Recently, however, numerous GPCRs have also been found to be associated with various intracellular membranes where, in certain cases, they activate intracellular signaling machinery leading to unique functional responses (1–5). One such organelle is the nucleus.

The nucleus is typically the largest organelle within the cell being separated from the cytosol by two membranes derived from the endoplasmic reticulum (ER), the inner nuclear membrane (INM) and the outer nuclear membrane (ONM) (6). Although these membranes are continuous, they are perforated by nuclear pore complexes and by unique protein complexes on either side. For example, proteins on the ONM are similar to those on the adjoining ER whereas the INM is enriched for many specific proteins involved in linking the nuclear envelope to the nuclear lamina and the chromatin (7, 8). Emerging data underscore the physical connections linking the cytoskeleton, the nuclear membrane, and the nucleoskeleton in order to transmit mechanical forces critical for nuclear movement, cell

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polarization and cell motility (7, 8). Given these critical roles, nuclear envelope proteins have been implicated in a broad range of diseases (9).

A growing number of GPCRs have been demonstrated to be present on both the INM and ONM by ultrastructural, immunohistochemical, pharmacological and molecular techniques (1--5). One such receptor is the metabotropic glutamate receptor, mGluR5, which in previous publications we have shown to function as a nuclear receptor (4, 5, 10, 11). Specifically, we have found 1) that mGluR5 is highly expressed on inner and outer nuclear membranes of heterologous and physiological cell types (4, 5); 2) that isolated nuclei expressing mGluR5 respond to permeable or transported agonists by increasing nucleoplasmic Ca^{2+} (4, 5); 3) that the ligand binding domain is within the nuclear lumen (4); 4) that sodium dependent and independent transporters/exchangers are present and responsible for transporting mGluR5 agonists across cell surface and nuclear membranes (5, 10, 11); 5) that endogenous nuclear mGluR5 couples to G_q and PLC to generate IP_3 -mediated Ca^{2+} release within the nucleus (10); and 6) that activation of nuclear mGluR5 generates distinct Ca^{2+} responses as well as downstream signaling cascades separate from cell surface counterparts (11,12). These observations and those by others challenge the notion that cells only interact with their environment at the plasma membrane to bring about long term changes.

Given the growing number of receptors being localized to the nuclear membrane and even within the nucleoplasm itself, this chapter focuses on how to prepare nuclear membranes from brain tissues that are pure yet still capable of carrying out complex signaling processes.

2. Materials

Prepare all solutions using purified deionized water and analytical grade reagents. Prepare and store all buffers at 4° C. Perform all purification procedures at 4° C. Make a 50X stock solution of protease inhibitors by dissolving one complete protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN) in 1 ml of water and freeze aliquots at -20° C. Add protease inhibitors to the buffers just prior to use.

PBS, PH 7.4 (phosphate buffered saline, Life Technologies, Grand Island, NY).

Hypotonic Buffer A: 2.0 mM MgCl_2 , 25 mM KCl, 10 mM HEPES (pH 7.5) and protease inhibitors.

Isotonic Buffer N: 2.0 mM MgCl_2 , 25 mM KCl, 10 mM HEPES (pH 7.5), 250 mM Sucrose and protease inhibitors.

2 M Sucrose Buffer: 2.0 mM MgCl_2 , 25 mM KCl, 10 mM HEPES (pH 7.5), 2 M Sucrose and protease inhibitors.

1.1 M Sucrose Buffer: 2.0 mM MgCl_2 , 25 mM KCl, 10 mM HEPES (pH 7.5), 1.1 M Sucrose and protease inhibitors

3. Methods

3.1 Preparation of nuclei from cultured cells or brain tissue regions

Isolate postnatal or adult rodent brain tissue from selected regions and keep on ice. Alternatively, harvest cultured cells by scraping cells after washing three times with PBS (see Note 1).

Weigh brain tissue or centrifuge down the cultured cell pellets at $1000 \times g$ for 10 minutes.

Finely mince the brain tissue on ice with a single-sided razor blade.

Re-suspend the brain tissue or the cell pellets in 12-20 volumes (per gram of tissue or volume of cell pellet) of Buffer A (see Note 2).

Swell the cells for 5-10 minutes on ice (see Note 3).

Homogenize the cells on ice in a Wheaton glass dounce homogenizer using 5-15 strokes (see Note 4) with either a tight-fitting or loose-fitting pestle depending upon the cell type (see Note 5). Greater than 90% of the cells should be disrupted, as judged by Trypan blue staining.

Re-adjust the osmolarity by adding 2 M Sucrose Buffer to the homogenate (see Note 6) to make the final concentration of sucrose in the buffer to be 250 mM (142.9 μ l of 2M sucrose buffer for 1 ml homogenate).

Filter the homogenate through 2 layers of sterile gauze at the bottom of a 5 ml syringe and centrifuge at $1000 \times g$ for 10 minutes.

Resuspended the nuclear pellet in Buffer N and gently layer the suspension over 2 ml of medium containing 1.1 M sucrose in Buffer A. Centrifuge at $1000 \times g$ for 10 min. The pellet from this step contains purified nuclei that are ready to be labeled in various ways.

Centrifuge the supernatant from step 8 at $100,000 \times g$ for 30 min to collect the membrane fraction (the pellet: lysosomes, mitochondria, peroxisomes, ER, Golgi, plasma membrane, endosomes) and cytosol (the supernatant).

Snap-freeze the samples for binding assays or western blot analysis in liquid nitrogen and store at -80°C .

¹The single most critical step in purifying healthy nuclei is to rapidly yet gently lyse cells such that nuclei are still intact and not overly exposed to low osmolarity. Therefore, the ratio of tissue weight to Buffer A volume, the length of time cells are exposed to hypotonic solutions and the number of strokes it takes to homogenize are all important variables. Too little lysis results in whole cell contamination of the nuclear preparation whereas too much lysis leads to damaged, broken, and unhealthy nuclei.

²The optimal weight of tissue or volume of cell pellet to volume of Buffer A is 1:10 for cultured HEK or striatal cells, 1:12 for striatum, 1:20 for cortex, 1:15 for hippocampus.

³The optimal time period to swell the cells is 10 minutes for cultured HEK or striatal cells, 5 minutes for striatum and hippocampus, 10 minutes for cortex.

⁴The optimal number of strokes is 15 for cultured HEK or striatal cells, 5 strokes for striatum, 10 strokes for cortex, 7 strokes for hippocampus.

⁵A tight-fitting pestle is best for HEK cells; use a loose-fitting pestle for cultured striatal neurons and brain tissue regions.

⁶Nuclei will continue swelling in hypotonic solutions. Therefore, rapidly re-adjusting the osmolarity once the desired purity is achieved is critical for healthy nuclei. The more swollen the nuclei appear, the more likely it is that they too have been hypotonically shocked. This makes functional recovery unlikely.

Follow procedures in section 3.4 for functional assays with isolated nuclei including fluorescent measurements of Ca^{2+} changes.

3.2 Check the efficiency and purity of isolated nuclei from cultured cells

Purity of nuclei can be assessed by loading monolayer cells (HEK or cultured neurons) with the cytoplasmic mitochondrial selective stain MitoTracker Red CM-H₂XRos (Life Technologies) as well as the DNA-specific vital dye, Hoechst 33342 (Sigma –Aldrich, St. Louis, MO).

Dilute the 1 mM MitoTracker Red CM-H₂XRos to 500 nM (for primary neuronal cultures) or 250 nM (for transformed cells) in each cell types' preferred growth medium without serum at 37° C.

Incubate the cells for 45 minutes at 37° C.

Gently wash the cells once with serum free medium and replace with Hoechst 33342 (0.5 µg/ml in serum free medium) for staining the nuclei.

Incubate for 20 minutes at 37° C.

Replace with serum free medium and monitor the mitochondrial and nuclear staining before and after purification procedures (Fig. 1).

3.3 Check the purity of isolated nuclear brain tissue via western blotting

Determine protein concentration of the fractions using the Bradford assay (Bio-Rad, Laboratories, Inc., Richmond, CA).

Heat aliquots of fractionated proteins at 55° C for 10 minutes in Sodium Dodecyl Sulfate Polyacrylamide (SDS) sample buffer (see Note 7).

Once lysed, the nuclear pellet can be sticky due to released DNA. Spin the nuclear lysate at $10,000 \times g$ for 5 minutes to pellet the DNA. Load the appropriate amount of supernatant on an 8.5% polyacrylamide gel.

Separate fractionated proteins by SDS-PAGE and blot onto a Sequi-Blot™ PVDF membrane (Bio-Rad Laboratories, Inc. Hercules, CA).

Probe the PVDF membrane with polyclonal anti-mGluR5 (1:1500; Upstate, Lake Placid, NY), a nuclear membrane marker such as monoclonal anti-lamin B₂ (1:1000, Life Technologies), and a plasma membrane marker such as polyclonal anti-Pan-Cadherin (1:2000, Cell Signaling Technology, Inc.) overnight at 4°C (see Note 8)

Wash the membrane with TBST (Tris-Buffered Saline Tween-20: 50 mM Tris, 150 mM NaCl, and 0.1% Tween 20 at pH 7.5) three times for 15 minutes each.

⁷GPCRs tend to aggregate at high temperature (100°) treatment. To avoid this, samples subjected to SDS-PAGE for GPCR detection are usually treated at 55° C for 10 minutes.

⁸Alternatively, another plasma membrane marker can be used: monoclonal anti-Na⁺-K⁺ ATPase (α6F, 1:1000, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa).

Probe the membrane with a horseradish peroxidase conjugated with goat anti-rabbit IgG (1:2000, Cell Signaling Technology, Inc. Danvers, MA) or anti-mouse IgG (1:2000, Sigma-Aldrich) at room temperature for one hour.

Wash the membrane with TBST three times for 15 minutes each.

Detect the protein bands with enhanced chemiluminescence detection kit (Amersham/GE Healthcare, Piscataway, NJ).

Perform densitometric analyses of detected proteins using Molecular Dynamic's Storm 860 Imager (Amersham/GE Healthcare) or ChemiDot™ MP System (Bio-Rad Laboratories, Inc.) (Fig. 2).

3.4 Fluorescent Measurement of nuclear Ca^{2+} in Isolated Nuclei

Load purified nuclei with 20 μM fluorescent Ca^{2+} indicator Oregon Green 488 BAPTA-1AM (Molecular Probes, Eugene, OR), 0.001% pluronic acid prepared in intracellular buffer (125 mM KCl, 2 mM KH_2PO_4 , 2 mM MgCl_2 , 0.3 mM CaCl_2 , 10 mM D-glucose, and 40 mM HEPES, pH 7.0) together with 1 mM CaCl_2 , 1 mM ATP, and protease inhibitors for 20-30 minutes on ice.

Wash the nuclei three times with the same buffer minus the Ca^{2+} indicator.

Plate the nuclei on poly-D-lysine-coated 35-mm dishes with glass grids.

Image immediately using a laser confocal microscope. For example, we use an Olympus BX 50WI together with Olympus LUMPlanFI/IR 40 $\times$ /0.80w or 60 $\times$ /0.90w objectives.

Capture baseline, real time images using the Olympus Fluoview FVX confocal laser scanning system together with Fluoview acquisition software (see Note 9).

Add drugs at 100 $\times$ concentrations to the dish with a gel-loading pipet tip (see Note 10).

Process the images with image analysis software. We use MetaMorph Professional Image Analysis software produced by Universal Imaging (Bedford Hills, New York).

The purity of the nuclear preparation and the putative presence of the predicted GPCR can be examined by post hoc identifying nuclei via immunocytochemistry followed by field relocation using the following procedures.

Wash the nuclei three times with PBS.

Fix the nuclei with 4% paraformaldehyde (PFA) for 15 minutes at room temperature.

Wash the nuclei three times with PBS (see Note 11)

Block nuclei in blocking buffer (1% BSA, 0.25% milk powder, 0.3 % Triton X-100 in TBS pH 7.4) for 1 hour at room temperature.

⁹Live images can be captured at 8.59 seconds per scan for striatal nuclei or 5.36 seconds per scan for HEK nuclei for 10-15 minutes.

¹⁰ Ca^{2+} changes can be monitored at room temperatures. A physiological chamber with a heating block can be used for temperature control.

¹¹Fluorescent signals from the Ca^{2+} indicator, Oregon Green 488 BAPTA-1AM are completely lost following fixation.

Incubate the nuclei with polyclonal anti-mGluR5 (1:200, Upstate) overnight at 4°C (see Note 12).

Wash the nuclei with PBS three times for 10 minutes each.

Incubate the nuclei with goat anti-rabbit Cy3 (1:300; Jackson ImmunoResearch, West Grove, PA) for one hour at room temperature.

Wash the nuclei with PBS three times for 10 minutes each.

Repeat steps 10-13 with monoclonal anti-Lamin B₂ (1:100, Life Technologies) and goat anti-mouse Alexa 488 (1:300, Life Technologies).

Perform post-hoc field relocation by an Olympus Fluoview FVX confocal laser scanning system using Fluoview acquisition software (Fig. 3).

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References

1. Tadevosyan A, Vaniotis G, Allen BG, Hébert TE, Nattel S. G protein-coupled receptor signalling in the cardiac nuclear membrane: evidence and possible roles in physiological and pathophysiological function. *J Physiol.* 2012; 590:1313–30. [PubMed: 22183719]
2. Vaniotis G, Allen BG, Hébert TE. Nuclear GPCRs in cardiomyocytes: an insider's view of β -adrenergic receptor signaling. *Am J Physiol Heart Circ Physiol.* 2011; 301:H1754–64. [PubMed: 21890692]
3. Bkaily G, Avedanian L, Al-Khoury J, Ahmarani L, Perreault C, Jacques D. Receptors and ionic transporters in nuclear membranes: new targets for therapeutical pharmacological interventions. *Can J Physiol Pharmacol.* 2012; 90:953–65. [PubMed: 22783834]
4. O'Malley KL, Jong YJ, Gonchar Y, Burkhalter A, Romano C. Activation of metabotropic glutamate receptor mGlu5 on nuclear membranes mediates intranuclear Ca^{2+} changes in heterologous cell types and neurons. *J Biol Chem.* 2003; 278:28210–9. [PubMed: 12736269]
5. Jong YJ, Kumar V, Kingston AE, Romano C, O'Malley KL. Functional metabotropic glutamate receptors on nuclei from brain and primary cultured striatal neurons. Role of transporters in delivering ligand. *J Biol Chem.* 2005; 280:30469–80. [PubMed: 15958386]
6. Isermann P, Lammerding J. Nuclear mechanics and mechanotransduction in health and disease. *Curr Biol.* 2013; 23:R1113–21. [PubMed: 24355792]
7. Rothballer A, Kutay U. Poring over pores: nuclear pore complex insertion into the nuclear envelope. *Trends Biochem Sci.* 2013; 38:292–301. [PubMed: 23639636]
8. Adams RL, Wentz SR. Uncovering nuclear pore complexity with innovation. *Cell.* 2013; 152:1218–21. [PubMed: 23498931]
9. Schreiber KH, Kennedy BK. When lamins go bad: nuclear structure and disease. *Cell.* 2013; 152:1365–75. [PubMed: 23498943]
10. Kumar V, Jong YJ, O'Malley KL. Activated nuclear metabotropic glutamate receptor mGlu5 couples to nuclear Gq/11 proteins to generate inositol 1,4,5-trisphosphate-mediated nuclear Ca^{2+} release. *J Biol Chem.* 2008; 283:14072–83. [PubMed: 18337251]

¹²Alternatively, incubate the nuclei with primary antibody for 2 hours at room temperature.

11. Jong YJ, Kumar V, O'Malley KL. Intracellular metabotropic glutamate receptor 5 (mGluR5) activates signaling cascades distinct from cell surface counterparts. *J Biol Chem.* 2009; 284:35827–38. [PubMed: 19840937]
12. Kumar V, Fahey PG, Jong YJ, Ramanan N, O'Malley KL. Activation of intracellular metabotropic glutamate receptor 5 in striatal neurons leads to up-regulation of genes associated with sustained synaptic transmission including Arc/Arg3.1 protein. *J Biol Chem.* 2012; 287:5412–25. [PubMed: 22179607]

Fig. 1.

Using the described nuclear preparation protocol, no MitoTracker Red (excluded from the nucleus) was seen in purified nuclear preparations stained with Hoechst (retained in the nucleus) in either HEK or striatal neurons verifying the purity of these organelles.

Fig. 2.

Using the described nuclear preparation protocol, subcellular fractionation of adult mouse striata shows that mGluR5 can be detected in fractions containing both the nuclear (N) and plasma membranes (PM). The purity of each fraction is assessed by blotting the same membrane using either the plasma membrane marker pan-cadherin (Pan-Cad) or the nuclear membrane marker lamin B₂.

Fig. 3. mGluR5 agonist-mediated Ca^{2+} changes in isolated striatal nuclei. A, left panel, Transmitted light image of purified nucleus from striatal culture loaded with Oregon Green BAPTA (middle panel). Right panel, mGluR5 (red) and lamin B₂ (green) co-staining of selected nucleus following drug treatment and post hoc field relocation. B, mGluR5 agonist quisqualate (Quis)-mediated representative trace. Quisqualate application increases nuclear calcium. The mGluR5 antagonist 2-Methyl-6-(phenylethynyl) pyridine (MPEP) blocked this response.

Table

Final concentration	Stock solution	M.W.	Buffer A (500 ml)	Buffer N (500 ml)	2 M Sucrose Buffer (250 ml)	1.1 M Sucrose Buffer (250 ml)
10 mM HEPES, pH 7.5	2.5 M		2 ml	2 ml	1 ml	1 ml
2 mM MgCl ₂	1 M		1 ml	1 ml	0.5 ml	0.5 ml
25 mM KCl	1 M		12.5 ml	12.5 ml	6.25 ml	6.25 ml
Buffer N 250 mM Sucrose		342.3		42.8 g		
2 M Sucrose Buffer		342.3			171.2g	
1.1 M Sucrose Buffer						94.1 g