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Mouse Neuroblastoma CB₁ Cannabinoid Receptor-Stimulated [³⁵S]GTPγS Binding: Total and Antibody-Targeted Gα Protein-Specific Scintillation Proximity Assays

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Abstract

G protein-coupled receptors (GPCRs) are important regulators of cellular signaling functions and therefore are a major target for drug discovery. The CB₁ cannabinoid receptor is among the most highly expressed GPCRs in neurons, where it regulates many differentiated neuronal functions. One model system for studying the biochemistry of neuronal responses is the use of neuroblastoma cells originating from the C1300 tumor in the A/J mouse, including cloned cell lines NS20, N2A, N18TG2, N4TG1, and N1E-115, and various immortalized hybrids of neurons with N18TG2 cells. GPCR signal transduction is mediated through interaction with multiple types and subtypes of G proteins that transduce the receptor stimulus to effectors. The [³⁵S]GTPγS assay provides a valuable pharmacological method to evaluate efficacy and potency in the first step in GPCR signaling. Here, we present detailed protocols for the [³⁵S]GTPγS-binding assay to measure the total G protein binding and the antibody-targeted scintillation proximity assay to measure specific Gα proteins in neuroblastoma cell membrane preparations. This chapter presents step-by-step methods from cell culture, membrane preparation, assay procedures, and data analysis.

1. INTRODUCTION

In seeking a model system to investigate cellular signaling using biochemical methods, the use of proliferating cloned cell lines in culture offers the advantages of the ability to grow large numbers of cells that express differentiated properties, are genotypically and phenotypically identical, and can be maintained under controlled experimental conditions. For studies of CB₁ cannabinoid receptor stimulation and signal transduction, cultured neuroblastoma cells exhibit many neuronal characteristics, but offer experimental advantages over primary neuronal cultures that do not proliferate and thereby have limited capacity, and are likely to be phenotypically heterogeneous. A number of cloned cell lines have been derived from the C1300 tumor, a spontaneous neuroblastoma found near the spinal cord of the A/J mouse (Augusti-Tocco & Sato, 1969; Schubert, Humphreys, Baroni,

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& Cohn, 1969). When injected into the A/J or nude mouse host, these cells form a tumor of cells appearing as neuroblasts, being round and devoid of fibers and having a high [nuclear:cytoplasmic] ratio (Augusti-Tocco & Sato, 1969; Heumann, Stavrou, Reiser, Ocalan, & Hamprecht, 1977; Schubert et al., 1969). When the cells adapt to a monolayer culture, a morphological change can be observed, characterized by elaboration of elongated neuritic processes forming a network of extensions, and uptake of Bodian stain (Augusti-Tocco & Sato, 1969; Schubert et al., 1969). Initial evaluation of a number of clones from the C1300 tumor revealed that most expressed acetylcholinesterase, and some expressed either tyrosine hydroxylase (e.g., N1E-115) or choline acetyltransferase (NS20), and some were nonadrenergic, noncholinergic (N18, N4) (Klee & Nirenberg, 1974). Some neuroblastoma cloned lines were shown to synthesize neuropeptides, such as met-enkephalin (N1E-115 and the NG108-15 hybrid cells) (Gilbert, Knodel, Stenstrom, & Richelson, 1982; Glaser, Hubner, & Hamprecht, 1982), or vasoactive intestinal peptide by N1E-115, NS20, and N18TG2 clones and NG108-15 hybrid cells (Brick, Howlett, & Beinfeld, 1985; Glaser, Besson, Rosselin, & Hamprecht, 1983; Said & Rosenberg, 1976). When selected and subcloned for thioguanine resistance, N18TG2 and N4TG1 cells were useful for hybridization with either the rat C6BU1 bromouridine deoxyribose (BUdR)-resistant glioma (NG108-15) (Gilman & Minna, 1973; Hamprecht, 1977; Klee & Nirenberg, 1974), and these hybrid cells have been utilized for studies of CB₁ receptors (Devane, Spain, Coscia, & Howlett, 1986; Howlett, Qualy, & Khachatrian, 1986; Mackie, Devane, & Hille, 1993). N18TG2 cells have also been used to “immortalize” neurons by hybridization: with mesencephalic neurons (MN9D) (Choi et al., 1991), which have been used for studies of interactions between CB₁ cannabinoid and D₂ dopamine receptors (Calipari et al., 2014; Eldeeb, Leone-Kabler, & Howlett, 2016); dorsal root ganglia cells (F-11) (Cruciani, Dvorkin, Morris, Crain, & Makman, 1993; Francel et al., 1987; Platika, Boulos, Baizer, & Fishman, 1985), which have been used for investigation of the endocannabinoid system (Fan et al., 2011; Fioravanti et al., 2008; Rimmerman et al., 2008; Ross et al., 2001) and other reports; and spinal cord neurons (NSC-34) (Cashman et al., 1992), which have been used for studies of neuroprotection by cannabinoids (Moreno-Martet et al., 2012). A 2017 PubMed search shows that since their initial characterization, cloned cell lines from the C1300 tumor have been the model system of choice for nearly 2000 publications. A number of cloned neuronal cell lines from the C1300 tumor express CB₁ cannabinoid receptors (Fig. 1), and over 50 publications indexed in PubMed have utilized N2A, N4TG1, N1E-115, or N18TG2 cells to investigate the endocannabinoid system.

Morphological, biochemical, and physiological differentiation of C1300-derived clones could be promoted by conditions that restricted the rate of cell division (Denis-Donini, Estenoz, & Augusti-Tocco, 1978). Following serum deprivation, cells elaborated neuritic processes and increased in size and protein content (Schubert et al., 1969; Schubert, Humphreys, Jacob, & de Vitry, 1971; Seeds, Gilman, Amano, & Nirenberg, 1970). Biochemically, the specific activity of acetylcholinesterase increased (Blume et al., 1970; Wilson et al., 1972); however, such conditions deprive the cells of nutrients normally obtained from serum. It has been demonstrated that cell viability decreases under such conditions (Blume et al., 1970). Morphological differentiation may also be induced by growth in BUdR or by inhibiting DNA synthesis with agents such as cytosine arabinoside,

FUDR, and mitomycin C (Denis-Donini et al., 1978; Schubert et al., 1971). Agents that increase intracellular cyclic AMP also promoted morphological differentiation in neuroblastoma and hybrid cells (reviewed by Hamprecht, 1977; Nirenberg et al., 1983; Prasad, 1975). Such agents include analogs of cyclic AMP (dibutyryl-cyclic AMP (db-cAMP), 8-bromo-cyclic AMP), cyclic AMP phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine, theophylline, Ro20-1724), and activators of adenylyl cyclase (forskolin). In the presence of db-cAMP, the doubling time of neuroblastoma cells increased (Charalampous, 1977), the average cell DNA content decreased, RNA and protein contents increased (Prasad, 1975), and key genes related to cell cycle were altered (Rudie, Nahreini, Andreatta, Edwards-Prasad, & Prasad, 2001). Treatment with db-cAMP increased protein kinase A activity and the content of cyclic AMP-binding proteins (Prasad & Rosenberg, 1978; Rosenberg et al., 1978; Walter, Costa, Breakefield, & Greengard, 1979). Functional differentiation also resulted from growth for several days in db-cAMP. The ability of norepinephrine and dopamine to regulate cyclic AMP accumulation was altered in neuroblastoma cells and in sympathetic ganglion X neuroblastoma hybrid cells (Myers, Blosser, & Shain, 1978; Prasad, 1975). Neuronal enzymes such as choline acetyltransferase, tyrosine hydroxylase, and dopamine β -hydroxylase were increased in neuroblastoma cells (Prasad, 1975; Waymire, Gilmer-Waymire, & Boehme, 1978; Waymire et al., 1979), and tyrosine hydroxylase and DOPA decarboxylase were increased in a sympathetic ganglion X neuroblastoma hybrid clone (Greene & Rein, 1977). Release of acetylcholine was increased in neuroblastoma—glioma hybrid cells by growth in db-cAMP (McGee et al., 1978). Because of the importance of the cyclic AMP pathway in differentiated neuronal functions, the investigation of CB₁ receptor coupling to G proteins that stimulate (Gs) or inhibit (Gi/o) adenylyl cyclase activity is of particular interest (Dalton, Bass, Van Horn, & Howlett, 2009; Howlett, 2004; Howlett et al., 2002; Mackie, 2008). In addition, CB₁ receptor coupling to Gq or G11 has been implicated in CB₁ receptor stimulation by certain agonists to stimulate a Ca²⁺ mobilization (Lauckner, Hille, & Mackie, 2005), and coupling to G12 and G13 are important in pathways that involve the small G proteins Rho and Rac (Dalton, Peterson, & Howlett, 2013; Howlett, 2005; Ishii & Chun, 2002).

The first interaction in agonist-stimulated CB₁ receptor cellular signaling is the interaction with the G α protein that results in dissociation of a GDP from the G α , thereby allowing GTP to bind within the guanine nucleotide-binding site, and for the G α to dissociate from the G $\beta\gamma$ dimer. If a poorly hydrolyzable analog of GTP, such as guanosine-5'-O-(3-[³⁵S]thio)triphosphate (GTP γ S) labeled with radioactive ³⁵S, is provided in an in vitro assay, the radioactive analog will bind to the vacated guanine nucleotide site on the guanine nucleotide-coupled receptor-stimulated G α protein. This method was first described for use with agonist-stimulated receptors and their associated G proteins in membranes from brain (Lorenzen, Fuss, Vogt, & Schwabe, 1993) and cultured neuroblastoma cells (Traynor & Nahorski, 1995). The use of this [³⁵S]GTP γ S-binding assay for CB₁ cannabinoid receptor-stimulated G proteins was described shortly thereafter (Griffin, Atkinson, Showalter, Martin, & Abood, 1998; Petitot, Jeantaud, Capet, & Doble, 1997; Sim, Selley, Xiao, & Childers, 1996).

One conundrum that we have faced in identifying G protein-coupled pathways is that the [³⁵S]GTP γ S-binding assay provides a net sum of all G proteins activated and is influenced

by the rates at which the GDP is able to dissociate from the various G α proteins. Identification of the relative activation of coupled G α proteins can be determined via [35 S]GTP γ S binding using an antibody-targeted GTP γ S scintillation proximity assay (SPA) (Delapp et al., 1999; Kahl & Felder, 2005), which quantitates [35 S]GTP γ S binding to each G protein individually. This procedure can mitigate concerns related to activation or inhibition of multiple G proteins and reduces the variability in sensitivity by different G proteins (Milligan, 2003; Strange, 2010). This assay has since been used to identify specific CB $_1$ receptor–G protein interactions (Blume, Eldeeb, Bass, Selley, & Howlett, 2015; Diez-Alarcia et al., 2016; Eldeeb et al., 2016; Erdozain, Diez-Alarcia, Meana, & Callado, 2012).

2. MEMBRANE PREPARATIONS FOR CULTURED NEURONAL CELLS

2.1 Equipment and Supplies

- CO $_2$ -incubator Panasonic MCO-19-PE incubator or similar.
- Biosafety Cabinet-Purifier Cell Logic Class II, Type A2 Biosafety Cabinets LabConco (Kansas City, MO) or similar.
- Centrifuges: Beckman Coulter Allegra 6R Allegra 6 Series, Beckman Avanti J-26XPI High Performance Centrifuge and Microcentrifuge—Eppendorf 5417R (max speed 13,000 $\times g$, refrigerated).
- Cell culture labware: sterile flasks, plastic pipets, centrifuge tubes, and microfuge tubes (Corning or Falcon products).
- Glass–glass homogenizer.

2.2 Buffers and Reagents

1. Dulbecco's Modified Eagle's Medium (DMEM):Ham's F-12 (1:1) (Gibco Life Technologies, cat #11330032, Gaithersburg, MD).
2. Penicillin (100 U/mL) and streptomycin (100 μ g/mL) (Gibco Life Technologies, cat #15140-63, Gaithersburg, MD).
3. Heat-inactivated bovine serum (Gemini Bioproducts, Sacramento, CA, cat #100-101) added at 10% volume of media.
4. TME (Tris–Cl, MgCl $_2$, EDTA) buffer (20 mM Tris–Cl, pH 7.4, 5 mM MgCl $_2$, and 1 mM Tris–EDTA).
5. Phosphate-buffered saline (PBS) 137 mM NaCl, 2.7 mM KCl, 10 mM NaH $_2$ PO $_4$, 1.8 mM KH $_2$ PO $_4$.
6. PBS–EDTA (0.625 mM) in PBS.
7. Protease inhibitor (protease inhibitor cocktail set III, EDTA free CALBIOCHEM, cat #539134).
8. Dithiothreitol (DTT) 100 mM (Sigma Chemical, St. Louis, MO).
9. Pierce™ BCA Protein Assay Kit (Thermo Scientific, cat #23225).

2.3 Neuroblastoma Cell Lines: Mouse N18TG2 Cell Culture Conditions

1. N18TG2 mouse neuroblastoma cells were cultured and maintained in complete media containing Dulbecco's Modified Eagle's Medium (DMEM):Ham's F-12 (1:1) with GlutaMax, sodium bicarbonate, and pyridoxine-HCl, supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) and 10% heat-inactivated bovine serum, and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ gas.
2. Cells were grown to confluence in a 175-cm² flask, harvested with 7 mL PBS-EDTA, and centrifuged at 1700 RPM for 5 min.
3. The cell pellet was used immediately, or stored at -80°C.

Notes: Various types of serum, including fetal bovine serum, have been compared for growth of N18TG2 cells, and the heat-inactivated bovine serum or heat-inactivated bovine calf serum provided the greatest viability and cellular signaling in cyclic AMP accumulation assays. Horse serum was toxic to the N18TG2 cells. For further discussion of serum for neuroblastoma cell culture, see Prasad, Spuhler, Arnold, and Vernadakis (1979).

2.4 Procedure

1. Resuspend cells with TME and protease inhibitor cocktail (10 µL/mL) and incubate at 0–4°C for 20 min to swell.
2. Homogenize using glass–glass homogenizer (7 mL).
3. Centrifuge at 600 × *g* for 3 min at 4°C and collect the supernatant.
4. Rehomogenize pellet and recentrifuge for 3 min at 600 × *g*.
5. Combine supernatants.
6. Centrifuge the supernatant at 40,000 × *g* for 20 min.
7. Resuspend the pellet (membranes) in TME containing DTT (1 mM).
8. Assay for protein content using the bicinchoninic acid (BCA) assay (Smith et al., 1985).

The BCA assay is a detergent-compatible formulation based on the colorimetric detection and quantitation of total protein. This method combines the reduction of Cu + 2 to Cu + 1 by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu + 1) using a unique reagent containing BCA.

1. Prepare BCA working reagent 10 mL of BCA and 200 mL copper sulfate.
2. Prepare standard curve dilutions from the stock 2 mg/mL bovine serum albumin (BSA) to the following concentrations (mg/mL): 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, and blank = 0.
3. Dilute samples in 50 mM Na HEPES. It can be expected that 10⁶ cells will yield 0.5 mg total cell protein. Dilutions can be made to be predicted to be on the standard curve.

4. Add 10 μ L of standard, blank, or samples per well into a 96-well plate, in triplicate.
5. Add 200 μ L of working reagent to each well.
6. Cover and incubate at 37°C for 60 min.
7. Cool plate and read at 562 nm using a Molecular Devices Spectra Max and quantifying using Soft Max software.
8. Calculation of protein concentration: The standard curve is a straight line $Y = mX + b$, where m is the slope and b is the y intercept. X is the protein concentration. $X = (Y - b)/m$.

3. METHODS OF DETERMINING [³⁵S]GTP_γS BINDING TO TOTAL G_α IN MEMBRANE PREPARATIONS

3.1 Equipment and Supplies (PerkinElmer)

1. Unifilter-96 Harvester part number C961960 or similar
2. Top Count NXT model C990200 or similar
3. UniFilter-96 GF/B, White 96-well Barex Microplate with GF/B filter of 1 μ m pore size part number 6005177
4. Scintillation cocktail MicroScintTM-20 cocktail or similar
5. Clear Polystyrene 96-well Microplates (Corning)
6. 96-well Filter bottom seals (PerkinElmer)
7. Top Seal-A PLUS Perkin Elmer Life Sciences 6050185

3.2 Buffers and Reagents

A. Stock buffers

1. Tris-Cl 500 mM Trizma base, adjust pH with HCl
2. MgCl₂ 100 mM
3. EDTA: 250 mM, adjust pH and solubility with NaOH
4. Na HEPES: 500 mM HEPES, adjust pH with NaOH
5. TME (20 mM Tris-Cl, 5 mM MgCl₂ and 1 mM Tris-EDTA)
6. Washing buffer (TME + 10 mM NaCl)
7. GTP_γS (Sigma G8634) MW = 562.98 10 mM stock: store in aliquots at -20°C
8. GDP (Sigma G127 100 mg) MW = 443.2 10 mM stock: store in aliquots at -20°C
9. Dithiothreitol MW = 154.25 100 mM stock: store in aliquots at -20°C

B. Assay buffers and drugs

1. Drugs (e.g., CP55940) are provided as solutions in ethanol by the Drug Supply Program of the National Institute on Drug Abuse (NIDA, Rockville, MD). Commercial sources include Cayman Chemical (Ann Arbor, MI) and Tocris (Bristol, UK). Cannabinoid agonists and antagonists are lipophilic and may not be soluble in aqueous solution above 5–10 μM . Aqueous solubility can be found for each ligand indexed in PubChem or other reliable source. To avoid solvents such as ethanol in the assays, the ethanol is removed and ligands are suspended in fatty acid-free BSA, with the rationale that albumin is the primary carrier of lipophilic small molecules in the serum. Stock solutions are stored as 10 mM in ethanol at -20°C . To prepare, blow dry 10 μL under a stream of filtered N_2 gas in a Regisil-treated glass test tube. Resuspend immediately in 1 mL 5 mg/mL fatty acid-free BSA by vortexing and then serially dilute to the desired concentration in 0.1 mg/mL fatty acid-free BSA in TME buffer. The suspension is more efficient if the fatty acid-free BSA is warmed to 37°C . Adherence to surfaces is avoided if the dilutions are made in Regisil-treated glass test tubes and the pipet tips are preconditioned by pipetting the volume 5–10 times before the aliquot is taken to the next dilution tube.
2. [^{35}S]GTP γS (Perkin Elmer Life Sciences NEG030250UC)

Apply 180 μL 50 mM Na HEPES to the stock vial containing [^{35}S]GTP γS and pipet solution $3 \times$ to mix; then transfer 200 μL to 1800 μL 50 mM Na HEPES and mix well. Aliquot and store at -20°C .

Use the PerkinElmer Radioactive Decay Calculator to calculate the necessary concentration and specific activities for the intended date of use: <https://www.perkinelmer.com/tools/CalculatorRAD#/product>

3.3 Procedures

1. Preparation of reaction mix (final concentrations in the assay are: 500 pM [^{35}S]GTP γS , 1 mM DTT, 10 μM GDP, and 100 mM NaCl). For example, to prepare 6 mL of reaction mix according to the calculation of the concentrations and specific activities for the intended date of use, add 1 nM from the [^{35}S]GTP γS stock, 120 μL 100 mM DTT, 12 μL 10 mM GDP, and 240 μL 5 M NaCl and then adjust the final volume to 6 mL with TME.
2. Pipet 50 μL reaction mix into all wells except the ones designated for nonspecific binding.
3. To determine basal activity, add 20 μL TME buffer to designated wells.
4. To determine up to two drugs per well, add the drugs as 10 μL of a $10 \times$ concentration in TME–BSA vehicle, up to two drugs per well. This can be adjusted to more than two drugs by reducing the volume added. Additional volume is made up in TME–BSA vehicle.

5. To determine nonspecific binding, dilute 100 μL GTP γ S (100 μM) into 600 μL reaction mix and add 70 μL /well into designated wells. The final concentration is 10 μM .
6. Initiate the assay by adding 5 μg cell membranes in 30 μL 0.1 mg/mL fatty acid-free BSA in TME to all wells. The final reaction volume is 100 μL .
7. Incubate plate for 1 h at 30°C.
8. Harvest the Unifilter plates by vacuum filtration on the Unifilter-96 Harvester and wash extensively using 4°C TME with 10 mM NaCl buffer
9. Seal the bottom of the filter plate and add 30 μL of scintillation cocktail to each well and seal the top.
10. Quantitate bound radioactivity using the Top Count scintillation spectrometer, counting at 1 min per well.
11. Data and statistical analyses.

For each data point, nonspecific binding measured in the presence of 10 μM unlabeled GTP γ S was subtracted from total bound DPMs to obtain specific binding. Ligand-stimulated values were transformed to “percent of basal” [% = (stimulated – basal)/(basal) * 100]. Graphs and statistical analyses were generated using GraphPad Prism V software (La Jolla, CA). For dose–response experiments, EC₅₀ values were determined by nonlinear regression analysis of the sigmoidal log [agonist] vs response, either one or two component, without constraint to the basal or the maximum. Other values obtained in the analyses include the Hill number (n_H) (often referred to as the slope factor), the maximum and the minimum (Fig. 2).

4. ANTIBODY-TARGETED SCINTILLATION PROXIMITY ASSAY FOR [³⁵S]GTP γ S BINDING TO SPECIFIC G α PROTEINS IN MEMBRANE PREPARATIONS

4.1 Equipment and Supplies

1. OptiPlate-96, White Opaque 96-Well Microplate (Perkin Elmer Life Sciences)
2. Top Seal-A PLUS (Perkin Elmer Life Sciences part number 6050185)
3. Centrifuge and Rotor for 96-well plates for SPA
4. Top Count NXT model C990200

4.2 Buffers and Reagents

1. Assay reagents are the same as in Section 3.2
2. Primary antibodies
 - G α o: Santa Cruz K-20 (sc-387) Antibody rabbit polyclonal IgG 200 $\mu\text{g/mL}$

- Gαi1: Santa Cruz (sc-56536) Antibody mouse polyclonal IgG 200 µg/mL
- Gαi2: Santa Cruz (sc-13534) Antibody mouse polyclonal IgG 200 µg/mL
- Gαi3: Santa Cruz (sc-262) Antibody rabbit polyclonal IgG 200 µg/mL
- Gαs: Santa Cruz (sc-823) Antibody rabbit polyclonal
- Gαq/11: Santa Cruz (sc-392) Antibody rabbit polyclonal
- Gα12: Santa Cruz (sc-409) Antibody rabbit polyclonal

3. SPA beads

Antirabbit PVT 500 MG (Perkin Elmer Life Sciences RPNQ0016)

Antimouse PVT 500 MG (Perkin Elmer Life Sciences RPNQ0017)

Dilute beads in 50 mL TME and keep covered in foil at 4°C

4.3 Procedures

Procedures are the same as for Section 3.3 steps 1 through 7.

8. After incubation (1 h at 30°C), then transfer plate to cold pack or ice at 4°C.
9. Add 20 µL 3% ice-cold IGEPAL in TME and incubate for 30 min in the refrigerator at 4°C.
10. Add 10 µL of primary antibody diluted 1:100 in TME and incubate for 1 h at 4°C.
11. Add 50 µL/well SPA beads covalently coupled to appropriate secondary antibody. Seal the top of the plate and incubate for 30 min at 4°C.
12. Centrifuge at $1000 \times g$ for 5 min.
13. Quantitate bound radioactivity using Top Count counting for 1 min/well.
14. Data and statistical analyses as in Section 3.3 (step 11; Fig. 3).

4.4 Antibody Verification by Western Blotting

Western blot equipment and supplies

1. Mini-PROTEAN TGX 4%–20% gradient SDS-PAGE gels (BioRad Hercules, CA).
2. Nitrocellulose membranes (Li-Cor, Lincoln, NE).
3. Mini-PROTEAN-Electrophoresis chambers (BioRad).
4. PowerPac™ Universal Power Supply-(BioRad).
5. Wet/Tank Blotting Systems or similar (BioRad).

Buffers and reagents

1. Laemmli's sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromophenol blue, 100 mM DTT) (BioRad).
2. 5% β -Mercaptoethanol (Sigma Chemical) added to Laemmli sample buffer before use.
3. Running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) (BioRad, cat #161-0732).
4. Tris-buffered saline (TBS; 20 mM Tris-Cl, pH 7.4, 137 mM NaCl).
5. Towbins buffer (25 mM Tris base, 192 mM glycine, and 20% methanol, pH 8.3).
6. TBST (TBS containing 0.1% Tween-20).
7. Odyssey blocking buffer (Li-Cor, Lincoln, NE, cat #92740000).
8. IRDye 800CW goat antirabbit or IRDye 680CW goat antimouse secondary antibodies (1:15,000) (Li-Cor, Lincoln, NE).
9. PBS

Procedure immunoblotting

1. Grow cells to confluence in a 75-cm² flask (expect approximately 10⁶ cells), harvest cells with PBS-EDTA, and sediment to obtain a cell pellet (see Section 2.3, steps 1–3).
2. Homogenize the cell pellet in (150–200 μ L) 1% Triton X-100 in PBS plus protease inhibitors (see Section 2.2, step 6).
3. Sediment lysed cell homogenate for 2 min at 1500 $\times g$ at 4°C to remove unbroken cells and nuclear debris.
4. Collect supernatant and perform BCA assay for protein as described in Section 2.4 (step 8).
5. Adjust protein concentration to 4 μ g/ μ L.
6. Dilute 1:1 with 2 $\times$ Laemmli buffer and heat 65°C for 8 min.
7. Apply 20–50 μ g protein/lane using a 4%–20% gradient gel.
8. Run PAGE at 180 V for 45 min at room temperature.
9. Transfer proteins to nitrocellulose membranes at 80 V for 50 min at 4°C.
10. Block in Odyssey blocking buffer for 45 min at room temperature.
11. Incubate with primary antibody overnight, gently shaking, covered, at 4°C.
12. Remove primary antibody and wash 4 $\times$ for 5 min each with TBST.
13. Incubate, covered from light, with secondary antibody in Odyssey buffer with 0.2% Tween-20. Gently shake at room temperature for 1–2 h.
14. Wash 4 $\times$ for 5 min each in TBST.
15. Wash 1 $\times$ PBS without Tween.

16. Scan using Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).
17. Quantitate band densities using Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

5. SUMMARY AND CONCLUSION

Our investigations demonstrate that in N18TG2 neuroblastoma cell membranes under our experimental conditions, the cannabinoid receptor agonist CP55940 stimulates [³⁵S]GTPγS binding in a concentration-dependent manner (Fig. 2). In order to investigate the contribution of different members of the G protein family to this effect, we employed the SPA assay using a specific antibody to each G protein type or subtype. Using Western blotting techniques, we found that N18TG2 cells express a variety of Gi/o family members and also express non-Gi/o proteins such as Gs, Gq/11, G12, and G13 (Fig. 4). This also demonstrates the specificity of the antibodies, with bands appearing at the appropriate apparent molecular weights. Note that the Gs antibody recognizes both Gs long and short forms.

Here, we demonstrate that CP55940 is able to stimulate [³⁵S]GTPγS binding mediated by Go, Gi1, Gi2, Gi3, Gq/11, Gi12, G13, and Gs. Allowing for the different basal values for different G proteins that we assessed, we were able to characterize the contribution of each member of the G protein family. To do so, the basal values of each subtype were counted toward the total of 100%. Then, each value was compared to this total. Using this analysis, we found that the Gi/o family represents the majority of the unstimulated [³⁵S]GTPγS-binding activity, with 72% of the total measured in N18TG2 cells (Fig. 3A). Within the Gi/o family, the order was: Gi3: 30%, Go: 19%, Gi2: 14%, and Gi1: 9%. The percentages of non-Gi/o family [³⁵S]GTPγS-binding activity was Gs: 11%, Gq/11: 9%, G12: 4%, and G13: 3%. This characterization will help us to properly interpret the contributions to basal [³⁵S]GTPγS-binding activity of different G protein subtypes, especially the non-Gi/o proteins. Of particular interest, the calculation of the agonist-stimulated CB₁ receptor [³⁵S]GTPγS-binding activity (above basal) followed a parallel distribution (Fig. 3B).

The [³⁵S]GTPγS-binding SPA provides valuable pharmacological information to better understand the role of G proteins in CB₁ receptor signaling and is helpful in understanding the CB₁ receptor interaction with other interacting proteins, such as the cannabinoid receptor-interacting protein 1a (CRIP1a) and β-arrestins (Blume et al., 2015). Our studies suggest a mechanism by which endogenous levels of CRIP1a modulate CB₁ receptor-mediated signal transduction by facilitating a Gi/o protein subtype preference for Gi1 and Gi2, accompanied by an overall suppression of G protein-mediated signaling in the N18TG2 cell model.

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ABBREVIATIONS

BCA	bicinchoninic acid
BSA	bovine serum albumin
DTT	dithiothreitol
GTPγS	guanosine-5'-O-(3-[³⁵ S]thio)triphosphate
PBS	phosphate-buffered saline
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPA	scintillation proximity assay

REFERENCES

- Augusti-Tocco G, & Sato G (1969). Establishment of functional clonal lines of neurons from mouse neuroblastoma. *Proceedings of the National Academy of Sciences of the United States of America*, 64, 311–315. [PubMed: 5263016]
- Blume LC, Eldeeb K, Bass CE, Selley DE, & Howlett AC (2015). Cannabinoid receptor interacting protein (CRIP1a) attenuates CB1R signaling in neuronal cells. *Cellular Signalling*, 27, 716–726. [PubMed: 25446256]
- Blume A, Gilbert F, Wilson S, Farber J, Rosenberg R, & Nirenberg M (1970). Regulation of acetylcholinesterase in neuroblastoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, 67, 786–792. [PubMed: 5289021]
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254. [PubMed: 942051]
- Brick PL, Howlett AC, & Beinfeld MC (1985). Synthesis and release of vasoactive intestinal polypeptide (VIP) by mouse neuroblastoma cells: Modulation by cyclic nucleotides and ascorbic acid. *Peptides*, 6, 1075–1078. [PubMed: 3010255]
- Calipari ES, Sun H, Eldeeb K, Luessen DJ, Feng X, Howlett AC, et al. (2014). Amphetamine self-administration attenuates dopamine D2 autoreceptor function. *Neuropsychopharmacology*, 39, 1833–1842. [PubMed: 24513972]
- Cashman NR, Durham HD, Blusztajn JK, Oda K, Tabira T, Shaw IT, et al. (1992). Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons. *Developmental Dynamics*, 194, 209–221. [PubMed: 1467557]
- Charalampous FC (1977). Differences in plasma membrane organization of neuroblastoma cells grown in the differentiated and undifferentiated states. *Archives of Biochemistry and Biophysics*, 181, 103–116. [PubMed: 195528]
- Choi HK, Won LA, Kontur PJ, Hammond DN, Fox AP, Wainer BH, et al. (1991). Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Research*, 552, 67–76. [PubMed: 1913182]
- Cruciani RA, Dvorkin B, Morris SA, Crain SM, & Makman MH (1993). Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 3019–3023. [PubMed: 8385355]
- Dalton GD, Bass CE, Van Horn CG, & Howlett AC (2009). Signal transduction via cannabinoid receptors. *CNS & Neurological Disorders Drug Targets*, 8, 422–431. [PubMed: 19839935]
- Dalton GD, Peterson LJ, & Howlett AC (2013). CB(1) cannabinoid receptors promote maximal FAK catalytic activity by stimulating cooperative signaling between receptor tyrosine kinases and integrins in neuronal cells. *Cellular Signalling*, 25, 1665–1677. [PubMed: 23571270]

- Delapp NW, McKinzie JH, Sawyer BD, Vandergriff A, Falcone J, McClure D, et al. (1999). Determination of [35S]guanosine-5'-O-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. *The Journal of Pharmacology and Experimental Therapeutics*, 289, 946–955. [PubMed: 10215674]
- Denis-Donini S, Estenoz M, & Augusti-Tocco G (1978). Cell surface modifications in neuronal maturation. *Cell Differentiation*, 7, 193–201. [PubMed: 699052]
- Devane WA, Spain JW, Coscia CJ, & Howlett AC (1986). An assessment of the role of opioid receptors in the response to cannabimimetic drugs. *Journal of Neurochemistry*, 46, 1929–1935. [PubMed: 3009717]
- Diez-Alarcia R, Ibarra-Lecue I, Lopez-Cardona AP, Meana J, Gutierrez-Adan A, Callado LF, et al. (2016). Biased agonism of three different cannabinoid receptor agonists in mouse brain cortex. *Frontiers in Pharmacology*, 7, 415. [PubMed: 27867358]
- Eldeeb K, Leone-Kabler S, & Howlett AC (2016). CB1 cannabinoid receptor-mediated increases in cyclic AMP accumulation are correlated with reduced Gi/o function. *Journal of Basic and Clinical Physiology and Pharmacology*, 27, 311–322. [PubMed: 27089415]
- Erdozain AM, Diez-Alarcia R, Meana JJ, & Callado LF (2012). The inverse agonist effect of rimonabant on G protein activation is not mediated by the cannabinoid CB1 receptor: Evidence from postmortem human brain. *Biochemical Pharmacology*, 83, 260–268. [PubMed: 22093909]
- Fan Y, Hooker BA, Garrison TR, El-Kouhen OF, Idler KB, Holley-Shanks RR, et al. (2011). Pharmacological and molecular characterization of a dorsal root ganglion cell line expressing cannabinoid CB(1) and CB(2) receptors. *European Journal of Pharmacology*, 659, 161–168. [PubMed: 21458448]
- Fioravanti B, De FM, Stucky CL, Medler KA, Luo MC, Gardell LR, et al. (2008). Constitutive activity at the cannabinoid CB1 receptor is required for behavioral response to noxious chemical stimulation of TRPV1: Antinociceptive actions of CB1 inverse agonists. *The Journal of Neuroscience*, 28, 11593–11602. [PubMed: 18987195]
- Francel PC, Harris K, Smith M, Fishman MC, Dawson G, & Miller RJ (1987). Neurochemical characteristics of a novel dorsal root ganglion X neuroblastoma hybrid cell line, F-11. *Journal of Neurochemistry*, 48, 1624–1631. [PubMed: 2435852]
- Gerald TM, Ward GR, Howlett AC, & Franklin SO (2006). CB1 knockout mice display significant changes in striatal opioid peptide and D4 dopamine receptor gene expression. *Brain Research*, 1093, 20–24. [PubMed: 16684513]
- Gilbert JA, Knodel EL, Stenstrom SD, & Richelson E (1982). Function and regulation of methionine5-enkephalin and its receptors in murine neuroblastoma cells. *The Journal of Biological Chemistry*, 257, 1274–1281. [PubMed: 6276378]
- Gilman AG, & Minna JD (1973). Expression of genes for metabolism of cyclic adenosine 3':5'-monophosphate in somatic cells. I. Responses to catecholamines in parental and hybrid cells. *The Journal of Biological Chemistry*, 248, 6610–6617. [PubMed: 4355495]
- Glaser T, Besson J, Rosselin G, & Hamprecht B (1983). Vasoactive intestinal polypeptide: Presence in neuroblastoma X glioma hybrid cells. *European Journal of Pharmacology*, 90, 121–123. [PubMed: 6873174]
- Glaser T, Hubner K, & Hamprecht B (1982). Production and regulation of enkephalinlike peptides in neuroblastoma x glioma cells. *Advances in Biochemical Psychopharmacology*, 33, 243–250. [PubMed: 7124500]
- Greene LA, & Rein G (1977). Dopaminergic properties of a somatic cell hybrid line of mouse neuroblastoma X sympathetic ganglion cells. *Journal of Neurochemistry*, 29, 141–150. [PubMed: 18554]
- Griffin G, Atkinson PJ, Showalter VM, Martin BR, & Abood ME (1998). Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5'-O-(3-[35S]thio)-triphosphate binding assay in rat cerebellar membranes. *The Journal of Pharmacology and Experimental Therapeutics*, 285, 553–560. [PubMed: 9580597]

- Hamprecht B (1977). Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *International Review of Cytology*, 49, 99–170. [PubMed: 16829]
- Heumann R, Stavrou D, Reiser G, Ocalan M, & Hamprecht B (1977). Tumorigenicity of neuroblastoma X glioma hybrid cells in nude mice and reintroduction of transplanted cells into culture. *European Journal of Cancer*, 13, 1417–1420. [PubMed: 598387]
- Howlett AC (2004). Efficacy in CB1 receptor-mediated signal transduction. *British Journal of Pharmacology*, 142, 1209–1218. [PubMed: 15308578]
- Howlett AC (2005). A short guide to the nomenclature of seven-transmembrane spanning receptors for lipid mediators. *Life Sciences*, 77, 1522–1530. [PubMed: 15953619]
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological Reviews*, 54, 161–202. [PubMed: 12037135]
- Howlett AC, Qualy JM, & Khachatryan LL (1986). Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Molecular Pharmacology*, 29, 307–313. [PubMed: 2869405]
- Howlett AC, Song C, Berglund BA, Wilken GH, & Pigg JJ (1998). Characterization of CB1 cannabinoid receptors using receptor peptide fragments and site-directed antibodies. *Molecular Pharmacology*, 53, 504–510. [PubMed: 9495818]
- Ishii I, & Chun J (2002). Anandamide-induced neuroblastoma cell rounding via the CB1 cannabinoid receptors. *Neuroreport*, 13, 593–596. [PubMed: 11973452]
- Kahl SD, & Felder CC (2005). Scintillation proximity assay. *Current Protocols in Neuroscience*. 30:715:7.15.1–7.15.55.
- Klee WA, & Nirenberg M (1974). A neuroblastoma times glioma hybrid cell line with morphine receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 71, 3474–3477. [PubMed: 4530316]
- Lauckner JE, Hille B, & Mackie K (2005). The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 19144–19149. [PubMed: 16365309]
- Lorenzen A, Fuss M, Vogt H, & Schwabe U (1993). Measurement of guanine nucleotide-binding protein activation by A1 adenosine receptor agonists in bovine brain membranes: Stimulation of guanosine-5'-O-(3-[35S]thio)triphosphate binding. *Molecular Pharmacology*, 44, 115–123. [PubMed: 8341267]
- Mackie K (2008). Signaling via CNS cannabinoid receptors. *Molecular and Cellular Endocrinology*, 286, S60–S65. [PubMed: 18336996]
- Mackie K, Devane WA, & Hille B (1993). Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Molecular Pharmacology*, 44, 498–503. [PubMed: 8371711]
- McGee R, Simpson P, Christian C, Mata M, Nelson P, & Nirenberg M (1978). Regulation of acetylcholine release from neuroblastoma x glioma hybrid cells. *Proceedings of the National Academy of Sciences of the United States of America*, 75, 1314–1318. [PubMed: 206894]
- McIntosh HH, Song C, & Howlett AC (1998). CB1 cannabinoid receptor: Cellular regulation and distribution in N18TG2 neuroblastoma cells. *Brain Research. Molecular Brain Research*, 53, 163–173. [PubMed: 9473654]
- Milligan G (2003). Principles: Extending the utility of [35S]GTP gamma S binding assays. *Trends in Pharmacological Sciences*, 24, 87–90. [PubMed: 12559773]
- Moreno-Martet M, Mestre L, Loria F, Guaza C, Fernandez-Ruiz J, & de LE (2012). Identification of receptors and enzymes for endocannabinoids in NSC-34 cells: Relevance for in vitro studies with cannabinoids in motor neuron diseases. *Neuroscience Letters*, 508, 67–72. [PubMed: 22206832]
- Mukhopadhyay S, McIntosh HH, Houston DB, & Howlett AC (2000). The CB(1) cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. *Molecular Pharmacology*, 57, 162–170. [PubMed: 10617691]
- Myers PR, Blosser J, & Shain W (1978). Neurotransmitter modulation of prostaglandin E1-stimulated increases in cyclic AMP. II. Characterization of a cultured neuronal cell line treated with dibutyl cyclic AMP. *Biochemical Pharmacology*, 27, 1173–1177. [PubMed: 29648]

- Nirenberg M, Wilson S, Higashida H, Rotter A, Krueger K, Busis N, et al. (1983). Modulation of synapse formation by cyclic adenosine monophosphate. *Science*, 222, 794–799. [PubMed: 6314503]
- Petit F, Jeantaud B, Capet M, & Doble A (1997). Interaction of brain cannabinoid receptors with guanine nucleotide binding protein: A radioligand binding study. *Biochemical Pharmacology*, 54, 1267–1270. [PubMed: 9416978]
- Platika D, Boulos MH, Baizer L, & Fishman MC (1985). Neuronal traits of clonal cell lines derived by fusion of dorsal root ganglia neurons with neuroblastoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, 82, 3499–3503. [PubMed: 3858835]
- Prasad KN (1975). Differentiation of neuroblastoma cells in culture. *Biological Reviews of the Cambridge Philosophical Society*, 50, 129–165. [PubMed: 240451]
- Prasad KN, Spuhler K, Arnold EB, & Vernadakis A (1979). Modification of response of mouse neuroblastoma cells in culture by serum type. *In Vitro*, 15, 807–812.
- Prashad N, & Rosenberg RN (1978). Induction of cyclic AMP-binding proteins by dibutyl cyclic AMP in mouse neuroblastoma cells. *Biochimica et Biophysica Acta*, 539, 459–469. [PubMed: 205271]
- Rimmerman N, Hughes HV, Bradshaw HB, Pazos MX, Mackie K, Prieto AL, et al. (2008). Compartmentalization of endocannabinoids into lipid rafts in a dorsal root ganglion cell line. *British Journal of Pharmacology*, 153, 380–389. [PubMed: 17965731]
- Rosenberg RN, Vance CK, Morrison M, Prashad N, Meyne J, & Baskin F (1978). Differentiation of neuroblastoma, glioma, and hybrid cells in culture as measured by the synthesis of specific protein species: Evidence for neuroblast-glioblast reciprocal genetic regulation. *Journal of Neurochemistry*, 30, 1343–1355. [PubMed: 670976]
- Ross RA, Coutts AA, McFarlane SM, Anavi-Goffer S, Irving AJ, Pertwee RG, et al. (2001). Actions of cannabinoid receptor ligands on rat cultured sensory neurones: Implications for antinociception. *Neuropharmacology*, 40, 221–232. [PubMed: 11114401]
- Rudie HA, Nahreini P, Andreatta CP, Edwards-Prasad J, & Prasad KN (2001). Identifying genes involved in regulating differentiation of neuroblastoma cells. *Journal of Neuroscience Research*, 64, 302–310. [PubMed: 11319775]
- Said SI, & Rosenberg RN (1976). Vasoactive intestinal polypeptide: Abundant immunoreactivity in neural cell lines and normal nervous tissue. *Science*, 192, 907–908. [PubMed: 1273576]
- Schubert D, Humphreys S, Baroni C, & Cohn M (1969). In vitro differentiation of a mouse neuroblastoma. *Proceedings of the National Academy of Sciences of the United States of America*, 64, 316–323. [PubMed: 4189500]
- Schubert D, Humphreys S, Jacob F, & de Vitry F (1971). Induced differentiation of a neuroblastoma. *Developmental Biology*, 25, 514–546. [PubMed: 5166556]
- Seeds NW, Gilman AG, Amano T, & Nirenberg MW (1970). Regulation of axon formation by clonal lines of a neural tumor. *Proceedings of the National Academy of Sciences of the United States of America*, 66, 160–167. [PubMed: 5273894]
- Sim LJ, Selley DE, Xiao R, & Childers SR (1996). Differences in G-protein activation by mu- and delta-opioid, and cannabinoid, receptors in rat striatum. *European Journal of Pharmacology*, 307, 97–105. [PubMed: 8831110]
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150, 76–85. [PubMed: 3843705]
- Song C, & Howlett AC (1995). Rat brain cannabinoid receptors are N-linked glycosylated proteins. *Life Sciences*, 56, 1983–1989. [PubMed: 7776822]
- Strange PG (2010). Use of the GTPgammaS ([35S]GTPgammaS and Eu-GTPgammaS) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors. *British Journal of Pharmacology*, 161, 1238–1249. [PubMed: 20662841]
- Traynor JR, & Nahorski SR (1995). Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Molecular Pharmacology*, 47, 848–854. [PubMed: 7723747]

- Walter U, Costa MR, Breakefield XO, & Greengard P (1979). Presence of free cyclic AMP receptor protein and regulation of its level by cyclic AMP in neuroblastoma-glioma hybrid cells. *Proceedings of the National Academy of Sciences of the United States of America*, 76, 3251–3255. [PubMed: 226964]
- Waymire JC, Gilmer-Waymire K, & Boehme RE (1978). Concomitant elevation of tyrosine hydroxylase and dopamine beta-hydroxylase by cyclic AMP in cultured mouse neuroblastoma cells. *Journal of Neurochemistry*, 31, 699–705. [PubMed: 28385]
- Waymire JC, Gilmer-Waymire K, Noritake D, Gibson G, Kitayama D, & Haycock JW (1979). Induction of tyrosine hydroxylase and dopamine beta-hydroxylase in cultured mouse neuroblastoma by 8Br-cAMP. Involvement of RNA and protein synthesis. *Molecular Pharmacology*, 15, 78–85. [PubMed: 34092]
- Wilson SH, Schrier BK, Farber JL, Thompson EJ, Rosenberg RN, Blume AJ, et al. (1972). Markers for gene expression in cultured cells from the nervous system. *The Journal of Biological Chemistry*, 247, 3159–3169. [PubMed: 5027747]
- Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, & Bonner TI (1999). Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 5780–5785. [PubMed: 10318961]

Fig. 1.

Verification of CB₁ cannabinoid receptor protein expression in cell membranes from mouse C1300 neuroblastoma clones. (A) CB₁ cannabinoid receptor protein immunoreactivity in membranes from NS20, N4TG2, N18TG2, and NIE-115 neuroblastoma cell lines. Protein sources are: lanes A&E, NS20 cells; lanes B&F, N4TG1 cells; lanes C&G, N18TG2 cells; lanes D&H, NIE 115 cells. (B) CB₁ cannabinoid receptor immunoreactivity in cell membranes from NG108-15 neuroblastoma-glioma compared with parental N18TG2 neuroblastoma cells. Protein sources are: lanes A&C, N18TG2 cells; lanes B&D, NG108-15 cells. The *left half* of each blot was stained with anti-CB₁1-14. The *right half* stained with nonimmune serum shows no protein staining, verifying the lack of nonspecific antibody binding to proteins during the procedure. Procedures and experimental protocols (Howlett, Song, Berglund, Wilken, & Pigg, 1998; McIntosh, Song, & Howlett, 1998; Mukhopadhyay, McIntosh, Houston, & Howlett, 2000; Song & Howlett, 1995) are summarized as follows: Neuroblastoma cells were homogenized in 10 volumes of hypotonic buffer (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2.5 mM DTT) with protease inhibitors (2 µg aprotinin/mL, 2.25 µg leupeptin/mL, 200 µM AEBSF, 10 mM benzamidine, and 1 µg pepstatin A/mL (Sigma Chemical Company, St. Louis, MO)). Nuclei and debris were removed by centrifugation (1500 × *g*) for 10 min. The supernatants were centrifuged at 40,000 × *g* for 30 min to collect membranes for analysis by PAGE and Western blot. Pellets were resuspended in 50 mM Tris-Cl, pH 7.4, and protein concentration was measured by the Coomassie blue-binding method of Bradford (Bradford, 1976). Proteins resuspended in Laemmli sucrose sample buffer with 5 mM EDTA and 10% SDS, and all lanes were loaded with 100 µg of membrane protein on 10% PAGE minigels. PAGE was performed, and proteins were transferred electrophoretically to polyvinylidene difluoride membranes (0.2 µm pore size) (BioRad, Hercules, CA). Western blots were probed with rabbit polyclonal antibody against the CB₁ N-terminal residues 1–14 (item #101500 Cayman Chemical, Ann Arbor, MI), shown to be specific for a protein that has electrophoretic mobility characteristic of the glycosylated form of the CB₁ cannabinoid receptor (Mukhopadhyay et al., 2000; Song & Howlett, 1995). Conjugated secondary goat-antirabbit-IgG-horseradish peroxidase was from Jackson Immuno-Research Laboratories (West Grove, PA). Detection of immunoblots was performed with enhanced chemiluminescence (ECL). Rainbow molecular weight markers, ECL reagents, and Hyperfilm were from Amersham, Inc. (Chicago, IL). Note: N-terminal amino acids 1–14 of the CB₁ receptor protein remain in G418 resistance protein that is produced by the Zimmer CB₁ receptor knockout mouse (Zimmer, Zimmer, Hohmann, Herkenham, & Bonner, 1999), and therefore caution must be used in interpreting the results of Western

blots in which antibodies to the N-terminal domain are used to verify the failure to express CB₁ receptor protein (Gerald, Ward, Howlett, & Franklin, 2006).

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Fig. 2.

A log concentration-response curve of the [³⁵S]GTPγS stimulation in N18TG2 membranes by CP55940. N18TG2 cell membranes were subjected to [³⁵S]GTPγS assay to quantitate ligand-mediated coupling to total G proteins. Nonspecific binding measured in presence of 10 μM GTPγS was subtracted from total binding to obtain specific binding. Ligand-stimulated values were transformed to “percent over basal” [% = ((stimulated * 100/basal) – 100)]. Data are shown as the mean ± SEM values of three or more independent experiments.

Fig. 3. Statistical analysis showing the percentage of individual G protein subtypes contributing toward total G protein activation in N18TG2 membranes. (A) The specific basal binding of each G protein subtype as a percentage of the sum of all measured subtypes. (B) The CP55940 (100 nM)-stimulated specific binding of each individual G protein subtype as a percentage of the sum of all measured subtypes.

Fig. 4.

Western immunoblot demonstrating the presence of each individual G protein subtype on N18TG2 cell membranes. Membranes from N18TG2 cells were analyzed by Western blotting for Gαo, Gαi1, Gαi2, or Gαi3 (*upper blot*) or Gαs, Gαq/11, Gα12, or Gα13 (*lower blot*). This demonstrates that (1) each subtype of G protein is expressed on membranes from N18TG2 cells and (2) the bands appear at the predicted apparent molecular weight for each G protein.