

The Use of Receptor-Specific Antibodies to Study G-Protein-Coupled Receptors

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Abstract

The identification of G-protein-coupled receptor (GPCR) cDNAs has facilitated a number of studies characterizing the biochemical properties of the receptor protein. Most of these studies have used antibodies directed against the epitope-tagged receptor expressed in heterologous cells, because of the lack of sensitive and selective antibodies capable of recognizing endogenous receptors in their native state. In order to facilitate studies with endogenous receptors, efforts have been made to generate receptor-type selective, sensitive antibodies that are able to recognize endogenous receptors. In this review, we discuss the strategies as well as the details of the techniques used for the generation of monoclonal and polyclonal antibodies with a focus on family A GPCRs.

Key Words: Opiate, opioid receptors, morphine, monoclonal.

Introduction

G-PROTEIN-COUPLED RECEPTORS (GPCRs) comprise the largest family of cell surface receptors and are characterized by the presence of seven transmembrane domains. This superfamily can be subdivided into three major subfamilies: family A (rhodopsin-like/beta adrenergic receptor family) characterized by relatively short N- and C-terminal regions; family B (glucagon-like/secretin-like) characterized by a large N-terminal region with multiple cysteines; and family C (metabotropic glutamate-like) characterized by a very large N-terminal region. Among these, family A is by far the largest and exhibits many of the structural features conserved in other GPCRs, such as the consensus sequence for N-linked glycosylation sites near the amino terminus, a palmitoylation site in the C-terminal tail, and phosphorylation sites in

the first and third intracellular domains as well as in the C-terminal tail (1). These receptors also elicit a wide range of responses (2–4). Recently it has been shown that family A GPCRs can associate with each other as well as with other GPCRs, leading to the modulation of their pharmacological, functional and trafficking properties (5–7).

Studies examining the biochemical and cell biological properties of endogenous receptors have been hampered by a lack of sensitive and selective antibodies that are able to recognize native receptors in live or fixed cells. To date, studies characterizing the biochemical properties of GPCRs have made use of the availability of receptor cDNAs to introduce an epitope tag at the N-terminus. Antibodies directed against this tag are then used to investigate epitope-tagged receptors expressed in heterologous cells. In order to study endogenous receptors in their native environment, antibodies would be useful. Receptor-type specific antibodies can be generated to peptides derived from the N-terminal region, which is relatively diverse between closely related receptors. A comparison of the N-terminal region of the three types of opioid receptors (μ , δ , κ) reveals significant differences that can be employed to generate receptor type specific antibodies. In this article we describe strategies that can be used in the generation and characterization of GPCR-type selective antisera.

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Methods

Generation of the Antibodies

Polyclonal antibodies in rats. The generation of polyclonal antibodies to a given antigen, such as a GPCR, is less time-consuming and labor intensive compared to the generation of monoclonal antibodies. In addition, it has other advantages, such as recognition of a mixture of epitopes on the antigen, which makes it less sensitive to changes in the nature of the antigen, such as polymerization or denaturation. The concentration of specific polyclonal antibodies in the serum is between 0.05 and 0.2 mg/mL, and in some instances it can be as high as 1–3 mg/mL. Polyclonal antibodies can be grown in animals as diverse as rats, goats, rabbits, etc. Here we describe the generation of polyclonal antibodies to opioid receptors in rats, a choice that has several advantages such as ease in handling the animals, low cost and relatively short time requirement.

Immunogen preparation: In order to raise polyclonal antibodies to GPCRs, antigenic peptides have to be generated to regions that are diverse when considering closely related GPCRs. For example, in the case of opioid receptors, the following sequences can be selected: amino acid residues 14–30 from mouse μ opioid receptor, 3–17 from mouse δ opioid receptor and 2–18 from rat κ opioid receptor. Antigenic peptides can be commercially synthesized on a multiple antigenic peptide (MAP) backbone (Research Genetics, Huntsville, AL) or conjugated to bovine serum albumin (BSA). When using MAP peptides, the immunogen is prepared by dissolving 1 mg of MAP peptide in 1 mL of sterile phosphate-buffered saline (PBS). In order to conjugate an antigenic peptide with BSA, 2 mg of the peptide and 10 mg of BSA are dissolved separately in 0.5 mL of PBS. The two solutions are mixed and transferred to a tube containing 10 mg EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] and mixed overnight at 4°C. The mixture is dialyzed against sterile deionized water for 24 h, and the dialyzate is concentrated using a Micron-30 column (Millipore Corporation, Bedford, MA) at 4°C. The retentate is diluted in 50 mM phosphate buffer (pH 7.2) to achieve a final concentration of the antigen of approximately 20 μ g/ μ L. Aliquots (50 μ L) are stored at –80°C till use.

Emulsion preparation: Approximately 0.3 mL Freund's adjuvant (complete or incomplete; GIBCO, Irvington, Carlsbad, CA) is mixed with 0.3 mL PBS containing antigen

(either the MAP or the BSA conjugated peptide) in an eppendorf tube and passed through an insulin syringe ten times. The mixture is mixed at 4°C at moderate speed for 20 min in a rotating shaker and passed through the syringe till emulsified. Emulsification is verified by placing a drop of the emulsion in 10 mL of water in a Petri dish. If the emulsification is incomplete, the drop will disperse in the water. If it does, the emulsification procedure is repeated as described above.

Serum preparation: The preimmune serum is collected prior to immunization. For this, Sprague-Dawley rats (200–250 g / male and approximately two months old) are placed in a small restraining cage or container. Their tails are warmed with an infrared lamp or hot water (50–60°C) to increase the flow of blood to the tails; the temperature should not hurt the animals (if it is too hot for the experimenter's hand, then it is too hot for the rats). A small incision is made at the tip of the tail and blood is collected (the bleeding will stop spontaneously). The preimmune serum is prepared (as described below) and stored in aliquots at –20°C till use (as control).

For serum preparation, the blood is allowed to clot for 30–60 min at 37°C for 2 h at room temperature, the clot is separated from the sides of the tube using a Pasteur pipette and the tube is kept at 4°C overnight to allow the clot to contract. The serum is collected by centrifugation at 10,000 \times g for 10 min at 4°C and stored in aliquots (1 mL) at –20°C till use. The working stock is prepared by adding 50 μ L of 0.5 M EDTA (pH 7.4) and 2 μ L of 50% thimerosal and kept at 4°C.

Injection schedule: For the initial injection, Freund's complete adjuvant is used, and for boosters an emulsion in Freund's incomplete adjuvant is used. Two booster injections are given every three weeks, and the animals are sacrificed a week after the second booster injection.

Monoclonal antibodies. The advantages of generating monoclonal antibodies are that they recognize a single epitope on the antigen, can be generated against complex mixtures of epitopes, are homogenous with respect to immunoglobulin subtype, and give highly reproducible results.

Preparation of antigen and injection: MAPs or peptides conjugated to BSA are emulsified in Freund's complete adjuvant, as described in the "Generation of the Antibodies—Polyclonal antibodies in rats" section above. Approximately 10 μ g of each emulsified peptide is injected intraperitoneally into Balb/c mice

(6-week-old female). Every 15 days, booster injections are given with 10 µg of antigen emulsified in Freund's incomplete adjuvant. Antibody production is monitored by ELISA (described below in the "**Characterization of the Antibodies—ELISA**" section) using blood collected from the tail of these mice as described earlier in the "**Generation of the Antibodies—Polyclonal—Serum preparation**" section.

Hybridoma generation: Mice producing the highest titer of antisera are given a booster injection of 50 µg of MAPs (or peptides conjugated to BSA) intravenously. Three days later the spleen is removed, splenocytes are prepared and 1×10^8 splenocytes are fused with 1×10^7 SP2/0-Ag-14 myeloma cells using PEG-1000, as described by Kohler and Milstein (8) and Gomes et al. (9). The cells are plated into 24-well plates in a hypoxanthine, aminopterin, thymidine (HAT) selection medium and allowed to grow for 15 days. The hybridoma-secreting clones are screened by ELISA as described below and sub-cloned by limiting dilution to achieve a single cell/well of a 96 well plate.

Purification and subtyping: The hybridoma supernatant is subjected to affinity purification using protein A-Sepharose CL-4B beads as described by Ey et al. (10). Fractions containing immunoglobulin are identified by absorbance at 280 nm, and protein concentration is determined by the method of Bradford (11). Purified antibodies are isotyped using an antibody isotyping kit as per manufacturer's instructions (Sigma Chemical Company, St. Louis, MO).

Large scale preparation:

1. **Tissue culture:** The simplest large scale method is to grow hybridomas in large tissue culture flasks (Integra Celline 1000, Integra Biosciences AG, Switzerland) and collect the supernatant according to the manufacturer's protocol. This will yield approximately 10 µg antibodies/mL of supernatant.

2. **Production of ascites:** Another method for obtaining a large quantity of antibodies is by producing ascites. For this, each animal is injected intraperitoneally (i.p.) with 0.5 mL of Pristane (2,6,10,14-tetramethylpentadecane). Seven days after the first Pristane injection, the cultured hybridoma cells are harvested, and centrifuged, and the supernatant is discarded. The pellet is resuspended in sterile PBS to a concentration of approximately $2-20 \times 10^6$ cells/mL. Approximately 0.5 mL of the cell suspension is injected i.p. into each animal

using a 21 G needle. Care must be taken to ensure that the animal is not in serious discomfort. When there is swelling of the abdomen (indicating ascitic fluid production), the animal is sacrificed, and the ascitic fluid is collected using a syringe with a 19 G needle and transferred to a 10 mL tube. Cells are removed by centrifugation for 10 min at $250 \times g$. The supernatant is frozen in small aliquots. The cells can be used to inoculate another animal or frozen in liquid nitrogen for future inoculation. This procedure can give approximately 10 mg/mL of antibodies.

Problems and troubleshooting: The most common problem during monoclonal antibody production is that of contamination. Even with the most extensive precautions, occasionally some contamination will occur; the most common types of contamination and methods of treatment are described below.

1. **Bacteria:** These are seen under the light microscope as rods or cocci and can be treated with antibiotics. If the growth media already contains penicillin and streptomycin, then 50 µg/mL gentamycin can be added. Alternatively, cells can be sub-cloned 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.

2. **Fungi:** These are easy to see under the microscope as thin strands of hyphae. Fungal growth can be prevented by addition of fungizone (2.5 µg/mL) to the growth media. However, once fungal contamination appears, it is best to discard the cells and sterilize the wells, because it can spread quickly to adjacent 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.

3. **Yeast:** These are easily recognizable under the light microscope as budding microorganisms. Yeast infection can be prevented by treatment with fungizone. However, once yeast contamination is detected in a well, it is best to discard the supernatant and sterilize the well, as described above for fungal contamination.

4. **Mycoplasma:** This contamination is difficult to diagnose, as the organisms cannot be seen under the light microscope, but typically they slow down cell growth. It is possible to rescue cells contaminated with mycoplasma by treat-

ing them with a mycoplasma removal agent (ICN Biomedicals, Inc., Costa Mesa, CA) according to the manufacturer's protocol.

Characterization of the Antibodies

Enzyme-linked immunosorbent assay (ELISA) is commonly used to screen and characterize the antibody specificity to the antigen. The antigen could be the immunogenic peptide itself or cells expressing the GPCR containing the immunogenic peptide. Appropriate controls are untreated peptides or cells expressing closely related GPCRs. Antigens are coated on the ELISA plate, and the hybridoma supernatant is used as a primary antibody. Several dilutions of primary or secondary antibody (horseradish peroxidase [HRP] conjugated) are used to assess the specificity of the interaction. We will describe in this section the different types of ELISA assays that can be performed using the monoclonal or polyclonal antibodies.

Enzyme-Linked Immunosorbent Assay (ELISA)

Peptide antigens. For routine screening of hybridoma supernatants the antigen (1 μg of the MAPs peptide in 50 μL PBS) is added to each well of an ELISA plate (96 well plate Flat-bottom Immuno plate, Nalge Nunc International, Rochester, NY) and allowed to air dry. Non-specific sites are blocked with 3% BSA (Sigma) in PBS for 1 h at 37°C. Wells are incubated with the hybridoma supernatant at 4°C for 16–18 h, washed 4 times with PBS, and incubated with peroxidase conjugated anti-mouse antibody (1:1000 in PBS) for 1 h at 37°C. Wells are washed 4 times with PBS followed by a 5 min incubation at room temperature with 100 μL of substrate (0.5 mg/10 mL of orthophenylenediamine in 0.15 M citrate-phosphate buffer, pH 5 containing 10 μL of H_2O_2). The reaction is terminated by the addition of 50 μL of 5N H_2SO_4 and the color is quantitated at 490 nm in an ELISA reader (Bio-Rad model 550; Bio-Rad Laboratories, Inc., Hercules, CA).

Cells in 24 well plates. For examining specificity and sensitivity of antibodies, cells that are untransfected or transfected with specific or closely related receptors are used for whole cell ELISA. Cell lines generally used for transfection include CHO (Chinese hamster ovary), COS (Simian kidney cells that are SV40 transformed) and HEK-293 (Human embryonic kidney). Cells are transfected with the receptors of interest using Lipofectamine 2000 reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA). After 48 h, approximately 2×10^5 cells/well are

plated onto a 24-well plate previously treated with poly-L-lysine. The medium is removed after 24 h (when the cells have attached), wells rinsed with PBS, and cells fixed with ice cold 100% methanol (at -20°C) for 8–10 min or 4% paraformaldehyde for 10 min (at 4°C). The wells are washed twice with PBS, and non-specific sites are blocked with 3% BSA in PBS for 1 h at 37°C . The wells are then incubated with the primary antibody (either monoclonal or polyclonal; 1:1000) at 4°C for 16–18 h. Next the wells are washed 4 times with PBS and incubated with peroxidase conjugated anti-rat or anti-mouse (1:1000; Vector Laboratories, Inc., Burlingame, CA) antibody in PBS for 90 min at 37°C . Finally, the wells are washed and antibody binding is determined as described above (“**Characterization of the Antibodies—ELISA—Peptide antigens**” section).

Cells in 96 well plates. For studies involving the screening of multiple samples, 96 well plates can be prepared and stored at 4°C . Cells heterologously expressing the receptor of interest or neuroblastoma cells that endogenously express the receptors are plated (1×10^5 cells/well in PBS buffer). We routinely use SK-N-SH, a human neuroblastoma cell line that expresses many family A GPCRs. Wells are air-dried using a commercial convection fan, sealed and kept at 4°C till use. ELISA is carried out as described above.

ELISA with membranes in 96 wells. For studies examining receptor expression in endogenous tissue, membranes are prepared either from cells expressing receptors or from endogenous tissues, as described elsewhere (12). Each well of an ELISA plate is coated with 10 μg of membrane protein, and the plates are air dried as described above (“**Characterization of the Antibodies—ELISA—Cells in 96 well plates**” section) and stored at 4°C till use. ELISA is carried out as described above.

ELISA with membranes in tubes. If the antibodies are sensitive to methanol fixation or air-drying, the assay can be carried out in tubes. Membranes from cells expressing receptors or from endogenous tissues are prepared as described by Gomes et al. (12). Then 10 μg of membranes are placed in eppendorf tubes (and 100–200 μg of HEK-293 cell membranes are added to the tubes as bulk for visualization). The same amount of HEK membrane protein is used as negative control. ELISA is performed as described above, except that membranes are not fixed with methanol and all of the washes are carried out by centrifugation at 3000 rpm for 3–5 min. Extreme caution must be exercised so as not to lose the sample between washes (narrow gel loading tips help in this regard).

Section ELISA. Section ELISA can be used to determine if the antibodies recognize endogenous receptors in tissue sections, and to compare region-specific differences in receptor expression. For this, brains are collected and dissected into different regions and are immediately frozen at -80°C till use. Frozen brain regions are embedded in M-1 Embedding Matrix (Thermo Electron Corporation, Pittsburgh, PA) and $10\ \mu\text{m}$ serial sections are cut using a MICROM HM 560 CryoStar (Richard-Allan Scientific, Kalamazoo, MI). Two sections from each region are placed on Fisher Brand Superfrost Plus slides (Fisher Scientific Research, Morris Plains, NJ) and circled immediately with ImmEdge PAP pen (Vector) to form a waterproof barrier; the resulting wells hold an approximately $200\ \mu\text{L}$ solution. ELISA is performed in these wells using the same protocol as described above. The reaction product is then transferred to wells of 96 well plates, and absorbance at $490\ \text{nm}$ is measured with a Bio-Rad ELISA reader.

Problems and troubleshooting: Problems commonly encountered during the ELISA assays are elevated background, poor signal or absence of a signal. These could be due to:

1. Inadequate washing and draining of wells: This can be addressed by draining the wells by completely inverting the plate on absorbent tissue at the end of each wash. And tapping the plate repeatedly to remove residual solution also helps.
2. Contamination of substrate solution with metal ions or oxidizing reagents: This can be avoided by using only distilled/deionized water in the preparation of the various solutions and by using clean plasticware.
3. Substrate exposed to light prior to use also leads to poor signal. Substrate should be freshly prepared about 10 min before use and should be kept in the dark till use (covering the tube with aluminum foil might help).
4. Evaporation of reagent from wells during incubation: To prevent the evaporation of reagents during incubation with primary and secondary antibodies, ELISA plates should be covered with a plastic lid.

Immunoprecipitation and Western Blotting

Immunoprecipitation of receptors from cells and tissues. Antibodies can be used to immunoprecipitate GPCRs from cells or tissues. For immunoprecipitation of opioid receptors we use HEK-293 cells, untransfected or transfected with Flag tagged μ , δ or κ opioid receptors, and solubilize them as described by Jordan and Devi (13). Briefly, cells are lysed for 1 h in NP-40 lysis buffer

($150\ \text{mM NaCl}$, $1\ \text{mM EDTA}$, 10% glycerol, and $1\ \text{mM CaCl}_2$) containing $10\ \text{mM}$ iodoacetamide and protease inhibitor cocktail (Sigma), and 100 – $200\ \mu\text{g}$ of solubilized protein is incubated with 1 – $2\ \mu\text{g}$ of receptor-specific monoclonal antibody overnight at 4°C . Immunocomplexes are isolated by incubation with 10% v/v protein A-Sepharose beads for 2 – $3\ \text{h}$. The beads are washed 3 times with NP-40 lysis buffer and resuspended in sample buffer followed by heating at 60°C for 15 – $30\ \text{min}$ to release the immunoprecipitated receptor into the sample buffer. The mixture is centrifuged at $10,000\ \text{rpm}$ for $20\ \text{min}$, and the supernatant is resolved under reducing or non-reducing conditions on 8% SDS-PAGE. Gels are transferred to $0.2\ \mu\text{m}$ nitrocellulose membranes, blocked in 5% non-fat dried milk in TTBS ($50\ \text{mM Tris-Cl}$, $150\ \text{mM NaCl}$, 0.1% Tween-20), and incubated with polyclonal anti-Flag antibody ($1:5000$ in TTBS) for $2\ \text{h}$ at room temperature. The membranes are washed 6 times ($5\ \text{min}$ each) in TTBS followed by incubation with anti-rabbit HRP conjugated polyclonal antibody ($1:5000$ in TTBS) for $1\ \text{h}$ at room temperature. The membranes are then washed 6 times with TTBS and treated with SuperSignal (Pierce Biotechnology Inc., Rockford, IL) for $3\ \text{min}$ followed by exposure to X-ray films (Eastman Kodak, Rochester, NY) for different time periods to obtain multiple exposures that differ in the intensity of signal.

Problems and troubleshooting: Two major problems during immunoprecipitation are blank blots or the presence of multiple bands on the blot. There are several possible reasons:

1. The antigen is not detected because of its small size, leading to transfer through the membrane. This can be prevented by reducing the transfer time and/or using a $0.20\ \mu\text{m}$ nitrocellulose membrane to trap the antigen (larger antigens would not be transferred under these conditions). To ensure that the transfer is complete, gel is stained with Coomassie blue, or the nitrocellulose membrane is stained with Ponceau S. If the antigen is not detected because of its large size, the transfer time is increased. Adding SDS to the transfer buffer or decreasing the methanol concentration in the transfer buffer also helps. It is also possible that the lack of signal is due to insufficient amount of antigen in the sample in which case the amount of sample, loaded on the gel should be increased.

2. Bands corresponding to the antigenic protein are not detected. This could be due to incomplete dissociation of immunoprecipitated material from the protein A-Sepharose beads,

in which case the time of heating with the sample buffer is increased. The temperature is increased to about 80°C, or 8M urea is used in the sample buffer. Alternatively, this could be due to the presence of large amounts of unrelated protein in the immunoprecipitate, in which case the protein A-Sepharose beads should be washed under more stringent buffer conditions such as higher salt and/or detergent concentration.

3. If the specific signal detected in the blot appears in the form of a smear, the amount of protein loaded on the gel can be decreased.

Flow Cytometry

Flow cytometry is used to characterize cell surface antigens, since this technique provides useful data for identifying and separating cell populations with different levels of receptor expression.

Analysis of cells. For this, approximately 3×10^5 cells/well are plated onto a 24 well plate, and the wells are washed with 0.5 ml of PBS. The cells are incubated with primary antibodies in 50% fetal bovine serum (FBS) in PBS at 4°C for 16 h, washed 3 times with 0.5 mL of 1% FBS in PBS and incubated with Alexa 488-conjugated goat anti-mouse/anti-rat IgG (1:400 in 50% FBS in PBS) for 2 h at 4°C. The wells are washed 3 times with 1% FBS in PBS and 3 times with PBS followed by 10 min incubation at 37°C with 250 μ L of 1% edetic acid (EDTA) in PBS. The cells are collected into tubes containing 25 μ L of 37% formaldehyde (care should be taken to ensure a single cell suspension by up and down pipetting), lightly vortexed and subjected to fluorescent-activated cell sorter (FACS) analysis (14) using a FACScan flow cytometer (Becton Dickinson Immunocytometry, Inc., Mountain View, CA).

Problems and troubleshooting: A common problem with FACS analysis is a weak signal, which could be due to a low expression of the antigen at cell surface, insufficient amount of primary antibody, or high background.

1. In the case of low expression of the antigen at the cell surface, the primary and secondary antibody concentration can be increased, and in the case of transfected cells the experiment can be repeated using a higher amount of cDNA for transfection, so as to increase receptor surface expression.
2. In the case of insufficient amount of primary antibody, the latter can be increased up to 1 μ g antibody / 10^6 cells.
3. High background can be due to insufficient washing following incubation with antibodies,

high concentration of primary or secondary antibody, or non-specific binding by the secondary antibody. The latter case can be addressed by using fluorescently coupled f(ab')₂ fragment of secondary antibody.

Background noise can also be reduced by incubating cells with 3% BSA or 5% normal goat serum prior to addition of primary antibody.

Some cells have high autofluorescence that may interfere with the specific signal. This can be addressed by looking at autofluorescence levels in cells not exposed to antibody treatment and adjusting the instrument settings so that the mean autofluorescent signal is between 0 and 10^1 (by consulting manufacturer's guidelines on altering instrument settings).

Immunofluorescence

Visualization of receptors on cells and tissues. This technique can be used to examine the subcellular localization of receptors in cells or tissue sections.

1. **Cell lines:** Poly-L-lysine coated coverslips (Fisher brand, Fisher Scientific, USA) are placed in 12 well plates (one/well). Cells (1×10^3) are plated on each well in complete media, and after 24 h the media are removed by suction, and the cells are washed twice with sterile PBS prior to fixation and immunofluorescence, as described below.

2. **Tissue preparation:** Sections from different regions of the brain are cut and placed into a small, soft-plastic, straight-sided ampoule (Fisher Scientific) with a push fit cap and enough saline (0.9%) to cover the tissue, and immersed in liquid nitrogen until frozen. Tissue samples are stored at -70°C till use. The saline protects against tissue damage caused by the formation of ice crystals when the ampoule is transferred from -70°C to -30°C in the cryostat for sectioning. Cryostat sections (5–8 μ m) are cut and transferred to microscope slides. The sections are allowed to air dry at room temperature for 2–24 h prior to fixation and immunofluorescence, as described below.

Cells/sections are fixed with 4% paraformaldehyde (pH 7.4) for 15 min at room temperature. Wells/slides are washed twice (5 min each) with Tris buffered saline (pH 7.4) and non-specific binding is blocked with 1% goat serum in TBS for 30 min. The cells/sections are incubated overnight at 4°C with the appropriate primary antibody. The

next day, wells/slides are washed four times (5 min each) with gentle shaking in TBS. Slides are incubated with the appropriate secondary antibody (rat or mouse FITC), for 60–90 min in the dark with gentle shaking. After incubation, the wells /slides are washed four times (5 min each) in TBS by gentle shaking. The cells/sections are sealed using Vector shield (Vector) and examined under a fluorescence microscope (Leica DM 6000B).

Problems and troubleshooting:

1. 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.
2. Some antibodies are sensitive to fixatives. In such cases, different fixatives (glutaraldehyde, methanol or 10% formalin) can be used.

Results and Discussion

In order to study the biochemical properties of endogenous opioid receptors, we generated antibodies to the N-terminal region of μ , δ , or κ receptors. The peptide sequences were chosen with the help of ExPASy software in order to avoid predicted N-glycosylation and phosphorylation sites. Monoclonal antibodies to μ (4D.6), δ (5A.2) and κ (7C.5) peptides were generated in mice, as described above. Here we present data using this synthetic peptide approach to antibody production. The N-terminal peptide sequences selected for the generation of antibodies to μ , δ or κ opioid receptors show a large diversity (least homology) among these receptors and can therefore be used in the generation of highly receptor specific antibodies. These peptides were synthesized on a poly-L-lysine backbone as MAPs leading to the formation of an immunogenic complex that does not require conjugation to a carrier molecule. This allowed the generation of antibodies to the peptide sequence of interest without the presence of contaminating anti-carrier antibodies. In addition, the presence of multiple copies of the desired antigenic sequence led to an increase in the immunogenic response of the animal.

We used the standard protocol described in the “**Generation of Antibodies—Monoclonal**” section to generate monoclonal antibodies to μ , δ or κ opioid receptors. Positive antibody-secreting clones were identified by anti-peptide ELISA and subjected to subcloning by limiting dilution to ensure that the antibodies were produced by cells derived from a single clone. This is important, because the presence of non-secreting cells will over time lead to the loss of the antibody-secreting pop-

ulation, since they divide much more rapidly compared to antibody-secreting clones. We selected clones 4D.6 (μ -mAb), 5A.2 (δ -mAb) and 7C.5 (κ -mAb) for further studies, since they exhibited the highest titers in peptide ELISA. The antibodies were isotyped using an isotyping kit according to manufacturer’s protocol (Sigma) and found to belong to the IgG2b subtype (Table). The antibodies showed receptor specificity at titers of 1: 750 (μ), 1: 800 (δ) and 1: 500 (κ) with an EC₅₀ for the selective peptide ranging from 470–700 ng (Table). The antibodies were purified on protein-A Sepharose CL-4B column to characterize their receptor selectivity. For this, the opioid receptors cDNAs were transfected into HEK-293 cells, since the transient expression of opioid receptors in these cells leads to fairly high levels of expression, and the cells do not normally express opioid receptors (12). As controls we used untransfected HEK-293 cells. As seen in Fig. 1, the monoclonal antibodies show receptor subtype selectivity. When the cells are incubated with μ -mAb as a primary antibody, only μ -receptors are recognized and there is no significant cross-reactivity with δ or κ opioid receptors. Similarly, δ -mAb and κ -mAb recognize only δ or κ -receptors, respectively, and do not exhibit significant cross-reactivity with other opioid receptor subtypes.

Next we examined if the monoclonal antibodies could be used to immunoprecipitate opioid receptors from cells expressing the receptors. To permit visualization of the immunoprecipitated receptors, we used N-terminally Flag epitope tagged μ , δ or κ receptors transiently expressed in HEK-293 cells (and untransfected HEK-293 cells as a control). Cell lysates were immunoprecipitated using μ , δ or κ affinity purified monoclonal antibodies, as described in the “**Characterization of Antibodies—Immunoprecipitation and Western**

TABLE
Characterization of Monoclonal Antibodies to μ , δ and κ Opioid Receptors.

	Isotype ¹	Titer ²	EC ₅₀ ³
μ -mAb	IgG _{2b}	1: 750	703 ± 109
δ -mAb	IgG _{2b}	1: 800	485 ± 40
κ -mAb	IgG _{2b}	1: 500	467 ± 81

¹Isotyping was carried out with an antibody isotyping kit according to manufacturer’s (Sigma) protocol.

²Titer was determined by ELISA; wells were coated with 1 μ g/mL of the antigenic peptide and secondary antibody dilution was 1:500.

³EC₅₀ (effective concentration for 50% binding) in ng of MAP was determined using 1:100 dilution of the primary antibody and 1:500 dilution of the secondary antibody.

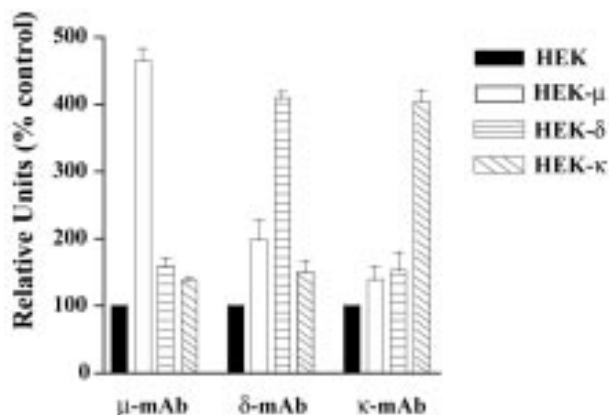


Fig. 1. HEK-293 cells transfected with either μ , δ or κ opioid receptors were used as antigens. ELISA was carried out as described in **Methods**, using either μ , δ or κ affinity purified mAb (1:1000 in PBS) as the primary antibody and HRP conjugated anti-mouse (1:1000 in PBS) as the secondary antibody. Untransfected HEK-293 cells were used as control and taken as 100%. Results are the Mean \pm SEM (n=3) *p<0.001 represents significant differences from control.

Blotting section. The immunoprecipitate was subjected to SDS-PAGE under reducing conditions and blotted with anti-Flag polyclonal antibody to visualize the receptors. We find that these antibodies are able to selectively immunoprecipitate the receptors. When cell lysates were subjected to immunoprecipitation with μ -mAb, we found a signal only in cells expressing μ but not δ or κ receptors. In the same manner, when cell lysates were immunoprecipitated with δ -mAb or κ -mAb, a signal was observed only from cells expressing δ or κ receptors but not from cells expressing other opioid receptors (Fig. 2). Since these antibodies are able to recognize endogenous receptors, we used the μ -mAb to immunoprecipitate μ - δ receptor complexes from mouse spinal cord tissue that is known

to express μ , δ and κ receptors (15). We probed the immunocomplexes isolated using μ -mAb with commercially available polyclonal antibody against δ receptors (Chemicon International Inc., Temecula, CA). We found the presence of μ - δ complexes in tissues from wild-type animals but not δ k/o animals (15). Thus, these antibodies are useful for investigating opioid receptor heterodimers *in vivo*.

We also examined the ability of these antibodies to measure endogenous receptors by ELISA, using membranes from the various mouse brain regions. We found that the μ -Ab is able to recognize μ receptors in all regions but not in the cerebellum, consistent with the lack of significant μ receptors in this brain region. The data for midbrain and cerebellum are shown in Fig. 3. We confirmed this by using polyclonal antibodies raised in rabbit against 18-amino acids (381–398) of the μ opioid receptor (16) or to a 50-amino acid C-terminal polypeptide expressed as GST-C50 fusion protein (17). These antibodies have previously been shown to selectively recognize μ -receptors present in cell lines and tissues (18). Using an ELISA for membranes coated onto a 96 well plate, we have been able to develop a rapid screening assay that reliably measures the levels of μ -receptors in various regions of the brain. Thus, peptide selective antibodies to opioid receptors are useful in quantitating receptor levels in endogenous tissues.

In summary, we have shown that N-terminally directed antibodies generated against GPCRs are highly selective and can recognize recombinant as well as endogenous receptors. Our results suggest that this strategy of targeting the N-terminal region can be useful for the generation of antibodies to other members of the GPCR superfamily. These antibodies are powerful tools

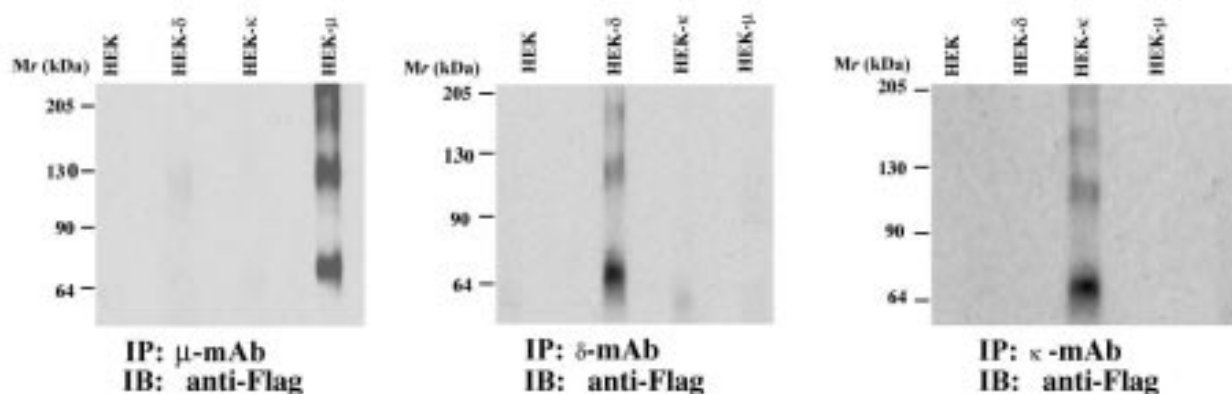


Fig. 2. HEK-293 cells transfected with either Flag tagged μ , δ or κ opioid receptors and untransfected HEK cells were solubilized with NP-40 lysis buffer and subjected to immunoprecipitation with 1 μ g of either μ , δ or κ -mAb, as described in **Methods**. Immunoprecipitates were subjected to SDS-PAGE under reducing conditions and immunoprecipitated receptors identified by Western blot analysis using anti-Flag antibodies. A representative blot of three experiments is shown.

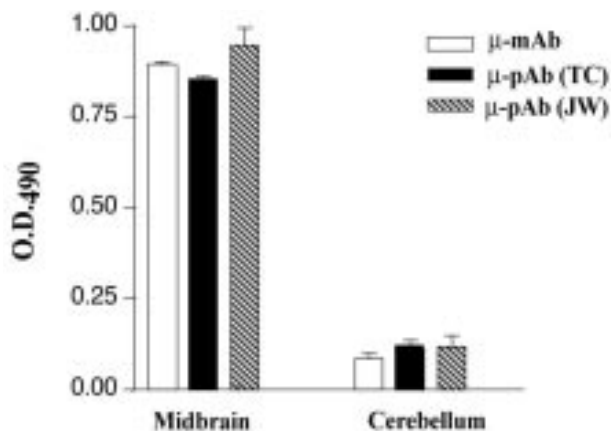


Fig. 3. Midbrain or cerebellar membranes (1 μ g) were plated on 96 well plates and the level of μ receptors was detected by ELISA. Results are the Mean \pm SEM (n=3). As a control against our antibodies (μ -mAb) we have used antibodies obtained from Tomas E. Cote (Bethesda, MD) and Jia-Bei Wang (Baltimore, MD). These are polyclonal antibodies generated against the C-terminal tail of μ opioid receptors.

for probing the biological function of GPCRs and could be of use in discovering new ligands and drugs, and in doing pharmacological research.

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