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Detection of Cell Surface Dopamine Receptors

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Abstract

Dopamine receptors are a class of metabotropic G protein-coupled receptors. Plasma membrane expression is a key determinant of receptor signaling, and one that is regulated both by extra and intracellular cues. Abnormal dopamine receptor signaling is implicated in several neuropsychiatric disorders, including schizophrenia and attention deficit hyperactivity disorder, as well as drug abuse. Here, we describe in detail the application of two complementary applications of protein biotinylation and enzyme-linked immunoabsorbant assay (ELISA) for detecting and quantifying levels of dopamine receptors expressed on the cell surface. In the biotinylation method, cell surface receptors are labeled with Sulfo-NHS-biotin. The charge on the sulfonyl facilitates water solubility of the reactive biotin compound and prevents its diffusion across the plasma membrane. In the ELISA method, cells surface labeling is achieved with antibodies specific to extracellular epitopes on the receptors, and by fixing the cells without detergent such that the plasma membrane remains intact.

Keywords

Schizophrenia; ADHD; DAPI; biotinylation; ELISA; plasma membrane

1. Introduction

Dopamine (DA) regulates movement, endocrine function, reward behavior, and memory processes by stimulating a family of five subtypes of G protein-coupled receptors (GPCRs) designated the D1 to D5 receptors (D1R-D5R). Disorders involving DA transmission include Parkinson's disease (PD), as well as a number of neuro-psychiatric illnesses including attention deficit hyperactivity disorder (ADHD) and schizophrenia. Several lines of evidence suggest that plasma membrane levels of D1Rs, in particular, are critically linked to working memory, an executive function impaired in schizophrenia (1–5). Since deficits in working memory and related executive functions are currently treatment-resistant, reagents which manipulate D1R cell surface expression could represent an effective therapeutic strategy.

A number of factors have been discovered in the past fifteen years or so which can regulate surface levels of D1Rs. For example, both hyper- and hypo-dopaminergic states produce

alterations in surface D1Rs *in vivo* (6), and similar effects are observed in cells in culture with D1R agonists and antagonists (7,8). Further, activation of glutamatergic N-methyl-D-aspartic acid (NMDA) receptors in neurons stimulates accumulation of D1Rs on synaptic membranes (9). This effect is regulated by physical interaction of NR1 NMDA receptor subunits with D1Rs (10). In addition, a variety of other mechanisms regulate D1R surface levels including endocytic recycling (11), receptor phosphorylation (12–14), as well as physical association with cytoskeletal proteins (15).

Biotinylation and enzyme-linked immunoabsorbant assay (ELISA) offer a number of advantages for detecting and quantifying cell surface receptors. With either methods, it is possible avoid the use of radioisotopes as is typically required in receptor ligand binding assays. Both methods are inherently quantitative. While immunofluorescent detection of DA receptor subtypes is also straightforward, quantification of surface levels by this method is not. The isolation of receptors on the cell surface devoid of contamination from other membrane compartments is troublesome with subcellular fractionation methods involving gradient centrifugation. However, the tools currently available for cell surface ELISA and biotinylation permit unambiguous assessment of receptors residing specifically on the plasma membrane.

We provide detailed protocols for biotinylation and ELISA based-methods to quantify the cell surface levels of DA receptors under basal conditions and agonist stimulation. We use D1Rs to illustrate application of these approaches. However, these tools can be easily adapted for other DA receptor subtypes. In the biotinylation method, cell surface receptors are labeled with non-cleavable Sulfo-NHS-biotin. At neutral pH, the sulfo-NHS ester reacts rapidly with any primary amine-containing protein such that the biotin label is attached via a stable amide bond. As the sulfonyl group is charged, the compound shows good water-solubility, and poor ability to cross intact plasma membranes. As a result, labeling is restricted to the extracellular domains of proteins spanning the plasma membrane. The sulfo-NHS-biotin compound can also be used to studying endogenous receptors in primary culture or in brain slices (16–17). Cleavable biotinylation reagents such as sulfo-NHS-S-S-biotin include a disulfide group positioned such that biotin label can be removed by treatment with reducing agents. These compounds are useful for quantifying agonist-stimulated receptor internalization as receptor remaining on the cell surface can be stripped prior to cell lysis (18). In the cells surface ELISA method, labeling is achieved by fixing the cells without detergent such that the plasma membrane remains intact. Receptors can be detected with epitope or subtype specific primary antibodies, followed by enzyme-linked secondary antibodies, and exposure to chromogenic substrates.

2. Materials

2.1. Biotinylation of Cell Surface DA Receptors

1. HEK293 cells.
2. FLAG-D1R cells: this is an HEK293 cell line which stably expresses human D1Rs carrying a FLAG epitope tag inserted at the N-terminus of the receptor coding sequence.

3. HEK293 culture medium: Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% penicillin-streptomycin (Roche Diagnostics, Indianapolis, IN).
4. FLAG-D1R stable cell line medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 450 µg/ml G418 (Invitrogen Life Technologies, Grand Island, NY).
5. PBS: 8.5 mM sodium phosphate, 1.5 mM potassium phosphate, 137 mM NaCl, pH 7.4.
6. Non-cleavable sulfo-NHS-Biotin (Pierce, Thermo Fisher Scientific, Rockford, IL).
7. 10 mM glycine in PBS.
8. Lysis buffer: 150mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% NP-40, 10% glycerol containing protease inhibitor cocktail (1 tablet/10 ml).
9. Protease inhibitor cocktail (Roche Diagnostics).
10. Sonic dismembrator (Fisher Scientific).
11. Streptavidin slurry (Pierce Biotechnology).
12. 1.5 M guanidine HCl.
13. 1x SDS loading buffer: 63 mM Tris HCl, 10% glycerol, 2% SDS, 0.0025% Bromophenol Blue (Sigma), pH 6.8.
14. 7.5% SDS-PAGE gels.
15. Electrophoresis power supply, SDS-PAGE and protein gel transfer equipment (Bio-Rad, Hercules, CA).
16. 10X transfer buffer: 0.25 M Tris base, 2 M glycine. Dilute with double distilled H₂O and add methanol to 20% for use.
17. PVDF membrane (Protran, GE Healthcare, Waukesha, WI).
18. Whatman 3M paper.
19. TBS-T buffer: 250 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 1% (v/v) Tween- 20. Dilute from 10X TBS stock with ddH₂O, and add Tween-20.
20. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
21. Anti-FLAG antibody M2 (Sigma).
22. Goat anti mouse-HRP antibody (Jackson ImmunoResearch, West Grove, PA).
23. ECL plus detection kit (Amersham, GE Healthcare, Waukesha, WI).
24. Kodak X-ray film (Kodak, Rochester, NY).

2.2. Detection of Cell Surface DA Receptors by ELISA

1. 24-well tissue culture plates.

2. 1 µg/ml of laminin: (BD bioscience, San Jose, CA).
3. 4% paraformaldehyde solution in PBS.
4. Non-permeabilizing blocking buffer: Tris-buffered saline (TBS) containing 5% non-fat dry milk and 5% normal goat serum.
5. Anti-FLAG monoclonal antibody M2 (Sigma-Aldrich).
6. Goat anti mouse antibody conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch).
7. Tetramethylbenzidine (TMB) substrate (Pierce).
8. Stop buffer: 2 M H₂SO₄
9. 20 nM DAPI solution (Invitrogen).
10. Standard fluorescence and absorbance multi-well plate reader or spectrophotometer.

3. Methods

3.1. Biotinylation of Cell Surface DA Receptors

1. Culture 1×10^6 FLAG-D1R cells and untransfected HEK293 cells in the appropriate medium in 10 cm dishes for two days at 37°C in a humidified 5% CO₂ incubator (*see* Note 1).
2. Place dishes on ice.
All of the following steps are performed on ice except if noted otherwise.
3. Wash cells 3 times briefly with 10 mL ice cold PBS (*see* Note 2).
4. Add 5 ml 0.5 mg/mL cell-impermeable, non-cleavable sulfo-NHS-Biotin in PBS to cells and incubate for 30 min on ice (*see* Note 3).
5. Wash cells 3 times with 10 mL glycine (10 mM) in PBS to quench the unbound biotin reagent (*see* Note 4).
6. After the last wash, wash cells once with ice-cold PBS.
7. Harvest cells in 1.5 mL of ice-cold PBS using a cell scraper, and collect cells by spinning the suspension at 800 x g for 5 min in a microcentrifuge.
8. Retain the pellet, and resuspend cells in 500 µL of lysis buffer.

¹Cells stably transfected with plasmids containing neomycin resistant markers such as the FLAG-D1R cells can also be maintained in HEK293 cell medium containing 250 µg/mL G418. However, the purity of the cell line is better maintained with higher concentrations of G418 (e.g. 450 µg/mL).

²The cells density should be about 90% confluence. Higher confluence will decrease the efficiency of cell surface protein biotinylation.

³Biotinylation reagents are susceptible to hydrolysis so the biotin compound should be prepared just prior to use. Optimal results are obtained when the cell labeling solution is prepared from newly opened bottles. Alternatively, a stock biotinylation solution (100–200 mg/mL) could be prepared in DMSO, and aliquots stored at –20°C until use.

⁴This step washes out protein in the culture medium which can be biotinylated as well as free sulfo-NHS-Biotin. Wash the cells gently since the plates are not coated with laminin, and HEK293 cells detach easily.

9. Sonicate cells for 10 sec on ice (*see* Note 5).
10. Incubate cells on ice for 30 min.
11. Centrifuge samples at 18,000 x g for 30 min at 4 °C.
12. Keep the supernatant.
13. Add the Streptavidin slurry (100 µL) to the supernatant, and mix by end-over-end rotation for 2 h at 4 °C.
14. Pellet the biotinylated protein bound streptavidin resin by centrifugation at 10,500 x g for 2 min (*see* Note 6).
15. Retain and wash the resin twice with lysis buffer, twice with 1.5 mL guanidine HCl, followed by two additional washes with lysis buffer (*see* Note 7).
16. Elute the bound proteins with 50µL SDS-PAGE loading buffer by boiling the beads for 3 to 5 min at 100 °C (*see* Note 8).
17. Load 20 µL of the samples and separate on a 7.5% SDS-PAGE gel by gel electrophoresis (*see* Note 9).
18. Transfer proteins to PVDF membrane in a gel transfer apparatus (*see* Note 10).
19. After transfer, wash the membrane 3 times (5 min each) with TBS-T on a rocking platform.
20. Incubate the membrane with 5% milk in TBS-T for 1–3 h at RT on rocking platform.
21. Discard block, and add anti-FLAG M2 m ab diluted 1:2000 in blocking solution. Incubate the membrane at 4 °C overnight (or for 2 h at RT) on a nutator (*see* Note 11).
22. Discard the primary antibody; wash the membrane 3 times (10 min each) with TBS-T at RT on rocking platform.
23. Incubate with the secondary antibody, rabbit anti mouse-HRP antibody (1:10,000) for 1 hour at RT on a nutator or rocking platform.
24. Discard the secondary antibody and wash the membrane 3 times (10 min each) with TBS-T.

⁵Keep the sonication probe moving slowly in the solution to avoid local fluxes in temperature, while keeping it submerged to avoid foaming.

⁶Carefully pipette off supernatant. Alternatively, use a spin filter to retain the streptavidin resin in the upper reservoir.

⁷The guanidine HCl wash helps reduce non-specific binding to streptavidin. This step does not affect recovery of the avidin-biotin complexes as the high (10^{-15} M) affinity of avidin and biotin renders them fairly insensitive to extremes of pH, detergent, solvents, and temperature.

⁸Boiling the samples is necessary to disrupt the non-covalent association of biotinylated proteins with the streptavidin beads.

⁹Run mini-gels at 100 volts for 10 min through stacking portion, and at 200 volts for 40 min through the separating region of the gels.

¹⁰Transfer can be carried out at 100 volts for 1h, or overnight at 25 volts, both at 4 °C.

¹¹The FLAG M2 mab is used to specifically detect D1Rs tagged with the FLAG epitope among all the biotinylated cell surface proteins eluted from the streptavidin slurry. If the DA receptor is not tagged, an alternative approach would be to immunoprecipitate with polyclonal receptor specific antibodies, and probe blots of material subsequently eluted from protein A/G resin with streptavidin-conjugated HRP. Resin washing conditions would need to be adjusted accordingly.

25. After the final wash, add 1 mL ECL reagent to cover the membrane, incubate for 1 min at RT. Wrap the membrane with saran wrap sheet, place in a developing cassette, and expose to x-ray film for a suitable time (typically, from 10 s to several minutes). Develop film in dark room (*see* Note 12) (Fig. 1).

3.2. Detection of Cell Surface DA Receptors by ELISA

1. Coat a 24-well plate by incubating plate with 1 µg/ml of laminin at least 2 h (*see* Note 13).
2. Discard laminin solution, and leave the plate in the hood for 30 min to dry.
3. Plate FLAG-D1R and untransfected HEK293 cells at 2×10^4 cells/well and culture cells for 2 days.
4. Wash the cells 3 times briefly with PBS; and then add 4% paraformaldehyde and incubate for 20 min at RT to fix cells (*see* Note 14).
5. Discard 4% paraformaldehyde and wash cells three times (5 min each) with PBS.
6. Block cells under non-permeabilizing conditions (PBS containing 5% non-fat dry milk, and 5% normal goat serum) for 1 h at RT (*see* Note 15, ¹⁶).
7. Discard blocking buffer and incubate cells with mouse anti-FLAG M2 mab (1:250) in blocking buffer (PBS containing 5% non-fat dry milk, and 5% normal goat serum) for 2 h at RT (*see* Note 17).
8. Discard the primary antibody, and wash cells with PBS on a rocking platform (*see* Note 18).
9. Incubate cells with the HRP-conjugate secondary antibody (1:5000) in blocking buffer for 1 h at RT (*see* Note 19).
10. Discard the secondary antibody, and wash cells four times (10 min each) with PBS on a rocking platform (*see* Note 20).
11. Add 500 µL of TMB to each well, and incubate the plate for 15 min at RT (*see* Note 21).

¹²Biotinylation efficiency will vary from protein to protein. If labeling efficiency seems low as gauged from the intensity of bands in streptavidin recovered lanes versus lysate lanes, consider performing a second round of biotinylation before lysing the cells. Alternatively, increase the pH of the biotinylation solution (pH 8-9) to improve labeling by increasing the proportion of lysine ε-amino groups conjugated (19).

¹³The laminin coating step helps decrease cell loss as the ELISA protocol involves several steps with extensive washes.

¹⁴This step can also be carried out following treatment with agonists such as shown in Fig. 2A. Agonist-induced receptor internalization can be inferred from the ratio of receptor surface levels detected before and after agonist treatment (Fig. 2B).

¹⁵This condition assures that anti-FLAG antibodies only bind the D1R on the cell surface. Non-specific binding of antibody to either the plates or the cells will increase background signal. Blocking buffer composition and volume or blocking time might need to be adjusted to reduce background noise. We also suggest plating HEK293 cells which do not express FLAG-D1Rs. Additionally, include FLAG-D1R negative control wells where the primary antibody is omitted and only the HRP-secondary is added; or where FLAG mab followed by *unconjugated* secondary antibodies. Negligible TMB signals coming from such negative control samples are necessary to validate the results from the experimental samples.

¹⁶An alternative strategy could be the use of D1R subtype selective antibodies directed at an extracellular epitope.

¹⁷Be cautious, no detergent!

¹⁸If background signal is high (e.g., non-transfected HEK293 and FLAG-D1R cells give equivalent signals), wash cells 6 times, or decrease the incubation time with the anti-FLAG Mab from 2 hours to 1 hour.

¹⁹Be cautious! No detergent!

²⁰If background is high, wash cells 6 times.

12. Stop the reaction by adding 50 μL H_2SO_4 (2M). The color will turn from blue into yellow.
13. Transfer 400 μL of the solution and measure the OD at 450 nm (*see* Note 22).
14. After HRP detection, add 100 μL DAPI (300 nM) for 5 min. Measure the DAPI fluorescence by exciting at 350 nm, and detecting at 470 nm. Cell number can be inferred from a standard curve of cells plated versus DAPI intensity as shown in Fig. 3.

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²¹Blue color should appear after 15 min. TMB is a chromogenic HRP substrate which absorbs at 450 nm. However, chemiluminescent and fluorescent HRP substrates are also available.

²²Remember to keep the plate for cell number counting.

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Fig. 1. Detection of cell surface D1Rs by biotinylation

48 h after plating untransfected HEK293 cells and FLAG-D1R cells expressing human D1Rs, cell surface proteins were labeled with sulfo-NHS-biotin. Biotinylated proteins were recovered by streptavidin resin. Cell surface Flag-D1Rs were detected by immunoblotting biotinylated proteins with HRP conjugated FLAG M2 antibody.

Fig. 2. Cells surface D1Rs measured 15 min after addition of D1R agonist SKF81297 (10 nM) or vehicle using the ELISA method

(A) Surface D1Rs by cell surface ELISA assay. Cells were fixed under non-permeabilizing conditions, and cell surface D1Rs detected with anti-FLAG and HRP conjugated secondary antibodies, followed by ELISA using the TMB substrate for HRP. Cell numbers were determined by DAPI staining. (B) The 'endocytosis ratio' was determined by the (surface D1Rs in vehicle treated cells- surface D1Rs treated with SKF for 15min/surface D1Rs in vehicle treated cells). The bar graphs show the mean $\pm$ SEM of three independent experiments each including four replicates per group.

Fig. 3. Determination of Cell Density using DAPI

HEK293 cells were plated at varying densities in a 24 well plate. After washing with PBS 4 times, 100 μ L of DAPI (300 nM in PBS) was added to each well, and the plate was incubated for 5 min at RT. After the incubation, wells were rinsed several times with PBS. Samples were excited in 358 nm and the emission at 461 nm was recorded.