

Preferential Interaction between the Dopamine D2 Receptor and Arrestin2 in Neostriatal Neurons

Tara A. Macey, Vsevolod V. Gurevich, and Kim A. Neve

Department of Behavioral Neuroscience, Oregon Health & Science University and Veterans Affairs Medical Center, Portland, Oregon (T.A.M., K.A.N.); and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee (V.V.G.)

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ABSTRACT

Dopamine D2 receptor interactions with arrestins and arrestin-dependent internalization have been characterized using heterologously expressed D2 receptor and arrestins. The purpose of this study was to investigate D2 receptor interaction with endogenous arrestins. Arrestin2 and arrestin3 in striatal homogenates bound to the third cytoplasmic loop of the D2 receptor, and purified arrestin2 and arrestin3 bound to the second and third loops and C terminus of the D2 receptor, in a glutathione S-transferase pull-down assay. In NS20Y neuroblastoma cells expressing an enhanced green-fluorescent protein-tagged D2 receptor (D2-EGFP), 2-h D2 agonist stimulation enhanced the colocalization of D2-EGFP with endogenous arrestin2 and arrestin3. These results suggest that the D2 receptor has the intrinsic ability to bind both nonvisual arrestins.

Agonist treatment of D2-EGFP NS20Y cells induced D2 receptor internalization (36–46%) that was maximal within 20 min, but that was prevented by small interfering RNA-induced depletion of arrestin2 and arrestin3. In neostriatal neurons, 2-h agonist treatment selectively increased the colocalization of the endogenous D2 receptor with arrestin2, whereas receptor colocalization with arrestin3 was reduced. Agonist stimulation caused translocation of arrestin2, but not arrestin3, to the membrane in neurons and selectively enhanced the coimmunoprecipitation of the D2 receptor and arrestin2. All three measures of receptor/arrestin interaction (colocalization, translocation, and coprecipitation) demonstrated selective agonist-induced interaction between the D2 receptor and arrestin2 in neurons.

Receptor desensitization is a phenomenon in which receptor responsiveness decreases after continued or repeated stimulation with an agonist. After termination of agonist stimulation, desensitization is followed by resensitization, the reinstatement of the ability to respond to ligands (Krupnick and Benovic, 1998). For G protein-coupled receptors (GPCRs), trafficking of the receptor through various subcellular compartments is an important part of desensitization and resensitization. A model of GPCR desensitization, best characterized for the β_2 -adrenergic receptor, has been developed in which the agonist-activated GPCR is phosphor-

ylated by GPCR kinases (GRKs) or second-messenger-dependent kinases such as protein kinase A (Krupnick and Benovic, 1998). Phosphorylation by GRKs enhances the interaction of the GPCR with additional proteins, termed arrestins. Binding of arrestin causes rapid desensitization of the receptor by inhibiting receptor binding to G proteins and also targets the receptor to clathrin-coated pits for internalization and either degradation or resensitization (Pippig et al., 1995; Tsao et al., 2001). Arrestin can also act as a scaffolding protein, promoting the stable association of signaling proteins with the receptor (Luttrell et al., 2001).

The rate of GPCR resensitization depends on the stability of the receptor/arrestin complex. Receptors that dissociate from arrestin near the cell membrane (called “class A receptors”) are rapidly dephosphorylated and recycled, whereas receptors that remain associated with arrestin during internalization (“class B receptors”) are dephosphorylated and

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GST, glutathione S-transferase; D2-EGFP, D2 receptor with enhanced green fluorescent protein attached to the C terminus; GRK, G protein-coupled receptor kinase; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; siRNA, small interfering RNA; TBS, Tris-buffered saline; 7-OH-DPAT, 7-hydroxy-2-(di-*n*-propylamino)tetralin; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; EGFP, enhanced green fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; D2-IC2, second cytoplasmic loop of the dopamine D2_L receptor; D2-IC3, third cytoplasmic loop of the dopamine D2_L receptor; D2-CT, C terminus of the dopamine D2_L receptor.

recycled more slowly (Shenoy and Lefkowitz, 2003). Class A and B receptors can also be differentiated by their affinities for arrestins, with class A receptors, including the D1 dopamine receptor, having higher affinity for arrestin3 than for arrestin2, whereas class B receptors have similar affinity for arrestin2 and arrestin3 (Oakley et al., 2000).

Desensitization and internalization of the dopamine D2 receptor have been described in a variety of cell lines and tissue preparations (Barton et al., 1991; Zhang et al., 1994; Boundy et al., 1995; Ng et al., 1997; Sibley and Neve, 1997; Vickery and von Zastrow, 1999; Kim et al., 2001, 2004). The interaction of arrestins and the dopamine D2 receptor and the contribution of this interaction to receptor internalization have also been investigated but chiefly in studies using heterologous expression of arrestins, GRKs, and the D2 receptor in non-neuronal cells. Activation of the heterologously expressed dopamine D2 receptor causes GRK-dependent receptor phosphorylation, translocation of GFP-tagged arrestin2 and arrestin3 to the cell membrane, and receptor internalization that is enhanced by overexpression of GRKs or arrestins and prevented by overexpression of a dominant-negative mutant of arrestin3 (Kim et al., 2001, 2004).

We report here that agonist stimulation caused rapid internalization of the D2 receptor heterologously expressed in NS20Y neuroblastoma cells and that depletion of endogenous arrestins prevented receptor internalization. In NS20Y cells, agonist-induced colocalization of the D2 receptor with both arrestin2 and arrestin3 suggests that the receptor interacts with both forms, as described for class B receptors, an interpretation supported by the direct binding of both forms of arrestin to the receptor second and third cytoplasmic loops and C terminus. In neurons, however, the endogenous dopamine D2 receptor preferentially interacted with arrestin2, as indicated by selective agonist-induced D2 receptor colocalization with, coimmunoprecipitation with, and translocation of arrestin2.

Materials and Methods

Materials. [³H]Spiperone was purchased from Amersham Biosciences Inc. (Piscataway, NJ), and [³H]sulpiride was from PerkinElmer Life and Analytical Sciences (Boston, MA). Serum was purchased from Hyclone Laboratories (Logan, UT). Other reagents, including culture media, 7-OH-DPAT, haloperidol, and (+)-butaclamol, were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies used include rabbit anti-dopamine D2L/S (1/250 dilution, AB5084P; Chemicon International, Temecula, CA), mouse anti-arrestin2 (1/300 dilution, A47520; Transduction Laboratories, Lexington, KY), mouse anti-arrestin3 (1/250 dilution, sc-13140; Santa Cruz Biotechnology Inc., Santa Cruz, CA), goat anti-GST (1/500 dilution, 27-4577-01; Amersham Biosciences), monoclonal anti-GFP (1/300, BD Biosciences Clontech, Palo Alto, CA), and monoclonal anti-GAPDH (1/50,000, MAB374; Chemicon). Secondary antibodies for confocal microscopy were purchased from Molecular Probes (Eugene, OR), and secondary antibodies for immunoblot analysis were from Santa Cruz Biotechnology Inc. The blocking reagent I-block was purchased from Tropic (Bedford, MA). Pregnant Sprague-Dawley rats at gestation day 13 were obtained from Harlan (Indianapolis, IN).

Generation of GST Fusion Proteins. For construction of the GST fusion protein, the second cytoplasmic loop of the dopamine D_{2L} receptor (D2-IC2), amino acids 119 to 154, the third cytoplasmic loop of the dopamine D_{2L} receptor (D2-IC3), amino acids 212 to 369, and the C terminus of the dopamine D_{2L} receptor (D2-CT), amino acids

419 to 444, were polymerase chain reaction-amplified, subcloned into BamHI-SalI sites in pGEX-4T-3 (Amersham Biosciences), and transformed into BL21 cells. Transformants were screened by induction with 50 μ M isopropyl β -D-thiogalactoside and immunoblot analysis using an anti-GST antibody. For larger-scale purification, the GST fusion protein was grown in LB containing ampicillin (100 μ g/ml) to $A_{600} = 0.5$ and stimulated with 50 μ M isopropyl β -D-thiogalactoside for 3 h at room temperature. Bacteria were pelleted and washed with phosphate-buffered saline. Pellets were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, and 1 mg/ml lysozyme, pH 8.0) and incubated for 1 h with gentle rotation at room temperature. The homogenates were clarified by centrifugation, and 600 μ l of supernatant, typically \sim 1 mg, was applied to the MicroSpin GST Purification Module (Amersham Biosciences) containing glutathione Sepharose 4B beads and purified according to manufacturer's instructions. Eluates were separated by SDS-PAGE, and the gel was stained with Gel Code Blue (Pierce, Rockford, IL) to determine the correct molecular weight of each fusion protein. In addition, a BCA protein assay was used to determine protein concentrations of the GST fusion proteins.

GST Pull-Down. For GST pull-down experiments, striata were dissected from Sprague-Dawley rats and homogenized in GST solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.05 mM EDTA, 10 mM CHAPS, and a complete protease inhibitor tablet per 50 ml) with five strokes of a glass-Teflon Dounce homogenizer. Samples were centrifuged at 38,000g for 30 min, and the protein concentration in the resulting supernatant was determined using the BCA protein assay kit. To obtain purified arrestins, plasmids were expressed in BL21 cells and arrestins purified using heparin-Sepharose chromatography, followed by Q-Sepharose chromatography (Han et al., 2001). Glutathione Sepharose 4B beads containing equal amounts of D2-IC3 GST, D2-IC2 GST, D2-CT GST, or GST without insert (\sim 1 mg of protein for each) were incubated with 500 μ g of striatal brain homogenate overnight at 4°C with gentle rotation or with 25 ng of purified arrestin2 or arrestin3 at 4°C for 2 h. The beads were washed three times with 20 mM Tris-HCl, pH 6.9, containing 70 mM NaCl. Samples were eluted with elution buffer (50 mM Tris-HCl and 10 mM glutathione, pH 8.0) for 20 min at room temperature with gentle rotation. Bound proteins were analyzed by immunoblotting with anti-arrestin2 or anti-arrestin3 as described below. In experiments using purified arrestins, the amount of bound arrestin2 or arrestin3 was calculated from a four-point standard curve generated using background optical density (i.e., no arrestin) and three concentrations of arrestin2 or arrestin3 between 0.625 and 2.5 ng. The amount bound to GST alone was subtracted from the total amount bound to each fusion protein to arrive at a value for the amount of arrestin2 or arrestin3 specifically bound to D2-IC3, D2-IC2, or D2-CT.

D2 Receptor-Expressing NS20Y Cells. The D2-EGFP receptor was constructed by cloning a rat D_{2L} receptor cDNA into the pEGFP-N1 N-Terminal Protein Fusion Vector (BD Biosciences Clontech). Three mutations were introduced into the wild-type D₂ receptor using the QuikChange mutagenesis kit (Stratagene, Cedar Creek, TX) to eliminate the stop codon from the D₂ receptor and add an ApaI restriction site for cloning into the EGFP vector in the proper reading frame: CTGCTGAGTCTG \rightarrow CTGCTGGGCCCG. Wild-type D_{2L} receptor (in pDNA3.1) and D2-EGFP were stably expressed in mouse neuroblastoma NS20Y cells by calcium phosphate coprecipitation (Neve et al., 1991). After selection for resistance to G418 (600 μ g/ml), pooled populations of D2-EGFP-expressing cells were isolated using a BD FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA) with excitation at 488 nm. Cells were maintained at 37°C in a humidified atmosphere with 10% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and 5% calf bovine serum, with 300 μ g/ml of G418 sulfate (Calbiochem, San Diego, CA). The EGFP-tagged D2 receptor and the wild-type D2 receptor had similar affinity for [³H]spiperone ($K_d = 49 \pm 22$ pM for D2-EGFP-NS20Y and 44 ± 4 pM for D2-NS20Y cells; data not shown). The integrity of the fusion protein was dem-

onstrated by colocalization of D2 receptor immunoreactivity and EGFP autofluorescence in D2-EGFP-NS20Y cells (data not shown).

Confocal Microscopy. D2-EGFP NS20Y cells and neostriatal neurons grown on glass coverslips were treated for 5, 10, 60, or 120 min at 37°C with 7-OH-DPAT (10 μ M) or vehicle (1% ethanol). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, and 68 mM NaCl, pH 7.4) for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and then blocked with 5% goat serum for 1 h at room temperature. All cells were incubated with mouse anti-arrestin2 or anti-arrestin3, washed, and incubated for 1 h with Alexa Fluor-Red–tagged goat anti-mouse IgG (1/400). Localization of the endogenous D2 receptor in neurons and of the wild-type D2 receptor in D2-NS20Y cells was done using rabbit anti-dopamine D2L/S followed by incubation for 1 h with Alexa Fluor-Green–tagged goat anti-rabbit IgG (1/400). Coverslips were washed, mounted onto a slide with Slowfade (Molecular Probes), and imaged with a Leica SP laser scanning confocal microscope (Leica, Wetzlar, Germany). The extent of colocalization of arrestin immunoreactivity with either D2 receptor immunoreactivity or EGFP autofluorescence (pixels expressing both red and green fluorescence) is expressed as a percentage of the total number of pixels expressing the green fluorescence of the dopamine D2 receptor. Colocalization was quantified for each image using IP Lab software (Fairfax, VA). Three independent experiments were done for each cell type and arrestin isoform, with an average of 20 cells per experiment analyzed for each time point.

Receptor Sequestration. Sequestration was measured using the intact cell [³H]sulpiride binding assay described by Kim et al. (2001). D2-EGFP– and D2-expressing NS20Y cells were grown to 80% confluence. Cells were rinsed and preincubated with serum-free minimal essential medium (MEM) containing 10 mM Na⁺-HEPES, pH 7.4, at 37°C. Cells were stimulated with 10 μ M 7-OH-DPAT for 0, 20, or 120 min as indicated. Stimulation was terminated by quickly cooling the plates on ice and washing three times with ice-cold serum-free MEM with 20 mM HEPES, pH 7.4. Cells were gently pipetted, collected, and incubated with 250 μ l [³H]sulpiride (final concentration, 2.2 nM) at 4°C for 150 min in the absence and presence of unlabeled competitive inhibitor (10 μ M of haloperidol). The assay was terminated by filtration (Whatman GF/C filters; Whatman, Clifton, NJ) using a 96-well Tomtec (Orange, CT) cell harvester. Filters were allowed to dry, and BetaPlate scintillation fluid (50 μ l) was added to each sample. Radioactivity on the filters was determined using a Wallac 1205 BetaPlate scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). Statistical comparisons were made using ANOVA followed by Dunnett's post hoc test.

Biotinylation Sequestration Assay. D2-EGFP NS20Y cells grown to 80% confluence on 10-cm tissue culture plates were treated with 7-OH-DPAT (10 μ M) or vehicle (1% ethanol) in DMEM for 20 min, after which the medium was decanted and the plates were placed on ice. The remaining cell-surface proteins were then biotinylated with 0.5 mg/ml EZ-Link NHS-SS-biotin (Pierce) and homogenized in solubilization buffer (25 mM Tris, 150 mM NaCl, and 1% CHAPS, pH 7.4), including a Complete protease inhibitor tablet (1 tablet per 50 ml; Roche Diagnostics, Mannheim, Germany) with a glass-Teflon homogenizer. Lysates were centrifuged at 16,000g, and supernatants containing equal amounts of total protein were incubated with ImmunoPure Immobilized streptavidin beads (Pierce) to capture biotinylated proteins. The protein concentration of each sample was determined using the BCA assay kit (Pierce). After washing in extraction buffer, biotinylated proteins were eluted from streptavidin beads by heating at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-GFP or anti-dopamine D2 receptor antibodies. A one-way ANOVA and Dunnett's post hoc comparison were used to analyze data.

Transfection of siRNAs. Chemically synthesized siRNAs with 19-nucleotide duplex RNA and 2-nucleotide 3' dTdT overhangs were purchased from Invitrogen (Carlsbad, CA). The siRNA sequences targeting mouse arrestin2 (NM_177231) and arrestin3 (NM_145429)

were 5'-AGCCUUCUGUGCUGAGAAC-3' and 5'-GGACCGAAAGU-GUUUGUG-3', respectively. Twenty-four hours before transfection, D2-EGFP–expressing NS20Y cells were plated in DMEM containing 10% fetal calf serum and were approximately 30% confluent the next day. LipofectAMINE 2000 (Invitrogen) was added to MEM according to the manufacturer's instructions while RNA mixtures at a final concentration of 25 nM were prepared in MEM. RNA mixtures were added dropwise to the LipofectAMINE 2000 mixture and incubated at room temperature for 30 min. After the incubation, the total mixture was added to cells in a six-well tissue culture cluster, for immunoblotting, or a 10-cm plate, for the internalization assay. Some wells received LipofectAMINE 2000 only as a negative control. Additional MEM was added to each well or plate 24 h after the addition of the RNA mixtures. Twenty-four hours later, the siRNA/MEM mixture was replaced by DMEM containing 10% fetal calf serum. Cells were harvested on the third day after transfection for quantification of arrestin and GAPDH immunoreactivity or D2 receptor sequestration as described above.

Neostriatal Neuronal Cultures. The striatal region was dissected from 4-day-old Sprague-Dawley rats and incubated in MEM containing 20 U/ml papain for 2 h at 37°C. The tissue was then triturated using fire-polished Pasteur pipettes in MEM supplemented with 10% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.5 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were plated on poly-D-lysine–treated glass coverslips at a density of 75,000 cells per coverslip. Neuronal medium containing 50% MEM, 39% Ham's F-12 medium, 10% horse serum, 1% fetal bovine serum, 0.45% glucose, 5 pg/ml insulin, 0.1 mg/ml apotransferrin, 0.5 mM kynurenic acid, and 1 μ g/ml glia-derived neurotrophic factor was added 1 h after initial plating. The medium was first conditioned with glial cells for 24 h. Cells were grown in a humidified 5% CO₂ incubator at 37°C and were used after 6 to 8 days in culture.

Arrestin Translocation. Striatal cultures were treated with 7-OH-DPAT (10 μ M) or vehicle for 20 or 120 min and then rinsed with calcium- and magnesium-free phosphate-buffered saline containing 25 mM EDTA. Cultures were scraped from plates and triturated, after which nuclei were pelleted by centrifugation at 500g for 10 min at 4°C. The supernatant was centrifuged at 100,000g for 30 min at 4°C. The pellets were resuspended in solubilization buffer (25 mM Tris and 150 mM NaCl with 1% CHAPS, pH 7.4), including a Complete protease inhibitor tablet, and sonicated for 10 s. Protein concentrations were determined using the BCA protein assay kit (Pierce), and 50 μ g of protein per sample was used for quantification of arrestin2 and arrestin3 by immunoblotting.

Immunoblots. Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel and transferred to polyvinyl membranes (Millipore, Bedford, MA). The membranes were blocked overnight with I-block [0.2% with 0.1% Tween 20 in Tris-buffered saline (TBS), pH 7.4] at 4°C, washed twice for 5 min, followed by two 10-min washes with TBS, and incubated with anti-arrestin2 or anti-arrestin3 antibody at room temperature for 2 h or with anti-dopamine D2 receptor antibody overnight at 4°C. The polyvinylidene difluoride membranes were again washed twice for 5 min and twice for 10 min in TBS, then incubated with secondary antibody at a dilution of 1:3000 (alkaline-phosphatase conjugated anti-mouse IgG or anti-rabbit IgG; Santa Cruz Biotechnology Inc.) at room temperature for 1 h. Membranes immunoblotted with anti-GAPDH were first stripped with 0.2M NaOH for 15 min at room temperature, followed by two 15-min washes with TBS, then incubated with I-block as described. Stripped blots were then incubated with anti-GAPDH antibody, washed as described, and incubated with alkaline-phosphatase conjugated anti-mouse IgG at a dilution of 1:25,000 as described. Immunodetection was accomplished using an ECF Western blotting kit (Amersham Biosciences). Proteins were visualized using the Typhoon phosphorimaging system and quantified with ImageQuaNT (Amersham Biosciences). A one-way ANOVA and Dunnett's post hoc comparison were used to analyze data.

Coimmunoprecipitation. Striatal cultures were treated with 7-OH-DPAT (10 μ M) or vehicle for 20 or 120 min, rinsed with

phosphate-buffered saline, and incubated at room temperature for 30 min with 2 mM disuccinimidyl suberate (Pierce) dissolved in phosphate-buffered saline containing a Complete protease inhibitor tablet. The cross-linking reaction was quenched with a final concentration of 10 mM Tris-HCl, pH 7.5, for 15 min at room temperature. Cultures were scraped and collected, and CHAPS was added at a final concentration of 1%. Lysates were incubated on ice for 1 h and then centrifuged at 17,500g for 15 min at 4°C. Protein concentrations of the supernatants were determined using the BCA protein assay (Pierce). Cell lysate (500 µg of protein), Antibody Capture Affinity Ligand, and 4 µg of anti-dopamine D2 antibody were added to prewashed Catch and Release beads and rotated overnight at 4°C. Beads were washed three times for 15 min, and samples were eluted according to the manufacturer's instructions, heated at 60°C for 20 min in sample buffer, separated by SDS-PAGE, and immunoblotted using anti-arrestin2 or arrestin3 antibodies. A one-way ANOVA and Dunnett's post hoc comparison were used to analyze data.

Results

Direct Interaction of the D2 Receptor with Arrestin2 and Arrestin3. D2-IC3-GST, D2-IC2-GST, and D2-CT-GST fusion proteins were constructed to identify direct binding of arrestins to intracellular domains of the D2 receptor. The fusion proteins were immobilized on glutathione Sepharose beads and incubated with rat striatal homogenates or purified arrestins. Arrestin2 and arrestin3 immunoreactivity in the eluates was determined by immunoblotting. Both arrestin2 and arrestin3 were detected in the eluates from the D2-IC3 incubated with striatal brain homogenates, suggesting a direct interaction of the third loop of the D2 receptor with both isoforms of arrestin (Fig. 1A). There was little or no specific binding of arrestin2 or arrestin3 to D2-IC2 and D2-CT, as indicated by the lack of arrestin immunoreactivity in eluates from both fusion proteins incubated with striatal homogenates (Fig. 1A). Similar experiments were carried out with purified arrestin2 and arrestin3 (Fig. 1B). D2-IC3 bound an average of 1.3 ± 0.3 and 1.6 ± 0.4 ng of purified arrestin2 and arrestin3, respectively ($n = 4$). D2-CT also bound purified arrestin2 (0.5 ± 0.04 ng, $n = 3$) and arrestin3 (0.3 ± 0.1 ng, $n = 3$). The D2-IC2 bound purified arrestin2 (0.4 ± 0.1 ng, $n = 3$) and to a greater extent arrestin3 (1.2 ± 0.3 ng, $n = 3$).

Agonist-Induced Colocalization of the D2 Receptor and Endogenous Arrestin2 and Arrestin3 in NS20Y Cells. Agonist-induced trafficking of the dopamine D2 receptor and arrestin was evaluated in D2-EGFP NS20Y cells.

Cells were grown on glass coverslips and treated with 7-OH-DPAT (10 µM) for 10, 60, or 120 min. Agonist-treated cells were compared with cells treated with vehicle to assess changes in the colocalization of D2-EGFP with arrestin2 or arrestin3 immunoreactivity. Treatment with 7-OH-DPAT for 120 min increased the colocalization of the receptor with arrestin2 and arrestin3 compared with untreated cells ($p < 0.01$, $n = 3$) (Fig. 2), whereas treatments for only 10 or 60 min had no significant effect.

We also used D2-NS20Y cells to confirm that similar results were observed for wild-type D2 and D2-EGFP receptors. Treatment with 7-OH-DPAT (10 µM) for 120 min increased the colocalization of the D2 receptor with arrestin2 and arrestin3 from basal levels of $10 \pm 2\%$ and $9.5 \pm 3\%$, respectively, to $46 \pm 5\%$ and $55 \pm 10\%$ after treatment with the D2 receptor agonist ($p < 0.001$, $n = 3$).

Agonist-Induced Internalization of the D2 Receptor in NS20Y Cells. Internalization of the D2 receptor was induced by treatment with the agonist 7-OH-DPAT (10 µM). Internalization was quantified as the loss of binding of the hydrophilic ligand [³H]sulpiride to intact NS20Y cells stably expressing D2-EGFP or the wild-type D2 receptor after agonist treatment for 20 or 120 min. Cells were treated with 7-OH-DPAT, washed, and incubated with [³H]sulpiride (2 nM) at 4°C for 90 min. The maximal loss of [³H]sulpiride binding was $36 \pm 6\%$ ($p < 0.001$, $n = 3$) for D2-EGFP and $45 \pm 1\%$ for wild-type D2 receptor after agonist treatment for 20 min ($p < 0.001$, $n = 3$) (Fig. 3). No further reduction in [³H]sulpiride binding was observed after treatment with agonist for 2 h. Carrying out the 7-OH-DPAT treatment at 4°C caused a modest reduction in binding that was not statistically significant (data not shown), indicating that the loss of binding of [³H]sulpiride reflected receptor internalization rather than persistent binding of the agonist. In addition, D2-EGFP NS20Y cell-surface proteins were biotinylated after a 20-min incubation with agonist. Treatment with 7-OH-DPAT for 20 min reduced D2-EGFP receptor immunoreactivity on the cell membrane by $36 \pm 8\%$ compared with untreated cells ($n = 3$, $p < 0.01$).

Prevention of D2 Receptor Internalization in NS20Y Cells. Treating D2-EGFP NS20Y cells with siRNAs directed against arrestin2 and arrestin3 decreased the abundance of both arrestin isoforms to levels barely detectable by immunoblotting without affecting GAPDH immunoreactivity (Fig. 4A). This siRNA-induced depletion of arrestins largely pre-

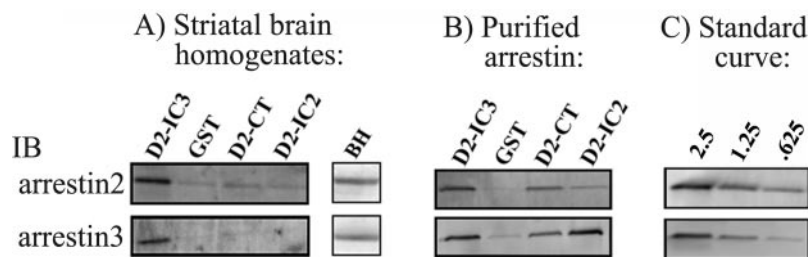


Fig. 1. Binding of arrestins to D2 receptor intracellular domains. Fusion proteins of GST and the D2 receptor third cytoplasmic loop (D2-IC3), second cytoplasmic loop (D2-IC2), or carboxy terminus (D2-CT), or GST alone (GST) were incubated with striatal homogenate (A) or purified arrestin2 or arrestin3 (B) and purified as described under *Materials and Methods*. Eluates were immunoblotted (IB) with anti-arrestin2 or anti-arrestin3 antibody, as indicated, with the binding of arrestin resulting in a band at ~55 kDa. The figures shown are representative of three to four independent experiments. A, each preparation was incubated with striatal homogenate (500 µg of protein). Aliquots (20 µg of protein) of the striatal homogenate (brain homogenate, BH) were run in two lanes to demonstrate the presence of both arrestin2 and arrestin3 in the striatal homogenate. B, each preparation was incubated with 25 ng of purified arrestin2 or arrestin3. C, the standard curve that was used to calculate arrestin binding in B is depicted. Each experiment with purified arrestins included a four-point standard curve (background and three concentrations of arrestin2 or arrestin3 as indicated, in nanograms).

vented agonist-induced sequestration of the D2 receptor. Thus, treatment with 10 μ M 7-OH-DPAT for 20 min decreased the binding of [3 H]sulpiride by $38 \pm 11\%$ ($p < 0.01$, $n = 3$) in D2-EGFP cells, but it decreased the binding of [3 H]sulpiride by only $5.5 \pm 5.5\%$ for D2-EGFP cells in which arrestins were depleted by transfection with arrestin2 and arrestin3 siRNAs (Fig. 4B).

Agonist-Induced Colocalization of Endogenous D2 Receptor and Arrestin2, but not Arrestin3, in Neostriatal Neurons. Agonist-induced trafficking of the endogenous dopamine D2 receptor and arrestin2 and arrestin3 in neostriatal cultures was investigated using cells that were treated with 7-OH-DPAT, fixed with paraformaldehyde, and immunostained with D2 receptor and arrestin antibodies as

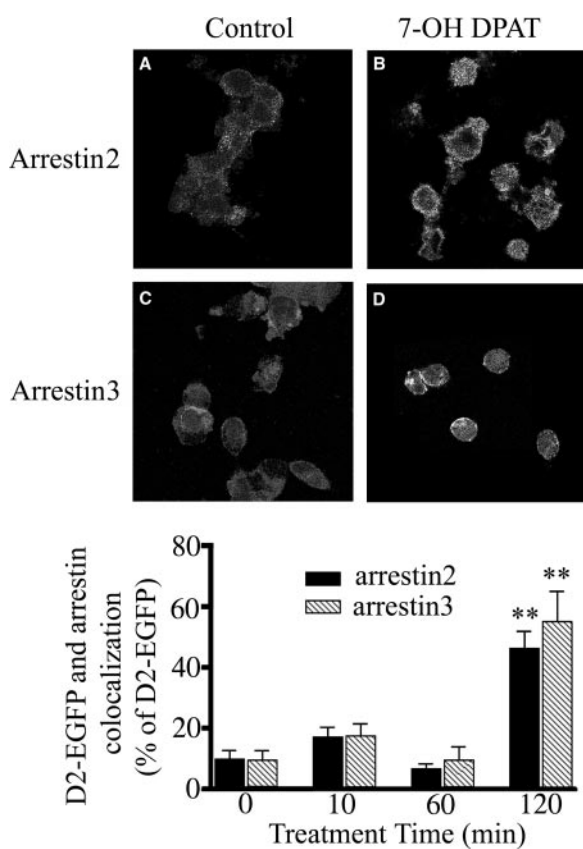


Fig. 2. Agonist-induced colocalization of D2-EGFP and endogenous arrestin2 and arrestin3 in NS20Y cells. NS20Y cells expressing D2-EGFP were grown on coverslips for 24 to 48 h and treated with 7-OH-DPAT (10 μ M) for 10, 60, or 120 min. Paraformaldehyde-fixed cells were incubated with mouse anti-arrestin2 or anti-arrestin3 antibody and imaged by confocal microscopy. Untreated cells were compared with treated cells at each time point to assess differences in colocalization of D2-EGFP and arrestin2 immunoreactivity. Images are representative confocal fluorescence images of the colocalization of D2-EGFP and endogenous arrestins in NS20Y cells. Cells treated as described above were used to assess colocalization of D2-EGFP fluorescence and arrestin2 (A and B) or arrestin3 (C and D) immunoreactivity in vehicle-treated (A and C) and agonist-treated cells (B and D). Green and red fluorescence images were merged, and pixels containing only green or red fluorescence were subtracted to show only pixels containing both green and red fluorescence. In the graph, the results shown are the mean \pm S.E.M. for colocalization of D2-EGFP autofluorescence and arrestin2 or arrestin3 immunoreactivity, expressed as a percentage of total D2-EGFP fluorescence, in cells treated with vehicle (0) or agonist for the indicated time. Treatment with 7-OH-DPAT significantly altered colocalization of D2-EGFP and arrestin2 and arrestin3 ($p < 0.0001$ by one-way ANOVA), with a significant increase observed after treatment for 2 h (**, $p < 0.01$ by Dunnett's post hoc comparison, $n = 3$).

described under *Materials and Methods*. Treatment with 7-OH-DPAT (10 μ M) increased the colocalization of arrestin2 and the endogenous D2 receptor from $20 \pm 5\%$ in untreated cells to $46 \pm 7\%$ after agonist treatment for 2 h ($p < 0.05$, $n = 3$) (Fig. 5). Shorter durations of treatment had no significant effect on colocalization with arrestin2. In contrast, the colocalization of the endogenous D2 receptor with arrestin3 decreased from $43 \pm 10\%$ to $13 \pm 2\%$, $10 \pm 4\%$, and $18 \pm 6\%$ after 7-OH-DPAT treatment for 5, 60, or 120 min, respectively (Fig. 5).

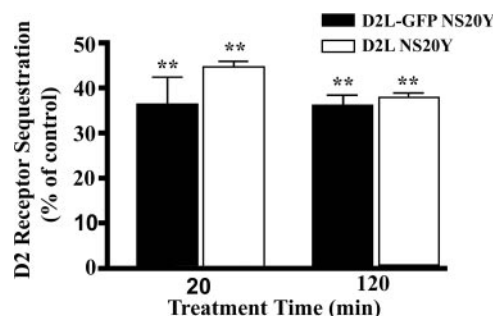


Fig. 3. Agonist-induced internalization of the D2 receptor in NS20Y cells assessed using [3 H]sulpiride. NS20Y cells expressing either wild-type D2 or D2-EGFP were treated with 7-OH-DPAT (10 μ M) for 20 or 120 min before quantifying internalization as loss of binding of [3 H]sulpiride, expressed as the percentage reduction from the value in vehicle-treated cells. Treatment with 7-OH-DPAT caused internalization of the dopamine D2 receptor ($p < 0.0001$ by one-way ANOVA). The four conditions shown were all significantly different from control (**, $p < 0.01$ by Dunnett's post hoc comparison, $n = 3$).

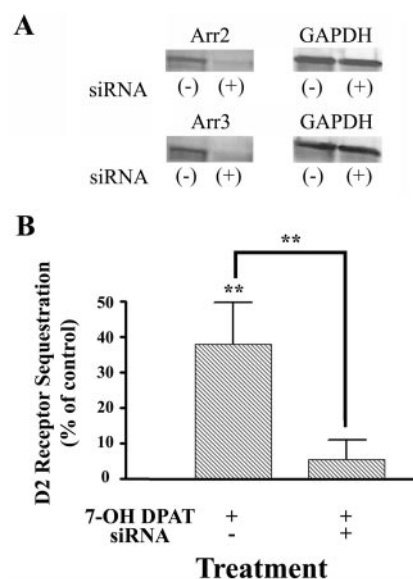


Fig. 4. Inhibition of D2 receptor internalization in NS20Y cells by siRNA-induced depletion of endogenous arrestins. A, immunoreactivity of arrestin2 (Arr2) and arrestin3 (Arr3) in lysates (50 μ g of protein) was determined in cells transfected with siRNAs specific to arrestin2 and arrestin3 as described under *Materials and Methods*. To control for equal loading of protein, membranes were stripped and immunoblotted with anti-GAPDH. The amount of arrestin in each sample was calculated from arrestin2 and arrestin3 standard curves similar to the representative curve depicted in Fig. 1C. B, NS20Y cells expressing D2-EGFP were transfected with siRNAs specific to arrestin2 and arrestin3 and treated with 7-OH-DPAT (10 μ M) for 20 min before quantifying internalization as loss of binding of [3 H]sulpiride, expressed as the percentage reduction from the value in vehicle-treated cells (control). Treatment with 7-OH-DPAT caused internalization of the dopamine D2 receptor that was significantly different from control and from siRNA-treated cells ($p < 0.01$ by Dunnett's t test for both comparisons).

Agonist-Induced Translocation of Arrestin2 in Neostriatal Neurons. To confirm that agonist-induced colocalization of D2 receptor and arrestin2 in neurons represents translocation of the adaptor protein to the membrane, the abundance of arrestin2 and arrestin3 was determined in membranes prepared from neostriatal neurons treated with 7-OH-DPAT (10 μ M) for 20 or 120 min. The abundance of arrestin2 in the membrane was enhanced by $91 \pm 31\%$ ($p < 0.05$, $n = 3$) after treatment with agonist for 2 h but not after 20 min (Fig. 6). There was no significant translocation of endogenous arrestin3 to the membrane at 20 or 120 min of 7-OH-DPAT treatment.

Agonist-Induced Coimmunoprecipitation of the D2 Receptor and Arrestin2 in Neostriatal Cultures. Cul-

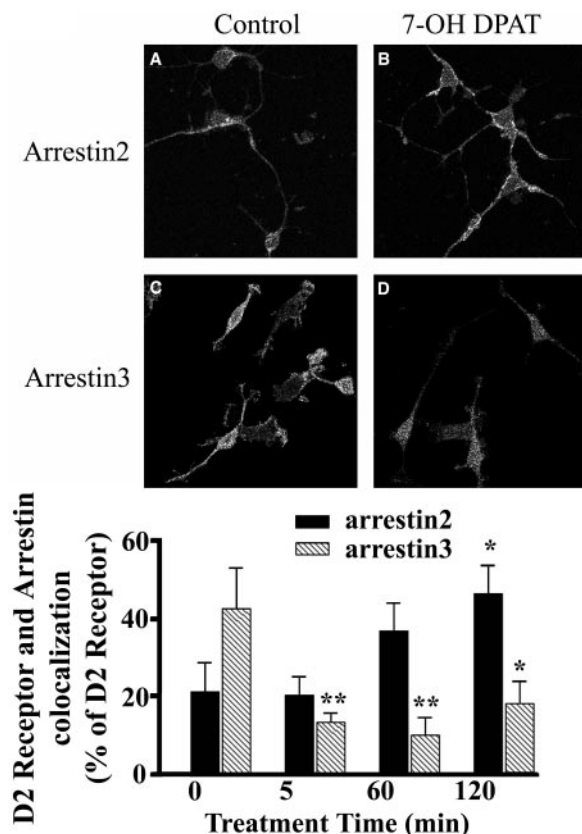


Fig. 5. Agonist-induced colocalization of the endogenous D2 receptor and endogenous arrestin2, but not arrestin3, in neostriatal neurons. Neuronal cultures prepared as described under *Materials and Methods* and treated as described in the legend to Fig. 3 were used to quantify colocalization of D2 receptor and arrestin2 or arrestin3 immunoreactivity. Representative confocal fluorescence images are shown for the colocalization of D2 and arrestin immunoreactivity in neostriatal neuronal cultures. The representative experiment shown depicts colocalization of endogenous D2 receptor immunoreactivity and immunoreactivity for arrestin2 (A and B) or arrestin3 (C and D) in vehicle-treated (A and C) or agonist-treated cells (B and D). Green and red fluorescence images were merged, and pixels containing only green or red fluorescence were subtracted to show only pixels containing both green and red fluorescence. In the graph, the results shown are the mean \pm S.E.M. for colocalization of D2 and arrestin2 or arrestin3 immunoreactivity, expressed as a percentage of total D2 receptor immunoreactivity, in cells treated with vehicle (0) or agonist for the indicated time. Treatment with 7-OH-DPAT significantly altered colocalization of D2 receptor and arrestin2 immunoreactivity ($p < 0.01$ by one-way ANOVA) with a significant increase observed after treatment for 2 h (\star , $p < 0.05$ by Dunnett's post hoc comparison, $n = 3$). Treatment with 7-OH-DPAT significantly altered colocalization of D2 receptor and arrestin3 immunoreactivity ($p < 0.01$ by one-way ANOVA), with significant decreases observed after treatment for 5, 60, and 120 min (\star , $p < 0.05$; $\star\star$, $p < 0.01$ by Dunnett's post hoc comparison, $n = 3$).

tures were treated with 7-OH-DPAT, cross-linked with disuccinimidyl suberate, immunoprecipitated with anti-dopamine D2 antibody, and immunoblotted with anti-arrestin2 or anti-arrestin3 antibody. Cross-linking of the D2 receptor and arrestin2 or arrestin3 formed a complex that was detected at ~ 100 kDa. Agonist treatment of neurons for 20 or 120 min increased the coprecipitation of the dopamine D2 receptor and arrestin2 by $33 \pm 11\%$ ($p < 0.05$, $n = 3$) and $36 \pm 8\%$ ($p < 0.05$, $n = 3$), respectively (Fig. 7). There was no significant effect of agonist treatment on the coprecipitation of the dopamine D2 receptor and arrestin3.

Discussion

The mechanisms of desensitization and resensitization of the dopamine D2 receptor have not been thoroughly elucidated, although studies using heterologously expressed arrestin and/or GRKs in non-neuronal cells suggest an important role for those proteins (Ito et al., 1999; Kim et al., 2001, 2004). Our aim was to investigate whether the dopamine D2 receptor differentiates between the two isoforms of endogenous nonvisual arrestins.

For many GPCRs, the third intracellular loop is the main site of interaction with arrestin (Krupnick et al., 1994; Wu et al., 1997; Gelber et al., 1999; Mukherjee et al., 1999; Kim et al., 2001; DeGraff et al., 2002), although the first and second intracellular loops and the carboxy terminus also contribute to the binding of arrestin to some GPCRs (Raman et al., 1999; Bennett et al., 2000; Nakamura et al., 2000; Cen et al., 2001; Hüttenrauch et al., 2002). Using a GST pull-down assay, we determined that the third intracellular loop of the D2 receptor bound both arrestin2 and arrestin3 in neostriatal homogenate and bound similar amounts of purified arrestin2 and arrestin3, suggesting that the third loop of the D2 receptor has no inherent selectivity for either form of arrestin. Both forms of purified arrestin bound to the C terminus and the second cytoplasmic loop of the D2 receptor, but they did so less avidly than to D2-IC3, which may account for the lack of specific binding when using neostriatal homogenate as the source of arrestins. D2-IC2 bound purified arrestin3 preferentially, but a lesser contribution of this receptor domain to arrestin binding might account for the lack of other evidence for preferential binding of arrestin3.

Arrestin binding is driven both by a conserved "phosphate

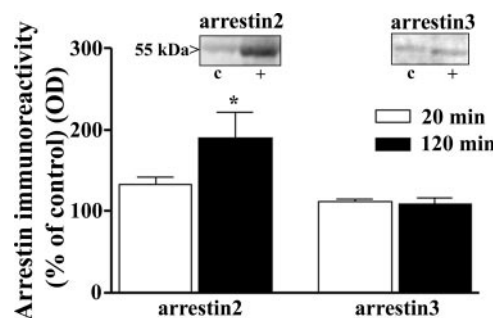


Fig. 6. Agonist-induced translocation of arrestin in neostriatal neurons. Neostriatal neurons were treated with 7-OH-DPAT (10 μ M) for 20 or 120 min, membranes were prepared, and levels of endogenous arrestin2 and arrestin3 were assessed using immunoblotting. Results are the mean \pm S.E.M. from three experiments expressed as a percentage of the band density in membranes from vehicle-treated cells. The inset depicts representative experiments in which cells were treated with vehicle (c) or agonist (+) for 120 min. (\star , $p < 0.05$ by Dunnett's post hoc comparison).

sensor”, involving salt bridges in the polar core of arrestin that are disrupted by the electrostatic interaction of phosphorylated residues in the GPCR with positively charged residues in arrestin, and by a theoretical “GPCR activation sensor” that interacts with residues exposed in the activated GPCR (Gurevich and Gurevich, 2004). Thus, although binding of arrestin to GPCRs is enhanced by receptor phosphorylation, there are also phosphorylation-independent determinants of binding that are sufficient for binding to occur between isolated GPCR cytoplasmic domains and arrestin (Wu et al., 1997; Shiina et al., 2000; Cen et al., 2001; DeGraff et al., 2002). Arrestin is presumably binding to sites on the fusion proteins that are occluded in the inactive GPCR and made accessible by receptor activation or, in this case, by removing them from the context of the intact receptor.

To evaluate the effects of D2 receptor stimulation on endogenous arrestins in a cell system, we first used confocal microscopy to quantify their agonist-induced colocalization. In NS20Y cells stably expressing D2-EGFP, colocalization of the receptor with both arrestin2 and arrestin3 was markedly enhanced by agonist treatment for 2 h but not by shorter treatments. Although colocalization is not proof of interaction, this is consistent with the well-established observation that agonist-activated GPCRs bind arrestins, and it is also consistent with the binding of both arrestin2 and arrestin3 to D2-IC3, D2-IC2, and D2-CT. Similar results were observed using GFP-tagged arrestins coexpressed with D2_L in Chinese hamster ovary cells (Kim et al., 2004). The apparent nonselectivity of the D2 receptor for the arrestin subtypes is typical of class B receptors such as the neurotensin NT1 and vasopressin V2 receptors (Oakley et al., 2000).

In neostriatal neurons, in contrast, agonist treatment for 2 h selectively enhanced the colocalization of the endogenous D2 receptor with endogenous arrestin2, whereas colocalization with arrestin3 was rapidly decreased by 7-OH-DPAT, an efficacious dopamine D2-like receptor-selective agonist (Chio et al., 1994). Selective translocation of arrestin2 in neurons was confirmed by quantifying arrestin immunoreactivity in membranes prepared from agonist-treated neuronal cul-

tures; the abundance of arrestin2, but not arrestin3, was enhanced after treatment for 2 h but not after 20 min. In addition, there was a selective agonist-induced increase in the direct interaction of the endogenous D2 receptor and arrestin2 as assessed by coimmunoprecipitation of the two proteins. These data suggest a preferential agonist-induced interaction of the endogenous D2 receptor with endogenous arrestin2 in neostriatal neurons.

The difference in results between D2 receptor and arrestin colocalization in D2-EGFP NS20Y cells (no apparent selectivity) and in neostriatal neurons (selectivity for arrestin2) could have been caused by the different cell types or by the use of a receptor-EGFP fusion protein in NS20Y cells. To eliminate the possibility that the presence of EGFP inhibited a selective interaction with arrestin2, we evaluated the colocalization of a recombinant wild-type D2 receptor with arrestins in D2-NS20Y cells and determined that agonist treatment enhanced the colocalization of D2 receptor immunoreactivity with both arrestin2 and arrestin3. Taken together with the results of the GST pull-down assay, we propose that the D2 receptor can bind both arrestin2 and arrestin3 but that an interaction with arrestin3 is prevented in neostriatal neurons. One possible mechanism for selective interaction with arrestin2 in neurons is separate compartmentalization of the D2 receptor and arrestin3. Another possibility is that arrestin2 is simply much more abundant in neostriatal tissue (Gurevich et al., 2002), although the ability to pull down similar amounts of arrestin2 and arrestin3 from neostriatal homogenates using D2-IC3-GST suggests that our results cannot be explained in this way. The high basal colocalization of arrestin3 and the D2 receptor in neurons may not reflect an interaction between the proteins, because any stimulus such as constitutive activity of other GPCRs that recruits arrestins to the cell membrane will alter the apparent colocalization of arrestins with all other membrane proteins.

We also evaluated the role of arrestins in D2 receptor internalization in NS20Y cells. Using direct binding of the hydrophilic ligand [³H]sulpiride, we observed substantial internalization of the D2 receptor that was maximal after 20 min of treatment with 7-OH-DPAT. A similar internalization time course was determined using a cell-surface protein biotinylation assay in both NS20Y cells and neostriatal neurons (T. A. Macey and K. A. Neve, unpublished observations). These results are similar to prior work demonstrating agonist-induced internalization of the D2_L receptor in Chinese hamster ovary, human embryonic kidney 293, and Neuro2A neuroblastoma cells (Itokawa et al., 1996; Vickery and von Zastrow, 1999), although others have observed little or no internalization in the absence of overexpressed GRK or arrestin (Ito et al., 1999; Kim et al., 2001, 2004). Suppression of the expression of arrestin2 and arrestin3 by transfection with siRNAs greatly decreased the internalization of the D2 receptor, indicating that D2 receptor internalization requires arrestin. This is consistent with prior work demonstrating that expression of a dominant-negative arrestin mutant inhibits D2 receptor internalization (Kim et al., 2004).

The time course of the trafficking of endogenous arrestins, however, was much slower than that determined using heterologously expressed arrestins (Shenoy and Lefkowitz, 2003). Translocation of endogenous arrestins to the membrane and agonist-induced colocalization of the D2 receptor and arrestins were not significantly increased for at least 60 min, and only coprecipitation of the D2 receptor and arres-

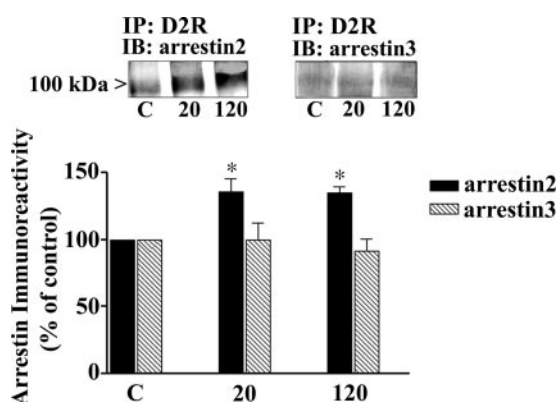


Fig. 7. Agonist-induced coimmunoprecipitation of the dopamine D2 receptor and arrestin2 and arrestin3 in neostriatal cultures. Striatal neurons were treated with 7-OH-DPAT or vehicle, cross-linked with disuccinimidyl suberate, and coimmunoprecipitated (IP) with anti-dopamine D2 antibody. Lysates were separated by SDS-PAGE and immunoblotted (IB) with anti-arrestin2 or arrestin3 antibody. Top, representative experiments in which cells were treated with vehicle (C) or 7-OH-DPAT for 20 or 120 min. Bottom, results are the mean \pm S.E.M. from three experiments, expressed as a percentage of the band density in membranes from vehicle-treated cells (*, $p < 0.05$ by Dunnett's post hoc comparison).

tin2 was significantly enhanced within 20 min of agonist treatment, when receptor internalization was maximal. According to the prevailing model of GPCR trafficking in which the binding of arrestin to agonist-activated, phosphorylated receptor mediates receptor desensitization and internalization (Krupnick and Benovic, 1998), agonist-induced translocation of arrestins should precede receptor internalization. One possibility is that constitutive interaction of arrestins with the D2 receptor that is suggested in Fig. 7 suffices to support receptor internalization. Another consideration is that D2 receptor colocalization with arrestin2 and arrestin3 in NS20Y cells, receptor colocalization with arrestin2 in neurons, and translocation of arrestin2 to the membrane in neurons all tended to be increased after 5 to 20 min of agonist treatment, although none of these effects reached statistical significance. If endogenous arrestin is much more abundant than D2 receptor expression, rapid receptor-induced changes in the distribution of a fraction of endogenous arrestin might be difficult to detect on the background noise of high arrestin concentrations. In the coprecipitation assay, on the other hand, in which rapid agonist-induced changes were observed, a high arrestin-to-receptor ratio would not affect the signal-to-noise ratio, because the only arrestin being measured is that which is bound to the receptor. Despite the seeming mismatch between the time courses of receptor internalization and some measures of arrestin translocation, the finding that siRNA-induced depletion of endogenous arrestins prevents receptor internalization provides strong support for the hypothesis that arrestin binding to the D2 receptor is required for receptor internalization.

The classification of GPCRs has been investigated chiefly using recombinant GPCR- and arrestin-overexpressing cell lines (Oakley et al., 2000; Shenoy and Lefkowitz, 2003), and other work suggests that not all rhodopsin-family GPCRs fit neatly within the A or B classification (Mukherjee et al., 2002; Tulipano et al., 2004). In this study, we determined that although the D2 receptor interacts with endogenous arrestin2 and arrestin3 in NS20Y cells and in vitro, characteristic of a class B receptor, the neostriatal D2 receptor interacts selectively with endogenous arrestin2 upon agonist activation.

Acknowledgments

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Address correspondence to: Dr. Kim A. Neve, VA Medical Center (R&D-30), 3710 SW US Veterans Hospital Rd, Portland, OR 97239-2999. E-mail: nevek@ohsu.edu