

Agonist-Induced Cell Surface Trafficking of an Intracellularly Sequestered D1 Dopamine Receptor Homo-Oligomer

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ABSTRACT

The role of oligomerization in D1 dopamine receptor trafficking to the cell surface was examined using conformationally distinct variants of this receptor. Substitution of the highly conserved aspartic acid (Asp103) in transmembrane domain 3 resulted in a constitutively active receptor, D103A, that did not bind agonists or antagonists but trafficked to the cell surface as oligomers. Coexpression of D103A with the wild-type D1 receptor in human embryonic kidney 293t cells resulted in inhibition of cell surface expression of the D1 receptor because of receptor oligomerization, causing intracellular retention of both proteins. Rescue of the intracellularly retained oligomer could be achieved only by membrane-permeable full and partial ago-

nists, which resulted in cell surface expression of the D1 receptor, whereas cell-permeable antagonists and cell impermeable agonists had no effect. Cell surface fluorescence resonance energy transfer studies of cells coexpressing D103A and D1 revealed no signal before agonist treatment but a robust signal after agonist treatment, indicating that the intact D1/D103A oligomer reached the cell surface only after agonist treatment but not under basal conditions. This suggests that rescue of the retained D1/D103A oligomer to the cell surface was a result of an agonist-induced change in the conformation of D1, permitting cell surface trafficking of the D1/D103A receptor oligomeric complex from the endoplasmic reticulum.

The D1 dopamine receptor belongs to the class A superfamily of G protein-coupled receptors (GPCRs) and activates adenylyl cyclase through the stimulatory G protein subunits G_s and G_{olf} in regions of the brain including the caudate nucleus, nucleus accumbens, and olfactory tubercle (Herve et al., 1993). This results in the accumulation of intracellular cAMP and subsequent activation of cAMP-sensitive effectors such as protein kinase A (Missale et al., 1998). The D1 dopamine receptor has been postulated to play a role in the etiology and treatment of various neuropsychiatric and neurodegenerative disorders including schizophrenia (Lidow and Goldman-Rakic, 1994; Okubo et al., 1997), cocaine addiction (Hummel and Unterwald, 2002), and Parkinson's disease (Emilien et al., 1999).

We have previously shown that D1 dopamine receptors exist as homo-oligomers (Ng et al., 1994; George et al., 1998); an increasing number of reports suggests that this is a gen-

eral characteristic of GPCR structural biology (George et al., 2002). Several studies have implicated the requirement of oligomerization for proper cell surface trafficking of certain receptors (Jones et al., 1998; Nelson et al., 2001; Uberty et al., 2003; Hague et al., 2004). Cell surface trafficking may be mediated by the masking of putative endoplasmic reticulum (ER) retention motifs (Margeta-Mitrovic et al., 2000) or the dissociation of ER export motif binding proteins (Bermak et al., 2001). In addition, the advent of biophysical techniques such as fluorescence resonance energy transfer (FRET) and bioluminescent resonance energy transfer (BRET) have demonstrated that GPCR dimers are formed constitutively and that the ER probably represents the site of dimer assembly (Issafras et al., 2002; Floyd et al., 2003). Despite these advances, little is known about the rules governing oligomer formation and the final conformational requirements for cell surface trafficking of receptor oligomers.

A number of studies have shown the importance of GPCR oligomer conformation in receptor trafficking by demonstrating dominant-negative effects of specific mutant receptors on wild-type receptor function, including deletion mutants of the V2 vasopressin (Zhu and Wess, 1998), α_{1A} -adrenergic (Coge et al., 1999), and gonadotropin-releasing hormone re-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; WT, wild-type receptor; TM, transmembrane domain; ER, endoplasmic reticulum; trFRET, time-resolved fluorescence resonance energy transfer.

ceptors (Grosse et al., 1997). These mutant receptors were possibly misfolded because they were individually retained in a subcellular compartment, probably the ER, and interaction with the wild-type receptor resulted in intracellular retention of the latter, thereby inhibiting trafficking and/or signaling of the cognate wild-type receptor. These examples demonstrate that both naturally occurring and artificially constructed receptor mutants can act as negative regulators of wild-type receptor function through GPCR oligomerization, indicating that this is an early event, probably occurring during or shortly after protein biosynthesis in the ER. We have determined previously that certain nonfunctional D2 dopamine receptor truncation and point mutants, including a point mutation in transmembrane domain 3 at Asp114, could act to attenuate the cell surface expression of the wild-type D2 receptor (Lee et al., 2000a). However, unlike the receptor mutants described above, each of these mutant receptors was able to traffic independently to the plasma membrane unimpeded. This suggests that certain regulatory mechanisms in the cell monitor the fidelity of GPCR oligomer formation and that improper conformations of an oligomer, such as may be generated by the wild-type receptor interacting with a mutant receptor, may be scrutinized by the ER and deemed unsuitable for cell surface transport.

In the current study, we investigated the regulatory mechanisms that govern the fate of a newly synthesized D1 dopamine receptor oligomer and the conformational requirements that enable or prevent a receptor oligomer from being trafficked to the cell surface. We created a constitutively active D1 receptor, D103A, by mutating the highly conserved aspartic acid residue in transmembrane domain (TM) 3, thus creating a conformationally distinct receptor. When coexpressed with the wild-type D1 receptor, D103A exerted an expression-dependent cell surface inhibition of the wild-type receptor through receptor oligomerization. We tested the hypothesis that this intracellularly retained complex could be rescued as an oligomer using cell-permeable dopaminergic ligands. We demonstrated that cell surface rescue of the intact D1/D103A oligomer could only be achieved using agonists but not antagonists or inverse agonists. We suggest that this occurs by achieving an activated conformation of the wild-type protomer within the oligomeric complex, which yields an oligomeric configuration that can bypass a quality control checkpoint, rendering it trafficking-competent.

Materials and Methods

DNA Constructs and Site-Directed Mutagenesis. Full-length human D1 receptor cDNA was used for site-directed mutagenesis studies. The Asp residue in TM3 (position 103) was mutated to Ala and Glu and designated D103A and D103E, respectively. Two Ser residues (positions 198 and 199) in TM5 were mutated to Ala and the mutant receptor was denoted as S198A/S199A. All receptor mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as suggested by the manufacturer and using the following pairs of oligonucleotides (sense and antisense): D103A: sense, GGGTGGCCTTTGCCATCATGTGCTCC; antisense, GGAGCACATGATGGCAAAGGCCACCC; D103E: sense, GGGTGGCCTTTGAAATCATGTGCTCC; antisense, GGAGCACATGATTTCAAAGGCCACCC; and S198A/S199A, sense, GGACATATGCCATCTCAGCCGCCGTAATAAGCTTTTACATCCC; antisense, GGGATGTAAAAGCTTATTACGGC GGCTGAGATGGCATATGTCC. The D103A, D103E, and S198A/S199A receptors had a cMyc

epitope subsequently inserted at the N terminus of the receptor with an appropriate Kozak sequence through a two-step polymerase chain reaction protocol using Pfu enzyme. Wild-type D1 cDNA also had an HA epitope inserted at the N terminus in a similar fashion. All receptor isoforms were prepared in the pcDNA3 mammalian expression vector and verified by DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada).

Cell Culture and DNA Transfection. HEK293t and COS-7 cells were maintained as monolayer cultures at 37°C in minimum essential medium (Invitrogen, Carlsbad, CA) and α minimum essential medium (University of Toronto, Toronto, ON, Canada), respectively, supplemented with 10% fetal bovine serum, antimycotic, and antibiotic. HEK293t cells were grown to 80% confluence before being transiently transfected using Lipofectamine (Invitrogen). For coexpression experiments in which the amount of one construct used was varied, the total amount of cDNA transfected was kept constant by the addition of a compensating amount of pcDNA3. Transfected cells were grown for 48 h before harvesting for functional assays.

Membrane Preparation. Cells were washed in PBS and centrifuged at 1500g to obtain a pellet. Cell lysates were prepared by disruption with a Polytron homogenizer (Kinematica, Basel, Switzerland) in ice-cold 5 mM Tris-HCl and 2 mM EDTA buffer containing protease inhibitors (5 μ g/ml leupeptin, 10 μ g/ml benzamidine, and 5 μ g/ml soybean trypsin inhibitor). Lysates were centrifuged at 1000g for 10 min to separate nuclei and unbroken cells. Crude membrane fractions were prepared by centrifuging the supernatant at 20,000g for 25 min. Membrane protein was determined by the Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Drugs. The following dopaminergic ligands (Sigma, St. Louis, MO) were used in 10 μ M concentrations and dissolved in either DMSO, ethanol, or water as indicated by the manufacturer. Ascorbic acid 0.1% was added when dopamine was used. Cells were treated with one of the following agonists (pergolide, SKF 81297, SKF 38393, apomorphine, or dopamine), antagonists (SCH23390), or inverse agonists [(+)-butaclamol, flupenthixol, or fluphenazine].

Radioligand Binding. Near-maximal saturation receptor binding was determined with 15 to 25 μ g of membrane protein with 1 nM [³H]SCH23390 to obtain an estimate of receptor densities. (+)-butaclamol (1 μ M) was used to determine nonspecific binding. Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Gaithersburg, MD), using GF/C filters.

Whole Cell Binding. Specific dopaminergic cell surface binding was determined by subtracting dopamine-displaced specific [³H]SCH23390 binding from specific [³H]SCH23390 binding. Specific binding to surface receptors with [³H]SCH23390 was first determined in the following manner. Transfected cells were trypsinized and incubated with 1 nM [³H]SCH23390 to determine total binding. Nonspecific binding was determined by incubating cells at 4°C with 1 nM [³H]SCH23390 and an excess of unlabeled close congener, (+)-butaclamol (1 mM). Specific binding was subsequently determined by total binding minus nonspecific binding. Specific dopamine displaced specific [³H]SCH23390 binding was then determined in the following manner. Transfected cells were trypsinized and incubated with 1 nM [³H]SCH23390 and an excess of 100 mM dopamine (in 0.1% ascorbic acid) to compete off total surface bound [³H]SCH23390; the amount of radioligand displaced was taken to represent total binding. Nonspecific binding was determined by incubating cells with 1 nM [³H]SCH23390, 100 mM dopamine, and 1 mM unlabeled (+)-butaclamol. Specific dopamine displaced [³H]SCH23390 binding was determined by total dopamine-displaced [³H]SCH23390 binding minus nonspecific dopamine-displaced [³H]SCH23390 binding. All reactions were mixed gently and incubated for 3 h on ice to prevent receptor internalization. Nonspecific binding accounted for approximately 10 to 15% of total bound ligand. Bound ligand was isolated by rapid filtration as mentioned previously.

Cell Surface Fluorometric Analysis. Ninety-six-well plates were coated with poly-L-ornithine before being plated with 50,000

cells/well. Cells were transfected 24 h later with HA-D1 and cMyc-D103A to yield expression in a 1:1 ratio. Cells were then fixed with 4% paraformaldehyde after 48 h and subsequently washed with PBS. Blocking was performed with 4% BSA at room temperature for 30 min, and cells were incubated with rat anti-HA (Roche, Penzberg, Germany) for 1.5 h. After primary antibody treatment, cells were washed with 4% BSA and incubated for an additional 1.5 h with FITC-conjugated anti-rat IgG (Sigma). Samples were excited at 485 nm and emission was measured at 530 nm by using the Cytofluor 4000 microplate reader (Applied Biosystems, Foster City, CA).

Coimmunoprecipitation. Transfected HEK293t cells were washed in PBS and scraped with homogenization buffer consisting of 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and protease inhibitors. Cells were harvested by disruption with a Polytron homogenizer (Kinematica, Basel, Switzerland) and solubilized by the addition of Nonidet P-40 to a final concentration of 1% (v/v) for 2 h at 4°C. Samples were centrifuged at 15,000g for 25 min, and the supernatant was collected for protein determination by the Bradford reaction; 2 mg of total cell lysate was used for immunoprecipitation. After preclearing for 30 min with 10 μ l of protein G agarose beads (Sigma), the lysates were incubated overnight with 3 μ g of rat monoclonal anti-HA (Roche). Immunocomplexes were subsequently incubated overnight with 50 μ l of protein G agarose beads and washed five times with ice-cold wash buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and protease inhibitors). The proteins were eluted in 80 μ l of Laemmli buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue) for 30 min at 37°C before immunoblotting.

Gel Electrophoresis and Immunoblotting. Membrane preparations were solubilized in Laemmli buffer with 5% β -mercaptoethanol for 20 min at room temperature (RT). Samples were separated on precast 10% polyacrylamide gels (Invitrogen) for 2 h. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 35 V for 3 h and blocked overnight in 10% skim milk powder and then incubated with either rat monoclonal anti-HA (Roche Diagnostics, Indianapolis, IN) or mouse monoclonal anti-cMyc (Upstate Biotechnology, Lake Placid, NY) for 3 h. Blots were rinsed with TBS and incubated with horseradish peroxidase-conjugated goat anti-rat or mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; Bio-Rad) for 1.5 h. Immunoreactivity was detected by enhanced chemiluminescence using an ECL Plus Kit (GE Healthcare).

Cell Surface Biotinylation. Biotinylation was carried out as described by Cao et al. (1998) with minor modifications. In brief, transiently transfected COS-7 cells were biotinylated with 0.5 mg/ml sulfo-NHS-biotin (Pierce, Rockford, IL) for 30 min at RT. Unreacted biotin was quenched with Tris-buffered saline three times for 5 min each. Cells were solubilized in extraction buffer (0.5% Triton X-100, 10 mM Tris-Cl, pH 7.5, 120 mM NaCl, 25 mM KCl, and protease inhibitors) and rotated for 3 h at 4°C. The lysate was clarified by centrifugation at 10,000 rpm for 20 min. The supernatant was collected and immunoprecipitations were carried out with 8 μ g of mouse anti-cMyc (Upstate Biotechnology, Lake Placid, NY) or rabbit anti-HA (BD Biosciences, San Jose, CA) overnight at 4°C. Samples were incubated with 50 μ l of protein G agarose PLUS (Pierce) for 3 h. Antigen complexes were subsequently washed sequentially in high-salt wash buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 0.1% SDS, and 0.5% Triton X-100), 1 M NaCl in HSB, pH 7.0, and low salt wash buffer (10 mM Tris-HCl, pH 7.5). Biotinylated proteins were eluted by incubation of beads in SDS-polyacrylamide gel electrophoresis sample buffer at 37°C for 15 min and resolved by 12% polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride and blocked overnight in 10% skim milk powder. Biotinylated proteins were then complexed with horseradish peroxidase by incubating membranes with Vectastain ABC Elite detection system (Vector Laboratories, Burlingame, CA). Biotinylated proteins were subsequently detected with ECL. The protein bands were scanned by densitometric analysis and relative intensities were quantified using NIH Image software version 1.33.

Whole Cell cAMP Assay. Basal cAMP levels were measured from HEK293t cells transiently expressing D1 or the mutated D1 receptors. Cells expressing D1 were incubated with 10 μ M dopamine for 15 min. Cells expressing D103A were incubated with 10 μ M concentrations of either dopamine, pergolide, SKF 38393, SKF 81297, fluphenazine, flupenthixol, or (+)-butaclamol for 15 min before cAMP accumulation was evaluated. Cells were lysed in 0.1 N HCl for 20 min, and the supernatant was assayed for cAMP accumulation using a highly sensitive enzyme-linked immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Immunofluorescence Microscopy. HEK293t cells were transiently transfected with either HA-D1/pcDNA3, cMyc-D103A/pcDNA3, or HA-D1/cMyc-D103A on glass coverslips that were pretreated with poly-L-ornithine (Sigma). Forty-eight hours after transfection, cells were washed twice with PBS and incubated for 2 h in the absence or presence of 10 μ M pergolide (Sigma) or 10 μ M SCH23390 (Sigma) at 37°C. After incubation, cells were washed twice with PBS and fixed in 4% paraformaldehyde at 4°C for 30 min. To detect the subcellular localization of HA-D1 and cMyc-D103A, cells were blocked and, where indicated, permeabilized with a buffer containing 0.1% Triton and 5% normal goat serum in PBS for 30 min at RT. The cells were then incubated with rat anti-HA monoclonal antibody (Roche) at a 1:200 dilution and mouse anti-cMyc monoclonal antibody (Santa Cruz Biotechnology) at a 1:150 dilution for 1 h at RT. After one wash with PBS for 30 min, the cells were incubated with FITC-conjugated anti-rat IgG (Sigma) at a 1:32 dilution and TRITC-conjugated anti-mouse IgG (Sigma) at a 1:64 dilution for 1 h at RT. After one wash with PBS for 30 min, coverslips were mounted, and FITC-labeled HA-D1 and TRITC-labeled cMyc-D103A were visualized with a Zeiss LSM-510 laser confocal microscope (Zeiss, Welwyn Garden City, UK). For detection of calnexin, cells were incubated with rabbit anti-calnexin polyclonal antibody (Stressgen Biotechnology, Victoria, BC, Canada) at a 1:200 dilution for 1 h at RT. Cells were subsequently incubated with Alexa-546-conjugated anti-rabbit IgG (Invitrogen) at a 1:500 dilution for 1 h at RT.

Colocalization Image Analysis. Dual-channel colocalization analysis was performed on randomly selected images using the NIH Image J software with the colocalization analyses plugin. The Mander's coefficients, M_{red} and M_{green} , represent the number of colocalized pixels expressed as a fraction of the total number of pixels within the red and green channel, respectively (Manders et al., 1993). The Mander's coefficients range from 0 (no colocalization) to 1 (complete colocalization) and are independent of the pixel intensities within each respective channel. Pixels with a value of zero in both channels were ignored as background.

Time-Resolved Fluorescence Resonance Energy Transfer. Cells coexpressing D1 and D103A (in the presence or absence of ligand) were trypsinized 48 h after transfection and counted. The FRET protocol was performed as described by McVey et al. (2001) using 15 nM Eu^{3+} -labeled anti-HA (donor) and 5 nM APC-labeled anti-cMyc (acceptor) (PerkinElmer Life and Analytical Sciences, Boston, MA). Time-resolved FRET was measured using a Victor3 microplate reader (PerkinElmer) with excitation of Eu^{3+} at 615 nm and emission of APC detected at 665 nm.

Statistical Analysis. All pharmacological data were analyzed using Prism (GraphPad Software, San Diego, CA). Saturation binding curves were generated using nonlinear least-squares regression curve fitting. One-tailed, unpaired Student's *t* tests were performed to compare groups of data sets. Statistical significance at the *p* < 0.05 level is denoted with *.

Results

Expression Analysis of Asp103 Substitution Mutants. The aspartic acid in TM3 (Asp103) was mutated to alanine and the receptor (D103A) was expressed transiently

in HEK293t cells. Membrane fractions were prepared and [3 H]SCH23390 saturation binding assays confirmed that this mutated receptor exhibited negligible binding compared with the wild-type D1 receptor (WT), which expressed at 5.83 ± 0.27 pmol/mg (Fig. 1A). To confirm that the lack of binding of D103A was not a result of reduced receptor expression, a cMyc epitope was inserted, and immunoblot analysis was performed. This revealed the existence of receptor protein species at 70 and 140 kDa, representing monomers and dimers, respectively (Fig. 1B), that were identical to WT. The majority of monomeric species detected at 70 kDa are a result of the disruption of D1 homodimeric complexes by SDS-polyacrylamide gel electrophoresis and reducing agents as described previously (Lee et al., 2000b). Glycosylation was confirmed by expression of an HA-tagged D1 receptor mutant,

denoted as HA-N5A/N175A, in which both putative glycosylation sites in the amino terminus and second extracellular loop were removed (Fig. 1B) (Karpa et al., 1999). A 15- and 30-kDa decrease in the sizes of monomeric and dimeric species, respectively, was observed with HA-N5A/N175A expression compared with HA-tagged D1 expression. We also examined whether substitution of the aspartic acid to glutamic acid (D103E), a similarly charged residue, was sufficient to allow [3 H]SCH23390 binding. The substitution of an acidic residue in this position did not restore ligand binding (Fig. 1A) despite normal expression as indicated by immunoblotting (Fig. 1B). This suggests that the function of the aspartic acid residue in this region of the ligand binding pocket is very specific and that charge preservation alone was insufficient to permit ligand binding.

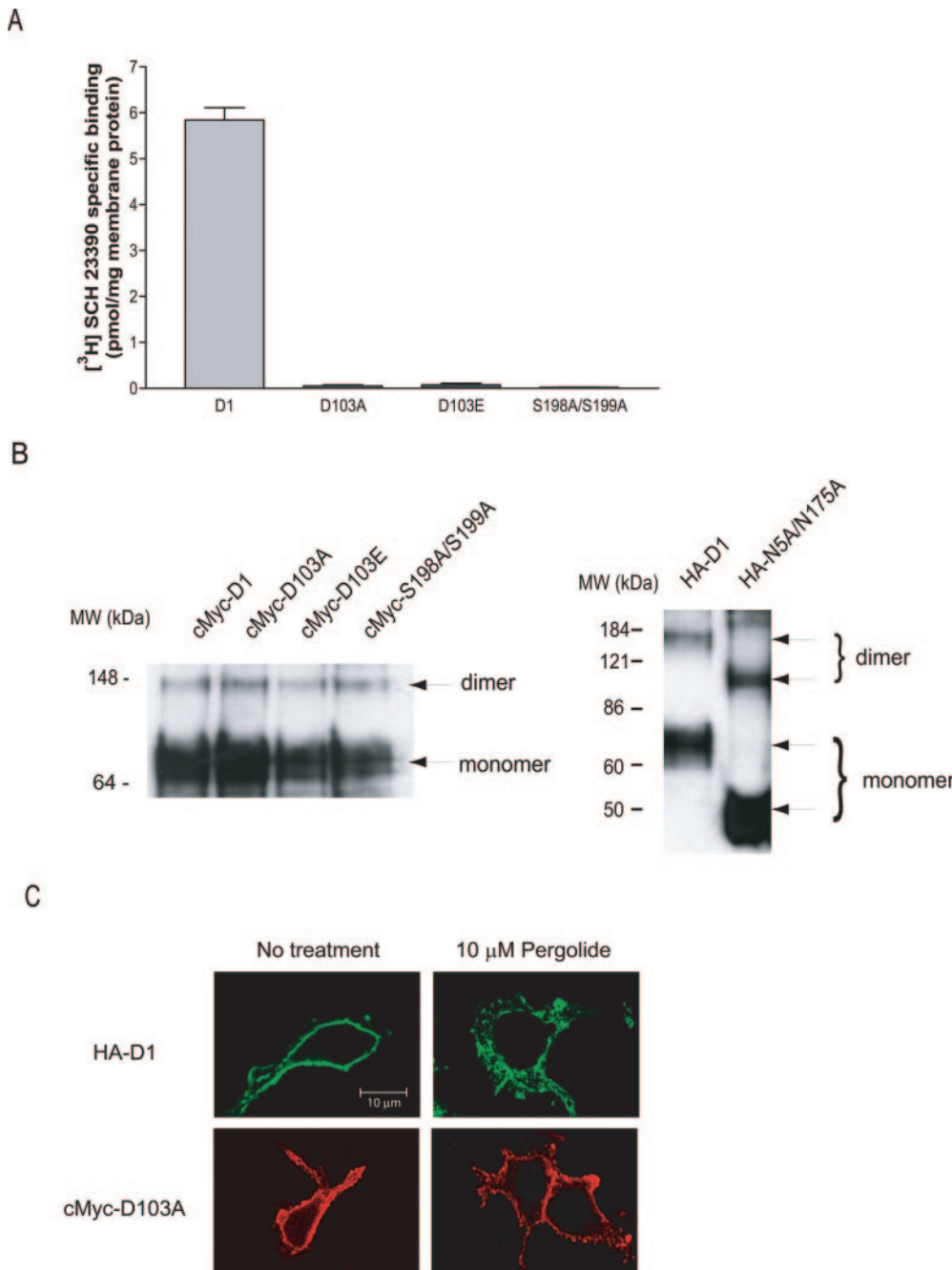


Fig. 1. Characterization of D1 receptor isoforms. A, receptor density estimated by radioligand saturation binding. [3 H]SCH23390 (1 nM) binding was performed on membrane fractions from HEK293t cells transiently transfected with D1, D103A, D103E, or S198A/S199A receptors. Nonspecific binding was defined with 1 μ M (+)-butaclamol. Data shown are the means of at least three independent experiments. B, immunoblot of membranes from HEK293t cells transiently transfected with cMyc-tagged D1, D103A, D103E, or S198A/S199A. Arrows at 70 and 140 kDa indicate the glycosylated monomeric and dimeric species, respectively. Glycosylation was confirmed by expression of a HA-tagged glycosylation deficient D1 receptor, HA-N5A/N175A. C, immunofluorescence imaging of HA-D1 visualized by FITC (green) and cMyc-D103A visualized by TRITC (red) showing basal cell surface localization and agonist-induced internalization of the D1 receptor but no effect on the mutant D103A receptor. HEK293t cells expressing either HA-D1 (top row) or cMyc-D103A (bottom row) receptors were exposed to 10 μ M pergolide for 20 min before fixing and permeabilization with Triton X-100.

To assess the cellular distribution of receptors in fixed HEK293t cells independently expressing HA-D1 and cMyc-D103A, immunofluorescence microscopy was conducted using FITC-conjugated anti-HA and TRITC-conjugated anti-cMyc antibodies (Fig. 1C). Both of these receptors exhibited a uniform cell surface distribution (Fig. 1C, left). Treatment with 10 μ M pergolide, a mixed D1/D2 agonist, triggered robust internalization of WT (Fig. 1C, top right) but had little effect on D103A (Fig. 1C, bottom right).

Substitution Mutants of Asp103 are Constitutively Active. Given the conserved nature of the aspartic acid residue in catecholamine receptors, we proceeded to determine whether Asp103 participated in other functional roles besides ligand binding. Because the mutation of comparable residues in other GPCRs has conferred constitutive activity to the receptor (Porter et al., 1996; Befort et al., 1999), we postulated that the Asp¹⁰³ mutation might have an equivalent effect. One of the hallmarks of a constitutively activated G_s-coupled GPCR is an increase in basal endogenous cAMP levels resulting from enhanced adenylyl cyclase activity. The D103A and the D103E receptors had $24.4 \pm 8.2\%$ and $21.5 \pm 2.2\%$ higher basal adenylyl cyclase activity, respectively, than unstimulated WT (Fig. 2) after normalizing to WT cell surface expression by densitometric analysis of biotinylated cell surface receptors (Fig. 2, inset). This represented approximately 45% of the cAMP response of wild-type receptor when maximally stimulated with 10 μ M dopamine ($51.3 \pm 18.3\%$ above basal), indicating that this specific TM3 mutation had resulted in a receptor that was in a partially activated, but not a fully activated, conformation. In addition, no change in constitutive cAMP production was observed upon incubation of D103A with agonists (dopamine, pergolide, SKF 38393, SKF 81297) or inverse agonists [fluphenazine, flupenthixol, (+)-butaclamol], indicating that removal of the aspartic acid residue also abolished binding to other dopaminergic ligands (data not shown). Another mutated D1 receptor that also did not exhibit antagonist binding, S198A/

S199A, did not exhibit a change in cAMP production different from D1 (Fig. 2) after normalizing for cell surface expression, and was also expressed as oligomers (Fig. 1B). These data demonstrated that the aspartic acid residue in TM3 participated in keeping the D1 receptor in an inactive conformation.

Interaction of D103A with Wild-Type D1 Results in Attenuated D1 Expression. When the wild-type D1 receptor was coexpressed with increasing amounts of D103A, there was a dose-dependent reduction of [³H]SCH23390 binding to cell membranes (Fig. 3A). When expressed singly, the WT density was determined to be 6.3 ± 0.1 pmol/mg. There was a 29% drop in the receptor density at a 1:0.5 transfection ratio of WT/D103A, and a 48% reduction at a 1:1 ratio, yielding WT expression levels of 4.5 ± 0.3 and 3.3 ± 0.2 pmol/mg, respectively. Receptor density decreased by approximately 63% to 2.3 ± 0.1 pmol/mg when maximal reduction was reached at a 1:1.5 transfection ratio. Further increments of D103A expression did not alter WT density. Coexpression of WT with the D103E receptor yielded similar results to that of the D103A receptor, demonstrating an inverse relationship between constitutively active receptor expression and WT expression (data not shown).

To evaluate whether these changes were reflective of changes in cell surface receptor expression, three additional strategies were employed: 1) whole-cell binding to dopamine, 2) biotinylation of cell surface receptors, and 3) whole-cell fluorometric analysis. For whole-cell binding experiments, we sought to use a cell-impermeable ligand to quantify cell surface expression. Dopamine is a highly polar compound rendering it highly hydrophilic and unable to cross the lipid bilayer (Barbier et al., 1997). Hence, cell surface receptor expression was estimated by the difference between specific [³H]SCH23390 binding and that displaced with an excess of dopamine as described under *Materials and Methods*. We determined that 100 μ M dopamine was sufficient to completely displace 1 nM [³H]SCH23390 from both high- and low-affinity sites of the wild-type D1 receptor in our compe-

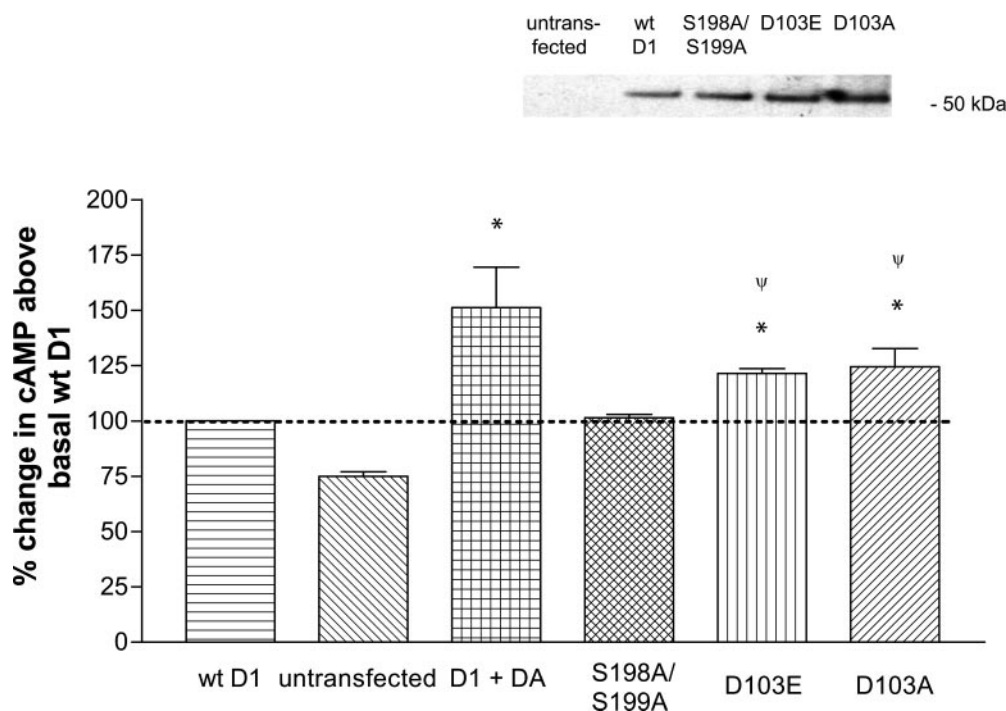


Fig. 2. Constitutive activity of the D1 receptor and mutated forms. Basal adenylyl cyclase activity was determined by quantifying cAMP accumulation. The average level of cAMP produced for S198A/S199A, D103A, D103E, and dopamine (DA)-stimulated D1 was 1.5 ± 1.5 , 24.4 ± 8.2 , 21.5 ± 2.2 , and $51.3 \pm 18.3\%$ above basal D1 cAMP levels, respectively. The inset compares cell surface expression of biotinylated receptors. All values are reported after normalization of receptor expression to WT by densitometric analysis. Data shown are from three individual experiments performed in triplicates. Data sets were normalized against the basal cAMP response in wild-type D1 transfected cells and expressed as a percentage change of this. Significance at $p < 0.05$ versus basal cAMP levels of cells expressing wild-type D1 is denoted by *. Significance at $p < 0.05$ versus stimulated D1 (D1 + DA) cAMP levels is denoted with ψ .

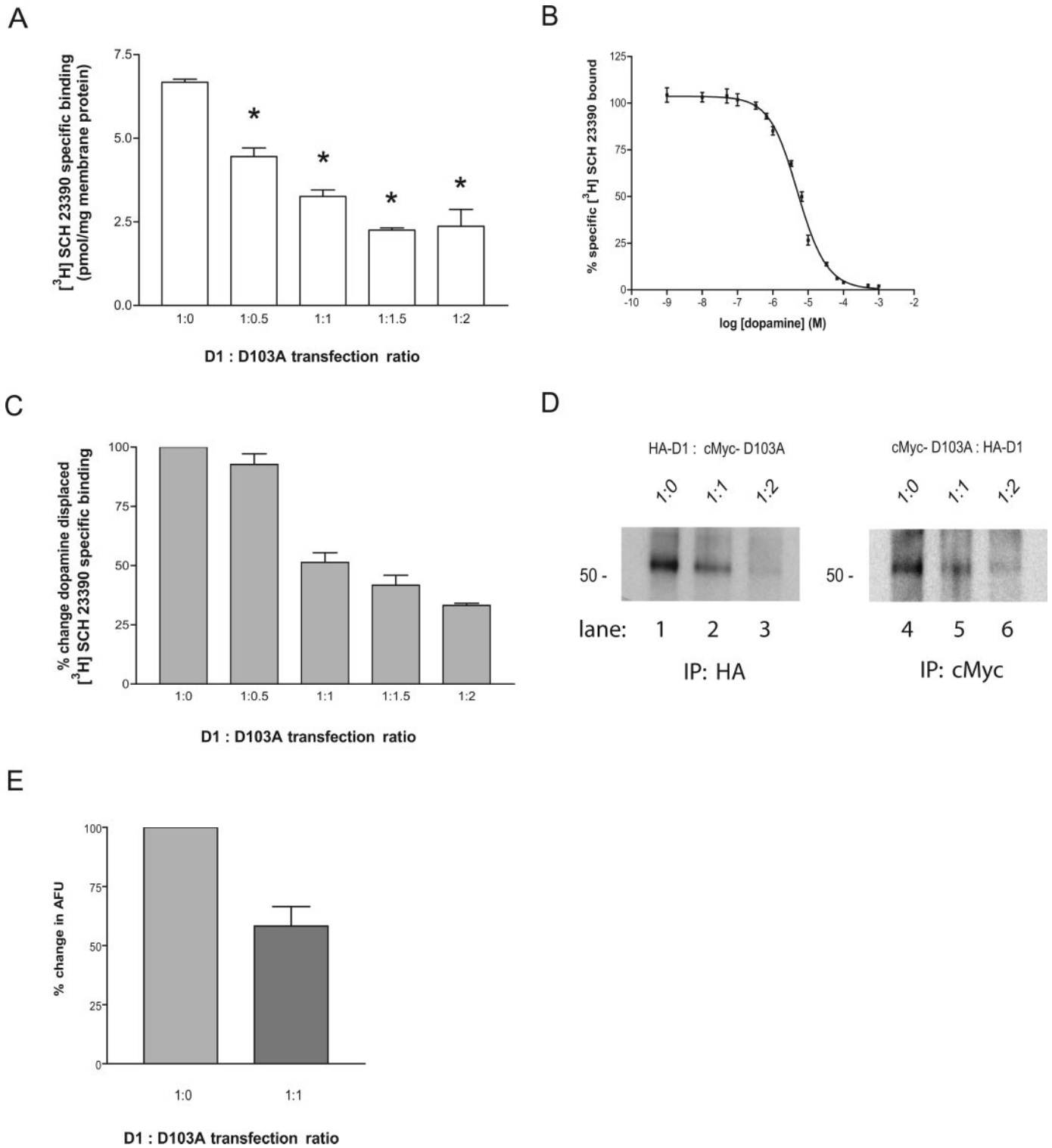


Fig. 3. Dominant-negative inhibition of D103A on wild-type D1 cell surface expression. **A**, wild-type D1 receptor density determined by binding of membranes to 1 nM [³H]SCH23390 from HEK293t cells cotransfected with increasing concentrations of D103A. Wild-type D1 receptor density was maintained constant (cDNA, 5 μ g) while D103A cDNA was transfected in varying amounts yielding transfection ratios of 1:0, 1:0.5, 1:1, 1:1.5, and 1:2, respectively. Wild-type D1 binding in the absence of D103A was 6.3 ± 0.9 pmol/mg (1:0) and gradually decreased with increasing expression of D103A by 29% (1:0.5), 48% (1:1), 63% (1:1.5), and 62% (1:2). The data shown are the means \pm S.E. of three to five experiments. Significance at $p < 0.05$ versus control D1 binding (at 1:0 transfection ratio) is denoted by *. **B**, competition of various concentrations of dopamine for 1 nM [³H]SCH23390 binding to WT from membrane preparations. **C**, whole-cell binding of 100 μ M dopamine displaced [³H]SCH23390 for WT in the presence of varying expression levels of D103A was performed. WT/D103A ratios of 1:0.5, 1:1, 1:1.5, and 1:2 yielded a 7.2 ± 4.5 , 48.7 ± 4.1 , 57.9 ± 4.5 , and $66.8 \pm 0.9\%$ reduction in WT cell surface density, respectively. The data shown are presented as the means \pm S.E. of three experiments. **D**, cell surface biotinylation of HA-WT in the presence of cMyc-D103A and vice versa. Biotinylated cell surface HA-WT receptor expression was determined in the presence of increasing expression levels of cMyc-D103A (lanes 1–3). The converse experiment was performed with cMyc-D103A (lanes 4–6). **E**, cells coexpressing cMyc-D103A and HA-WT (1:1 ratio) were incubated with FITC-conjugated anti-HA and cell surface D1 expression was detected by whole-cell fluorometry. Coexpression with cMyc-D103A (1:1) resulted in a decrease in HA-WT expression (as measured in arbitrary fluorescence units, AFU) to $58.3 \pm 8.3\%$ of individually expressed D1 (1:0).

titution binding studies from membranes (Fig. 3B) and thus used these conditions for the whole-cell binding assays. Nonspecific binding was defined by [3 H]SCH23390 binding in the presence of 1 μ M (+)-butaclamol, as described under *Materials and Methods*. As shown in Fig. 3C, there was a $7.2 \pm 4.5\%$ decrease in WT cell surface expression at a D1/D103A transfection ratio of 1:0.5 compared with expression at a 1:0 ratio. Further increases in D103A expression yielded 48.7 ± 4.1 , 57.9 ± 4.5 , and $66.8 \pm 0.9\%$ reductions in WT cell surface receptor density at 1:1, 1:1.5, and 1:2 transfection ratios, respectively. These results are comparable with data obtained through saturation binding of membrane preparations.

Cell surface expression of D103A was also assessed by determining the proportion of cell surface biotinylated D103A receptors in response to increasing WT expression in COS-7 cells. This cell line is more adherent than HEK 293t cells, rendering it more suitable for the cell surface biotinylation experiments. As shown in Fig. 3D, lanes 1–3, the proportion of biotinylated D1 receptors decreased in the presence of increasing levels of D103A expression. The D1 receptor migrates at approximately 55 kDa in COS-7 cells because of differences in glycosylation. The converse experiment revealed a similar pattern of reduced biotinylated D103A expression with increasing levels of D1 receptor expression (Fig. 3D, lanes 4–6) consistent with our whole-cell binding data in Fig. 3C.

Finally, we performed cell surface fluorometric analysis on nonpermeabilized whole cells coexpressing amino terminally tagged HA-D1 and cMyc-D103A. As shown in Fig. 3E, WT cell surface labeling in the presence of D103A (1:1 expression ratio) was reduced to $58.3 \pm 8.3\%$ of WT when expressed alone. This is consistent with the data obtained through the methods described above, suggesting that the WT/D103A mixed oligomer is selectively hindered from trafficking to the cell surface.

To control for translational efficiency and ensure that the biosynthesis of the WT was not compromised by the cotranslation of the mutated receptor, the S198A/S199A mutant was coexpressed with WT in a 1:1 ratio and whole cell analysis of

dopamine displaced [3 H]SCH23390 binding was determined as described above. This receptor did not exhibit enhanced constitutive activity (Fig. 2) and expressed comparably to D1 when transfected alone (Fig. 1B). There was negligible inhibition of cell surface expression of WT when coexpressed with S198A/S199A (Fig. 4A), indicating the translational efficiency of the WT was not compromised during coexpression with a second receptor. Similar results were obtained when the WT was cotransfected with the apelin receptor (Fig. 4A).

To confirm whether D103A existed within an oligomeric complex with the WT, cMyc-D103A, and HA-D1 were coexpressed in a 1:1 ratio and the total cell lysates were immunoprecipitated with either antibodies directed to cMyc or HA. Immunoprecipitates were subsequently analyzed by immunoblot for HA immunoreactivity. As shown in Fig. 4B, cMyc-D103A and HA-D1 were coimmunoprecipitated with the other, each visualized as a 70-kDa species, indicating that the receptors were within an oligomeric complex containing the D103A and wild-type D1 receptor.

Cell Permeable Agonists Specifically Rescue Wild-Type D1 Cell Surface Expression. Despite the ability of the wild-type D1 and D103A receptors to traffic as oligomers independently, we hypothesized that the WT/D103A receptor oligomer was sequestered by the quality control machinery in the cell because of an oligomeric configuration that was recognized by the cell as unsuitable for cell surface trafficking. We predicted that treatment of cells coexpressing these two receptors with various dopaminergic ligands might stabilize the sequestered oligomer into a conformation permissive for trafficking. Thus, we evaluated the potential of a series of cell-permeable dopaminergic agonists, antagonists, and inverse agonists to reverse the cell surface inhibition of the WT imposed by oligomerization to the D103A mutant. Wild-type and D103A receptors were cotransfected in a 1:1 ratio, and cells expressing these receptors were incubated for 2 h with an array of cell permeable D1 ligands (pergolide, SKF 81297, SKF 38393, apomorphine, SCH23390, (+)-butaclamol, fluphenazine, and flupenthixol) and the nonpermeable agonist, dopamine, and washed thoroughly before membrane receptor

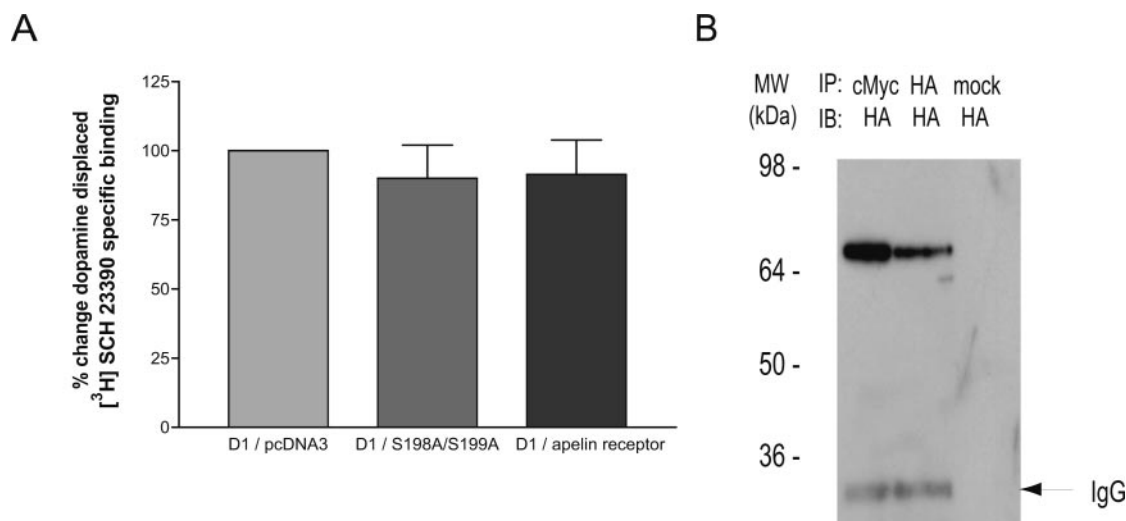


Fig. 4. A, cell surface WT expression is not compromised by coexpression with S198A/S199A or the apelin receptor. Whole-cell binding of cells cotransfecting WT and S198A/S199A or apelin receptor in a 1:1 ratio yielded cell surface WT expression levels that were reduced by 9 ± 7 and $12 \pm 8\%$, respectively, compared with untreated cells. B, HEK293t cells were cotransfected with cMyc-D103A and HA-WT receptors for 48 h, and whole-cell lysates were subjected to coimmunoprecipitation. Immunoprecipitation with either anti-cMyc or anti-HA precipitated HA-WT, detected at 70 kDa. Mock coimmunoprecipitations were done on transfected lysates in the absence of immunoprecipitating antibody.

density was evaluated. The cell permeable compounds indicated have previously been reported to cross the plasma membrane (Barbier et al., 1997) over the time period tested. As shown in Fig. 5A, the full D1 agonists, pergolide and SKF 81297, induced 66.2 ± 26.7 and $71.7 \pm 19.4\%$ increases, respectively, in the amount of WT expression detected. The partial agonist, SKF 38393, induced a much greater increase in WT expression by approximately $165 \pm 32.5\%$. Apomorphine and dopamine exerted little change on WT density. In contrast, the D1 antagonist SCH23390 had no significant effect on WT expression. A similar nonsignificant change in WT density was also observed for the inverse agonist (+)-butaclamol, and little change in WT expression was found

with two other inverse agonists, fluphenazine and flupenthixol (Fig. 5A). Thus, the ability to rescue the intracellularly retained wild-type D1 receptors was specific only to the ligands that were cell-permeable agonists. To determine whether this rescue was representative of wild-type D1 expression at the cell surface, whole-cell binding was performed on cells coexpressing WT and D103A. As shown in Fig. 5B, pretreatment of cells with pergolide caused a significant $46.4 \pm 6.5\%$ increase in wild-type receptor density. In contrast, preincubation with SCH23390 had only a negligible effect on cell surface expression of the wild-type D1 receptor.

The cellular distribution of coexpressed (in a 1:1 ratio) HA-D1 and cMyc-D103A receptors was visualized by immunofluores-

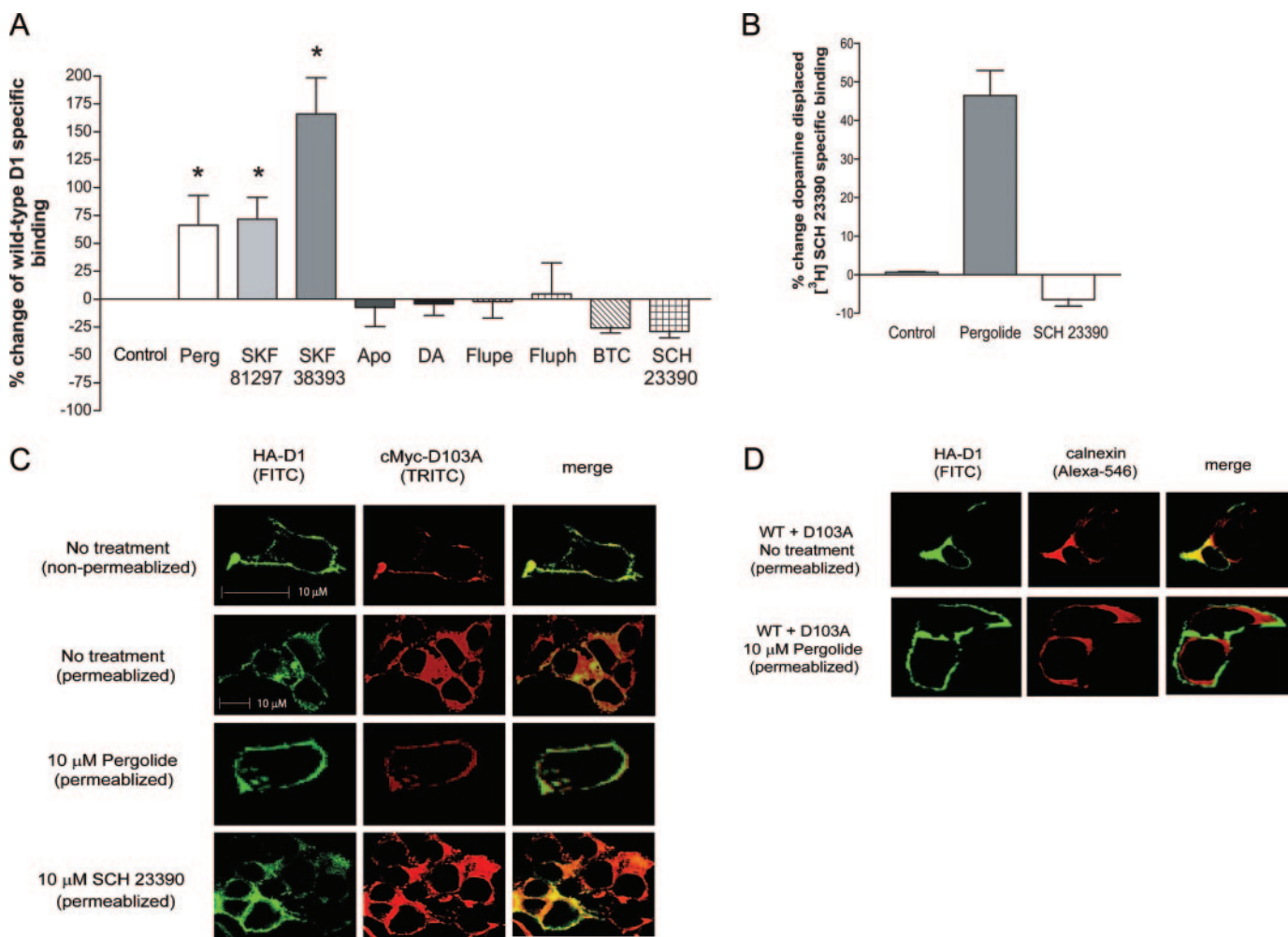


Fig. 5. Agonist-induced rescue of the intracellularly sequestered WT/D103A oligomer. **A**, HEK293t cells cotransfected with WT and D103A in equal amounts were treated with 10 μ M of the dopaminergic ligands indicated for 2 h at 37°C. The ligands used were: pergolide (Perg), SKF 81297, SKF 38393, apomorphine (Apo), dopamine (DA), flupenthixol (Flupe), fluphenazine (Fluph), (+)-butaclamol (BTC), and SCH23390. Plasma membrane fractions were prepared and D1 receptor density was assessed by saturation binding with 1 nM [³H]SCH23390. Data sets were normalized against D1 binding in cells coexpressing WT and D103A (control) and expressed as a percentage change of this. Pergolide, SKF 81297, and SKF 38393 induced a 66.2 ± 26.7 , 71.7 ± 19.4 , and $165 \pm 32.5\%$ increase in WT receptor density, respectively. Results are presented as the means \pm S.E. of three to five independent experiments. Significance at $p < 0.05$ versus control binding is denoted with *. **B**, cotransfected HEK293t cells were pretreated with the indicated ligands for 2 h, and whole-cell dopamine displacement of [³H]SCH23390 binding was performed as described under *Materials and Methods*. Data sets were normalized against D1 binding in cells coexpressing D1 and D103A (control) and expressed as a percentage change of this. Pergolide treatment caused a $46.4 \pm 6.5\%$ increase in WT cell surface density. Pretreatment with SCH23390 had no significant effect on WT density. Results are presented as the mean \pm S.E. of three independent experiments. Significance at $p < 0.05$ versus control binding is denoted with *. **C**, the subcellular distribution of wild-type HA-D1 and cMyc-D103A coexpressed in a 1:1 ratio was revealed by fluorescence microscopy in HEK293t cells. Immunostaining with anti-HA and anti-cMyc antibodies visualized by FITC- (green) and TRITC- (red) conjugated antibodies, respectively, was performed under nonpermeabilized (row 1) and permeabilized conditions (rows 2–4). Ligand treatments with 10 μ M pergolide and 10 μ M SCH23390 were performed for 2 h at 37°C before fixing. **D**, HEK 293t cells cotransfected with HA-D1 and cMyc-D103A were immunostained with anti-HA and anti-calnexin antibodies visualized by FITC (green) and Alexa-546 (red), respectively, before (row 1) and after 10 μ M pergolide treatment (row 2), as described previously.

cence microscopy. In nonpermeabilized cells, both receptors were localized at the cell surface under basal conditions (Fig. 5C, row 1). In permeabilized cells, both receptors were seen to have pronounced intracellular localization within the cell, probably involving the ER and/or Golgi (Fig. 5C, row 2). This distribution was in contrast to the cell surface distribution that was observed when each of these receptors was expressed individually (Fig. 1C) and confirms that intracellular retention of both receptors occurred. Pergolide treatment caused a change in the intracellular distribution of both receptors to a predominantly cell surface distribution (Fig. 5C, row 3). This is consistent with the data obtained through quantitative ligand binding. Treatment with SCH23390 did not seem to alter the intracellular distribution of receptors (Fig. 5C, row 4); this was confirmed by the absence of any changes in cell surface expression as demonstrated by the whole-cell binding experiments (Fig. 5B). Incubation with dopamine also had no effect on the cellular distribution of both receptors (data not shown). To identify the intracellular site of WT/D103A oligomeric retention, we used antibodies directed to both the wild-type D1 receptor and the ER-resident transmembrane protein, calnexin, in permeabilized cells coexpressing wild-type receptor and D103A receptor. As shown in Fig. 5D (row 1), under basal conditions, WT and calnexin exhibited extensive colocalization in the ER in the presence of D103A. In contrast, pretreatment of cells with 10 μ M pergolide, triggered a clear release of WT from the ER compartment, as evidenced by a lack of colocalization with calnexin and a subsequent redistribution to the cell surface (Fig. 5D, row 2). Wild-type D1 receptors expressed in the absence of D103A exhibited a robust cell surface distribution without colocalization with calnexin (data not shown). A random selection of images were quantified in Table 1 by determining Mander's colocalization coefficients for each channel (as described under *Materials and Methods*). A significant portion ($M_{\text{green}} > 0.75$) of WT (green) was found colocalized with D103A (red) under all conditions when the antigen pair D1/D103A was assessed. In contrast, colocalization of WT with calnexin was much reduced when WT/D103A expressing cells were treated with pergolide as shown for the antigen pair D1/calnexin (M_{green} , 0.99 without pergolide versus M_{green} , 0.15 after pergolide treatment).

Although not shown, we also tested the possibility that differential phosphorylation of WT and D103A by PKA might be the regulatory step in the ER quality control that is scrutinized for oligomer trafficking. Pretreatment of cells with the PKA inhibitor H-89 did not affect the extent of pergolide-induced WT/D103A rescue, suggesting a phosphorylation-independent mechanism of oligomer restoration to the cell surface.

TABLE 1

Colocalization analysis of different antigen pairs in permeabilized cells coexpressing D1 and D103A

Antigen Pair	M_{green}	M_{red}
D1/D103A (green/red)		
No treatment	0.86	0.70
10 μ M Pergolide	0.94	0.96
10 μ M SCH23390	0.77	0.53
D1/calnexin (green/red)		
No treatment	0.99	0.97
10 μ M Pergolide	0.15	0.19

$M_{\text{green}}/M_{\text{red}}$, Mander's coefficient for green or red channel (represents the number of colocalized pixels in the green or red channel expressed as a fraction of the total number of nonzero-pixels in the respective channel).

Agonist-Rescued Wild-Type D1 Receptors Remain Oligomeric with D103A on the Cell Surface. To test the possibility that prolonged agonist treatment may have triggered conformational changes in the sequestered WT that would cause it to dissociate from the WT/D103A oligomer and allow independent trafficking to the surface, analysis of the continued association between the rescued wild-type and D103A receptors was performed. To evaluate restored trafficking of the WT/D103A oligomer to the plasma membrane, cell surface time-resolved fluorescence resonance energy transfer (trFRET) between the two receptors was assessed in the absence and presence of agonist. Time-resolved fluorescence resonance energy transfer is a biophysical approach used to determine the proximity of proteins to each other. Energy transfer between two proteins implicates an intermolecular distance of 50 to 100

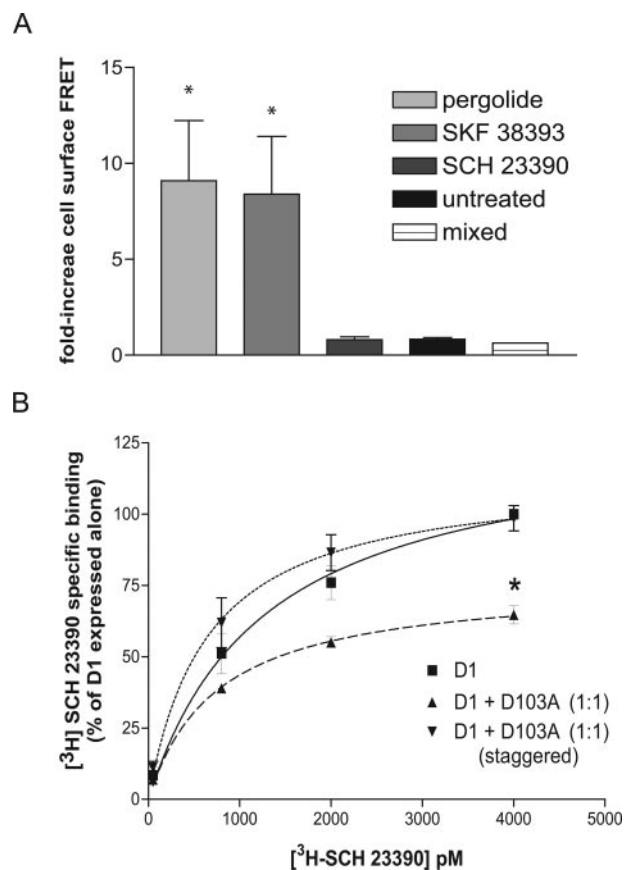


Fig. 6. Cell surface time-resolved fluorescence resonance energy transfer after agonist treatment. A, HEK293t cells coexpressing HA-WT and cMyc-D103A (1:1 ratio) were incubated in the absence or presence of either 10 μ M pergolide, SKF 38393 or SCH23390 for 2 h. The ligand was washed off and cells were then counted and incubated with Eu³⁺-labeled anti-HA (donor) and APC-labeled anti-cMyc (acceptor) antibodies for 2 h at 37°C and trFRET was determined by measuring APC emission after Eu³⁺ excitation. Separate pools of independently transfected cells were mixed to measure nonspecific trFRET. Significance at $p < 0.05$ versus control trFRET in the absence of ligand (untreated), is denoted with *. B, saturation isotherm of wild-type D1 coexpressed with D103A in a 1:0 (solid line) and 1:1 (dashed line) ratio. The B_{max} dropped by $35.3 \pm 3.2\%$ at a 1:1 transfection ratio. The total amount of cDNA transfected was maintained constant by addition of pcDNA3 vector. Membrane protein was prepared as described. Staggered expression of the two receptors (24 h apart) in a 1:1 ratio (dotted line) resulted in a nonsignificant drop in B_{max} by $1.4 \pm 4.4\%$. The data shown are expressed as a percentage of D1 binding (when expressed alone) and presented as the mean \pm S.E. of three experiments. Significance at $p < 0.05$ versus B_{max} of cells coexpressing D1 and D103A is denoted by *.

Å, which is indicative of a direct protein-protein interaction. Cells coexpressing wild-type and D103A receptors were incubated for 2 h in the presence of either pergolide, SKF 38393, or SCH23390. Intact cells were incubated with Eu^{3+} -labeled anti-HA (donor) and APC-labeled anti-cMyc (acceptor). trFRET was determined by monitoring light emission at 665 nm from APC. Under basal conditions, no FRET was detected in whole cells coexpressing both receptors indicating that only noninteracting receptor species were present at the cell surface (Fig. 6A). This suggests that the receptors at the cell surface under non-permeabilized conditions do not represent WT/D103A heterooligomers (Fig. 5C, row 1); rather, they separate WT and D103A receptor homo-oligomers. Treatment of cells coexpressing both receptors with pergolide or SKF 38393 yielded a 9.1- and 8.4-fold increase in energy transfer, respectively, indicating that the WT/D103A mixed oligomer was now present on the cell surface. Preincubation of cells with SCH23390 or mixing of cells independently expressing wild-type or D103A receptors yielded a negligible FRET signal. Therefore, we conclude that the rescued wild-type D1 receptor was part of a mixed oligomeric complex that remained intact on the cell surface after agonist rescue.

Staggered Expression of D103A with Wild-Type D1 Receptors Does Not Compromise D1 Cell Surface Expression. To further test the hypothesis that the inhibition of wild-type D1 surface expression was dependent on its close association with the D103A receptor, expression of both receptors were staggered by transfecting them 24 h apart. We have independently confirmed that 85 to 90% of D1 receptor protein is synthesized 24 h after transfection, thus achieving near maximal expression at this time point (G. Varghese, unpublished observations). Wild-type receptor cotransfected with D103A in a 1:1 ratio resulted in a significant drop in B_{max} by $35.3 \pm 3.2\%$ (Fig. 6B). However, no significant de-

crease in B_{max} was observed between WT expressed independently or when coexpressed with D103A in a 1:1 ratio, staggered 24 h apart (reduction in B_{max} by $1.4 \pm 4.4\%$). This suggests that the two receptors trafficked independently when biosynthesis of both receptors was not cotranslational and that the dominant-negative effect of the D103A occurred only under cotranslational conditions.

Discussion

The quality control mechanisms that scrutinize GPCR oligomer conformation in the ER are largely unknown. The examples of misfolded mutant receptors retaining wild-type receptors in the ER, although indicative of GPCR oligomerization (Grosse et al., 1997; Zhu and Wess, 1998; Coge et al., 1999), do not address the intracellular mechanisms mediating such dominant-negative effects. In the present study, using two receptors fully capable of cell surface expression when individually expressed, we demonstrate that the conformational arrangement of D1 dopamine receptor oligomers is a critical determinant of their ability to traffic to the cell surface. A constitutively active D1 receptor was generated by a single point mutation in TM3. Coexpression of this conformationally distinct variant with the wild-type D1 receptor yielded an oligomeric complex that was sequestered intracellularly and rescued selectively by treatment with dopaminergic agonists. Cell surface expression of the WT and constitutively active receptor (WT/D103A) oligomer was restored specifically by cell-permeable agonists but not by antagonists or inverse agonists.

Based on our results, we propose that occupancy by D1-selective agonists intracellularly "switched" the WT receptor to an activated conformation, matching that of the constitutively active D103A receptor, thus yielding a uniform oligomeric configuration that restored trafficking of the sequestered WT/

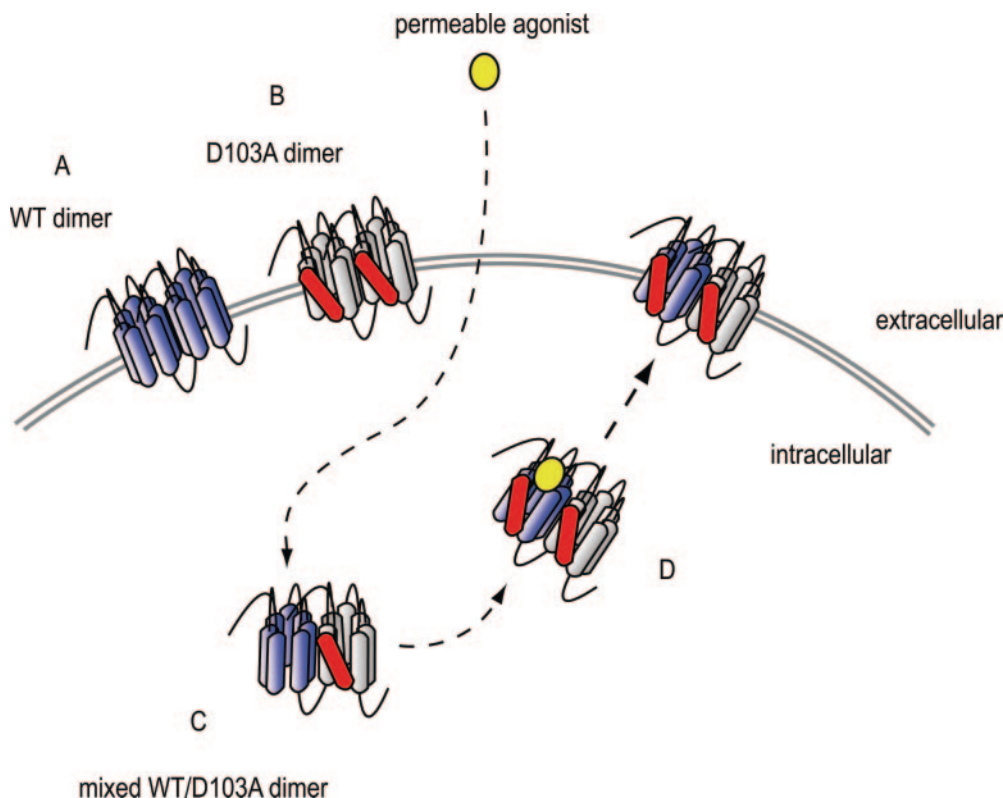


Fig. 7. Schematic of the hypothetical mechanism of intracellular rescue of WT/D103A dimers. Proposed model showing that the configuration of a D1 receptor dimer is a critical determinant of its ability to traffic to the cell surface. The wild-type D1 dimer trafficked normally (A) as did the non-binding, constitutively active D103A dimer (B). Coexpression of wild-type D1 with D103A results in a heterogeneous configuration of the dimeric complex that had an impaired ability to traffic (C). An activated conformation of the wild-type D1 receptor induced by a cell permeable agonist may alter its conformation to resemble that of the D103A receptor (D), resulting in a homogenous dimeric configuration (similar to B), further resulting in restored trafficking.

D103A oligomer to the plasma membrane. Ligand treatment revealed that the effect was selective and limited only to those agonists that were cell-permeable, indicating an intracellular locus of action. This was substantiated by the fact that dopamine, a permanently charged molecule incapable of penetrating the lipid bilayer, was found to not elicit a rescue effect. Because agonist rescue occurred within 2 h, it suggested that there was a release of the WT/D103A oligomer from an intracellular compartment rather than receptor synthesis *de novo*. This concept is depicted schematically in Fig. 7. We demonstrated that the binding pocket of the WT protomer was the target of hydrophobic agonist treatment. Two lines of evidence exclude the D103A protomer as a site of action: 1) there was no change in the cellular distribution of D103A in response to pergolide treatment and 2) there were no changes in the basal cAMP accumulation of D103A in response to agonists (pergolide, SKF 81297, SKF 38393). We also attempted the converse [i.e., to convert the D103A into an unactivated conformation matching that of the WT using the inverse agonists fluphenazine, flupenthixol, and (+)-butaclamol (Cai et al., 1999)], which would also be predicted to facilitate cell surface transport of this oligomeric complex, now rendered homogenous. However, cell surface trafficking of the WT/D103A oligomer with inverse agonist treatment was not observed experimentally; this is consistent with the fact that D103A was incapable of interacting with any dopaminergic ligand, including inverse agonists.

An alternative hypothesis that was tested was whether a cell-permeable agonist could trigger intracellular dissociation of the WT/D103A oligomer, resulting in separated WT and D103A receptor that could traffic to the cell surface independently, as shown when expressed individually. However, the trFRET data showing a robust energy transfer between WT and D103A suggested that WT/D103A oligomer dissociation did not occur by agonist treatment because FRET was detected on the cell surface only after agonist treatment. The absence of cell surface FRET in cells cotransfected with WT and D103A indicates that no WT/D103A mixed oligomers were present on the cell surface under basal conditions and that the receptors present on the cell surface were only the noninteracting WT and D103A receptor homooligomers. Thus, these data confirm that the sequestered oligomer was trafficked intact as a complex from an intracellular compartment, not as dissociated receptors. This intracellular compartment is likely to correspond to the ER as suggested by colocalization of WT with calnexin in the presence of D103A. Although agonist-dependent dissociation of GPCR oligomers has been suggested to occur for certain GPCRs (Cheng and Miller, 2001), the consensus of the majority of other studies indicates that cell surface oligomers remain intact after agonist activation (Lee et al., 2000b; Babcock et al., 2003; Dinger et al., 2003).

The dopaminergic agonists pergolide and SKF 81297 and the partial agonist SKF 38393 all induced a significant increase in WT/D103A oligomer density in plasma membrane preparations with varying degrees of rescue of the retained oligomeric complex. Extended treatment with each of these agonists (2 h) not only achieved cell surface expression but also triggered varying degrees of internalization of the wild-type receptors. This would lead to a loss in WT density from the cell surface that might, in part, be compensated for by the resensitization and recycling mechanism. All of the full agonists used induce internalization and recycling of the surface WT. This process, for the D1 recep-

tor, occurs in less than 30 min (Vickery and von Zastrow, 1999) and may perpetuate over a 2-h period with persistent agonist stimulation. This may partially explain why full agonists such as SKF 81297 and pergolide did not seem to promote WT cell surface expression as dramatically as SKF 38393. We hypothesize that restored cell surface trafficking as a result of intracellular adoption of an activated conformation by the WT receptor assumes that the fully activated conformation of WT and the D103A conformation are similar. However, SKF 38393 pretreatment yielded the highest recovery of WT cell surface receptor density, possibly resulting from its induction of an activated conformation in the WT that most closely mimicked that of D103A. Indeed, SKF 38393 is a partial agonist implicating its ability to put the wild-type receptor into a partially activated conformation. It is noteworthy that D103A was found to be only partially activated compared with the maximal stimulation of WT by dopamine as indicated by its more modest degree of constitutive cAMP generation and suggests that the SKF 38393-induced WT conformation may have most closely matched the conformation of the D103A receptor. This may provide evidence for a conformational checkpoint in the cell that selectively permits certain oligomeric arrangements to be trafficked to the cell surface. These data also indicate that multiple conformational states of the receptor may be achieved in response to occupancy by different agonists as suggested previously (Gether and Kobilka, 1998; Ghanouni et al., 2001). The apparent lack of efficacy of apomorphine was unpredicted but may be attributed to its structural characteristics. Compared with the SKF series of compounds, apomorphine has a very rigid structure and limited conformational mobility (Ryman-Rasmussen et al., 2005); this may limit its ability to trigger the appropriate oligomeric conformational requirements necessary for trafficking. This is currently undergoing further investigation.

The mechanism of selective agonist release by conformational manipulation that we describe is distinct from the ligand-mediated "rescue" described for GPCRs retained in the ER as a result of misfolding or the dominant-negative effects imparted by these receptor mutants (Morello et al., 2000; Janovick et al., 2003). The majority of these retained receptors undergo ligand-assisted folding upon interaction with pharmacological chaperones that were receptor-specific but nondiscriminatory among agonists, antagonists, or inverse agonists, all of which can be equally efficacious to promote folding. In our study, neither the WT nor the D103A was misfolded, in that they both exited the ER, expressed robustly at the cell surface as oligomeric units, and were functional, as demonstrated by their ability to generate cAMP.

The quality control mechanisms governing assembly of GPCR dimers and oligomers requires further understanding. A novel finding of the present study is that the relative conformation of protomers within an oligomer is recognized by a quality control mechanism that monitors the structural integrity of oligomers before cell surface trafficking. Two conformationally distinct receptors that were found to traffic normally as homo-oligomers when expressed individually were retained intracellularly when interacting with each other. However, because cell surface restoration of the oligomer could be achieved by pharmacological intervention only with the use of cell-permeable agonists, we propose that a specific cellular checkpoint monitors for certain conforma-

tional prerequisites of a homo-oligomer before its trafficking through the distal secretory pathway. One of these prerequisites may be the requirement for a homogenous conformational arrangement of an oligomeric complex in which all the constituent protomers must be in either an activated or an unactivated state. This may provide a novel approach for reversing potentially unwanted dominant-negative effects on cell surface expression resulting from conformationally heterogeneous receptor oligomers, such as may result from the expression of two different alleles in an individual heterozygous for a receptor gain of function mutation. Further studies using receptors with a spectrum of constitutively activated states will more firmly establish whether the conformational uniformity of a receptor oligomer is an absolute structural necessity for complete cellular processing. In addition, it would be of interest to study these trafficking phenomena outside heterologous cell expression systems and in the native neuronal environment in which these receptors are normally expressed. In particular, it would be interesting to determine the role of various proteins relevant to D1 receptor export in brain, such as DRiP78 (Bermak et al., 2001) and neurofilament-M (Kim et al., 2002), in oligomer biosynthesis and maturation.

We have provided, for the first time, evidence for the existence of a mechanism for scrutiny of formed GPCR oligomers, which occurs just before release for trafficking to the cell surface. The oligomer composed of structurally dissimilar but normally folded receptor units was blocked from export to the cell surface and could be released intact after specific conformational changes induced within the oligomer by pharmacological manipulation. The ability of the intracellular quality control mechanisms to recognize this structural variation within an oligomer is indicative of the high degree of stringency imposed on the final receptor product exported to the cell surface.

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