Reciprocal Regulation of Dopamine D1 and D3 Receptor Function and Trafficking by Heterodimerization

Chiara Fiorentini, Chiara Busi, Emanuela Gorruso, Cecilia Gotti, PierFranco Spano, and Cristina Missale

Section of Pharmacology, Department of Biomedical Sciences and Biotechnology (C.F., C.B., E.G., P.F.S., C.M.), and Centre of Excellence on Diagnostic and Therapeutic Innovation (P.F.S., C.M.), University of Brescia, Brescia, Italy; and Consiglio Nazionale delle Ricerche Institute of Neuroscience, Milano, Italy (C.G.)

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ABSTRACT

Colocalization of dopamine D1 (D1R) and D3 receptors (D3R) in specific neuronal populations suggests that their functional cross-talk might involve direct interactions. Here we report that the D1R coimmunoprecipitates with the D3R from striatal protein preparations, suggesting that they are clustered together in this region. Using bioluminescence resonance energy transfer (BRET²), we further suggest the existence of a physical interaction between D1R and D3R. Tagged D1R and D3R cotransfected in human embryonic kidney (HEK) 293 cells generated a significant BRET² signal that was insensitive to agonist stimulation, suggesting that they form a constitutive heterodimer. D1R and D3R regulate adenylyl cyclase (AC) in opposite ways. In HEK 293 cells coexpressing D1R and D3R, dopamine stimulated AC with higher potency and displaced [³H] DA-(±)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390) binding with higher affinity than in cells expressing the D1R. In HEK 293 cells individually expressing D1R or D3R, agonist stimulation induces internalization of D1R but not of D3R. Heterodimerization with D3R abolishes agonist-induced D1R cytoplasmic sequestration induced by selective D1R agonists and enables internalization of the D1R-D3R complex in response to the paired stimulation of both D1R and D3R. This mechanism involves β-arrestin binding because it was blocked by mutant β-arrestinV53D. These data suggest that as a result of dimerization, the D3R is switched to the desensitization mechanisms typical of the D1R. These data give a novel insight into how D1R and D3R may function in an integrated way, providing a molecular mechanism by which to converge D1R- and D3R-related dysfunctions.

Dopamine (DA) controls various physiological functions, including locomotor activity, learning and memory, and motivation and reward; dopaminergic dysfunctions have been implicated in the development of Parkinson’s disease, schizophrenia, and drug abuse. DA acts through five receptors, including locomotor activity, learning and memory, and movement; dopaminergic dysfunctions have been implicated in the development of Parkinson's disease, schizophrenia, and drug abuse. DA acts through five receptors, each displaying unique properties, including affinity for DA and specificity for G protein coupling and signaling and showing a peculiar neuronal distribution (Missale et al., 1998). The D1 receptor (D1R) is the most abundant and widespread DA receptor in the brain, where it is found at high density in both motor and limbic areas (Missale et al., 1998). The D3 receptor (D3R) is less abundant and exhibits a more restricted pattern of distribution with high concentrations in the ventral striatum, partially in the shell of the nucleus accumbens and islands of Calleja (Sokoloff et al., 1990; Lévesque et al., 1992) and lower expression in other brain regions (Sokoloff et al., 1990; Lévesque et al., 1992; Schwartz et al., 1998). Both D1R and D3R have been implicated in the regulation of rewarding mechanisms and motivated behavior and in the modulation of emotional and cognitive processes (Xu et al., 1997;...
responses of both D1R and D3R to agonist stimulation. Biochemical and behavioral evidence suggests that D1R and D3R may functionally interact. For example, D1R stimulation induces D3R mRNA expression in rat striatum and medulloblastoma cells (Levi-Sivan et al., 1998; Bordet et al., 2000), and coactivation of D1R and D3R in the shell of nucleus accumbens synergistically enhances substance P gene expression (Ridray et al., 1998; Schwartz et al., 1998). Moreover, D3R-deficient mice exhibit increased behavioral sensitivity to the stimulation of D1R and D2R (Xu et al., 1997) and decreased D1R-induced c-fos expression (Jung and Schmauss, 1999); furthermore, D1R and D3R interactions are apparently involved in the rewarding properties of low doses of cocaine and in cocaine-mediated inhibition of cAMP response element-binding protein phosphorylation (Karasinska et al., 2000, 2005). The cross-talk between D1R and D3R could occur either at the level of neuronal networks or within the same neuron. This latter type of interaction is supported by the observation that D1R and D3R mRNAs are colocalized in a large number of neurons within the shell of the nucleus accumbens (Le Moine and Bloch, 1996; Ridray et al., 1998; Schwartz et al., 1998) and the striatum (Surmeier et al., 1999) and that t-DOPA administration to hemiparkinsonian rats induces the overexpression of D3R in striatonigral neurons that constitutively express the D1R (Bordet et al., 2000; Guilin et al., 2001). Interaction between D1R and D3R in single neurons might involve either the convergence of their signaling pathways or the formation of heterodimeric complexes. It has been shown, in fact, that a general property of GPCR is to form heterodimeric receptor complexes with peculiar pharmacological, signaling, and trafficking characteristics (Angers et al., 2002), suggesting that receptor heterodimerization may represent a new integrative mechanism at the synaptic level. On this line, it has been shown that the D3R directly interacts with the D2R (Scarselli et al., 2001) and with the adenosine A2AR (Torvinen et al., 2005), and that the D1R interacts with the D2R (Rashid et al., 2007), with the adenosine A1R (Gines et al., 2000), and with the glutamate N-methyl-d-aspartate receptor (Lee et al., 2002; Fiorentini et al., 2003; Scott et al., 2006), and that the formation of these novel signaling units may represent the molecular basis for the functional interactions between these receptors.

The aim of this study was to investigate whether D1R and D3R may form a heterodimeric receptor complex and to define the functional properties of this complex. The results show that D1R and D3R directly interact in both striatal membranes and cotransfected cells and that this interaction influences D1R coupling to adenylyl cyclase and the adaptive responses of both D1R and D3R to agonist stimulation.

Materials and Methods

Materials. Human embryonic kidney (HEK) 293 cells were provided by Deutsche Sammlung von Mikroorganismen und Zelzkulturen GmbH (Braunschweig, Germany). Tissue culture media and fetal bovine serum were purchased from Euroclone Colbio (Milano, Italy). SKF 81297, quinpirole, and SCH23390 were purchased from Tocris (Bristol, UK); dopamine, (-)-sulpiride, and the monoclonal anti-D1R antibody (clone 1-1-F11-S.E6) were purchased from Sigma (Milano, Italy). The anti-D3R antibody and the horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz (Santa Cruz Biotechnology Inc., Heidelberg, Germany). The anti-hemoagglutinin (HA) antibody was from Sigma, and the anti-GFP antibody was from Invitrogen (Carlsbad, CA). The Cy3-labeled secondary antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). [3H]Sulpiride (78.2 Ci/mmol), [3H]Sch23390 (86 Ci/mmol), and [3H]Halochlorid (62.2 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Waltham, MA). Human D3R-GFP, human D3R, human D1R, β-arrestin-1V5D3, and dynamin-IK44A were kindly provided by Dr. Marc Caron (Duke University, Durham, NC); the ChemR23 chemokine receptor was kindly provided by Dr. Silvano Sozzani (University of Brescia, Brescia, Italy).

Generation of a Rabbit Anti-D1R Polyclonal Antibody. A polyclonal antibody directed to the peptide GSSEDLKKEEGIAPKPLEKS, corresponding to the rat D1 receptor (D1R) amino acids 396 to 417 (anti-D1R822), was produced in rabbits and was affinity-purified as described previously (Valiati et al., 1999). The sequence used does not match with the other DA receptor subtypes.

Protein Preparation, Immunoprecipitation, and Western Blot. The rat striatum was homogenized with a glass-window homogenizer in ice-cold 10 mM Tris-HCl containing 5 mM EDTA and a complete set of protease inhibitors (Roche, Milano, Italy), pH 7.4, and was centrifuged at 700 g for 10 min. The supernatant containing the total cell proteins was added with 1% SDS and stored at -80°C. To isolate the membrane fraction, the striatum was homogenized in 5 mM Tris-HCl containing 2 mM EDTA and a mixture of protease inhibitors, pH 7.8, and was centrifuged at 80 g for 10 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 30,000 g for 20 min at 4°C to pellet the membrane fraction. Protein concentration was determined by using the DC Protein Assay Reagent (Bio-Rad, Milan, Italy). To detect the D1R, 60 µg of protein preparations was resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and blotted for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat powdered milk. Membranes were incubated overnight at 4°C with the anti-D1R822 antibody (1:700 dilution) or the anti-D3R antibody (1:200 dilution). Detection was performed by chemiluminescence (Chemilucent; Chemicon, Milano, Italy) with HRP-conjugated secondary antibodies (1:3000 dilution). In the immunoprecipitation (IP) experiments, 60 µg of striatal protein preparations were incubated overnight at 4°C with either the anti-D1R822 antibody (1:50 dilution) or the anti-D3R antibody (1:50 dilution) in 200 mM NaCl, 10 mM EDTA, 10 mM Na2HPO4, 0.5% Nonidet P-40, and 0.1% SDS (buffer A). Protein-A-agarose beads were added, and incubation was continued for 2 h at room temperature. The beads were collected and extensively washed with buffer A. The resulting proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and blotted for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat powdered milk. Membranes were incubated overnight at 4°C with the anti-D3R antibody (1:200 dilution) or the anti-D1R822 antibody (1:700 dilution). Detection was performed by chemiluminescence with HRP-conjugated secondary antibodies (1:3000 dilution). In another series of experiments, HEK 293 cells were transfected with HA-tagged D1R and GFP-tagged D3R. Total cell proteins were immunoprecipitated with either the anti-HA (1:200 dilution) or the anti-GFP (1:200 dilution) antibody, and the resulting proteins were immunoreacted with either the anti-GFP (1:500 dilution) or the anti-HA (1:500 dilution) antibody, respectively, and detected as described above.

Generation of Bioluminescence Resonance Energy Transfer Fusion Constructs. The preparation of the D1R-luciferase construct (D1R-Rluc) was described previously (Fiorentini et al., 2003). The coding sequence of human D3R was amplified out of its original vector using primers containing unique HindIII and BglII sites and

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the native Pfu DNA polymerase (Stratagene, Milano, Italy) to generate a stop codon-free fragment. This fragment was cloned in-frame into the pGFP-N2(h) vector containing the green fluorescent protein (GFP) (PerkinElmer) to generate the plasmid D3R-GFP. The coding sequence of ChemR23 receptor was amplified out of its original vector using primers containing unique BamHI and EcoRI sites and inserted into the pGFP-N2(h) vector to generate the plasmid ChemR23-GFP.

**Cell Culture, Transfection, and Bioluminescence Resonance Energy Transfer Assay.** HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Semiconfluent cells were cotransfected with D1R-Rluc (0.2 μg) and increasing concentrations of either D3R-GFP (0.2–2 μg) or ChemR23-GFP (0.2–2 μg) using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The total amount of DNA was kept at 2.2 μg. In competition experiments, cells were transfected with D1R-Rluc (0.1 μg) and D3R-GFP (0.5 μg) in the absence or presence of different amounts of either untagged pcDNA-D1R (0.1–1.5 μg) or untagged pcDNA-D3R (0.1–1.5 μg) or pcDNA-D2R (0.1–1.5 μg) or pcDNA-ChemR23 (0.1–1.5 μg). Twenty-four hours after transfection, cells were harvested, centrifuged, and resuspended in PBS containing 0.1 mg/ml CaCl2, 0.1 mg/ml MgCl2, and 1 mg/ml D-glucose. Approximately 15,000 cells/well were distributed in a 96-well microplate (white Optiplate; PerkinElmer). DeepBlueC coelenterazine (PerkinElmer) was added at the final concentration of 5 μM, and bioluminescence resonance energy transfer (BRET) signals were determined using a Fusion universal microplate analyzer (PerkinElmer), which allows sequential integration of signals detected at 390/400 and 505/510 nm. To define the D1R-Rluc/D3R-GFP expression ratio in each sample, HEK 293 cells transfected with increasing amounts of either D1R-Rluc or D3R-GFP or ChemR23-GFP were evaluated for total luminescence or total fluorescence and for D1R-Rluc or D3R-GFP or ChemR23-GFP protein level expression. D1R-Rluc and D3R-GFP levels were determined by radioreceptor binding with [3H]SCH 23390 and [3H]raclopride, respectively. ChemR23-GFP levels were determined by flow cytometry. In brief, cells were labeled using an anti-ChemR23 monoclonal antibody (IgG3; R&D System Inc., Minneapolis, MN) or an isotype control (mouse IgG3; Biologend, San Diego, CA) followed by a goat anti-mouse-PE secondary antibody (Invitrogen). Samples were acquired on a Fas II (Partec GmbH, Münster, Germany) and analyzed using FlowJo version 7.2 (Tree Star, Ashland, OR). Luminescence was plotted against D1R-Rluc expression levels, and fluorescence was plotted against D3R-GFP or ChemR23-GFP expression levels. Because the relationship between measured luminescence or fluorescence and the corresponding receptor was linear, the acceptor/donor ratio was expressed as the fluorescence/luminescence ratio. To test the effects of agonists, cells cotransfected with D1R-Rluc and D3R-GFP at the 1:5 ratio were distributed in a 96-well microplate and incubated in the absence or in the presence of 1 μM SKF 81297, 1 μM quinpirole, or 1 μM sulpiride for 5 to 60 min at 37°C with 1) the D1R agonist SKF 81297 (10 nM to 10 μM); 2) the D3R agonist quinpirole (0.5 nM to 1 μM); 3) a combination of 1 μM SKF 81297 and 1 μM quinpirole; 4) DA (100 nM to 10 μM); and 5) 1 μM DA in the presence of either the D1R antagonist SCH 23390 (1 μM) or the D3R antagonist (−)sulpiride (1 μM). To study receptor recycling to the plasma membrane, cells were exposed to a combination of 1 μM SKF 81297 and 1 μM quinpirole for 60 min at 37°C to promote sequestration. Agonists were removed by extensive washes with ice-cold PBS, and cells were incubated in the standard medium at 37°C for 5 to 60 min. Receptor sequestration and recycling to the plasma membrane were evaluated by both immunofluorescence and radioreceptor binding.

**Immunofluorescence.** Cells expressing D1R and D3R-GFP were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS containing 5% bovine serum albumin and 5% normal goat serum. Cells were incubated with the monoclonal rat anti-D1R antibody (Sigma; 1:800 dilution in PBS containing 1% normal goat serum) overnight at 4°C and then with the Cy3-conjugated secondary antibody (1:1000 dilution) for 45 min at room temperature. The immunolabeled cells were recorded with a fluorescence microscope (IX51; Olympus, Tokyo, Japan) at a 100× magnification. Nontransfected cells and omission of the primary antibody were used as negative controls.

[3H]Sulpiride Binding in Intact Cells. Sequestration of D3R was measured according to Kim et al. (2001) exploiting the hydrophilic properties of [3H]sulpiride. HEK-D3R and HEK-D1R/D3R cells were plated at the density of 2 × 10^5 cells/well in 24-well plates, allowed to recover for 24 h, and stimulated with agonists as described previously. Incubation was blocked by cooling plates on ice and extensively washing cells with ice-cold serum-free medium containing 20 mM HEPES, pH 7.4. Intact cells were incubated at 4°C for 150 min with [3H]sulpiride at the final concentration of 2.2 nM. The nonspecific binding was defined with either 10 μM (−)sulpiride or 10 μM haloperidol. The incubation was stopped by three washes with the same medium, and 1% Triton X-100 was added. The amount of radioactivity in each sample was determined on a liquid scintillation analyzer.

**Membrane Preparation and Radioreceptor Binding.** Transfected HEK 293 cells were rinsed, harvested, and centrifuged at 100 g for 10 min. Cells were homogenized with an Ultra Turrex homogenizer in 5 mM Tris-HCl containing 2 mM EDTA and a mixture of protease inhibitors, pH 7.8, and centrifuged at 80 g for 10 min. The supernatant was centrifuged at 30,000 g for 20 min at 4°C, and the resulting pellet, containing total cell membranes, was resuspended in 50 mM Tris-HCl containing 5 mM MgCl2, 1 mM EGTA, and the protease inhibitors, pH 7.8, layered on a 35% sucrose cushion and centrifuged at 150,000 g for 90 min to separate the light vesicular and heavy membrane fractions as described previously (Fiorentini et al., 2003). The heavy fraction, at the bottom of the sucrose cushion, was
resuspended in 50 mM Tris-HCl containing 5 mM EDTA, 1.5 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl, and 120 mM NaCl, pH 7.4, and used for binding assay. Protein concentration was determined by using the DC Protein Assay Reagent (Bio-Rad). Aliquots of membrane suspension (50 µg of protein/sample) were incubated at room temperature for 90 min with a saturating concentration (4 nM) of [³H]SCH23390. The nonspecific binding was defined with 1 µM d-butyralamol. To define the Kᵦ and Bₑₘₐₓ of D1R and D3R and D1R-RLuc and D3R-GRFP² in HEK 293 cells, aliquots of total cell membranes (50 µg protein/sample) were incubated with increasing concentrations of [³H]SCH23390 (0.05–25 nM) or increasing concentrations of [³H]raclopride (0.5–7.5 nM) for 30 min at 37°C. The nonspecific binding was defined with 1 µM d-butyralamol in the case of [³H]SCH23390 and with 1 µM (-)-sulpiride in the case of [³H]raclopride. The reactions were stopped by rapid filtration under reduced pressure through Whatman GF/C filters (Whatman, Clifton, NJ).

Measurement of Adenylyl Cyclase Activity. Cells were homogenized in ice-cold 10 mM Tris-maleate, pH 7.4, containing 1.2 mM EGTA. Adenylyl cyclase (AC) activity was assayed in a 500-µl reaction mixture containing 80 mM Tris-maleate, 16 mM MgSO₄, 0.5 mM 3-3-isobutyl-1-methylxanthine, 0.6 mM EGTA, 0.02% ascorbic acid, pH 7.4, 2 mM ATP, 5 mM phosphocreatine, 50 U/ml creatine kinase, and various concentrations of DA (10 nM to 10 µM) in the absence and presence of selective D1R (1 µM SCH23390) or D3R (1 µM (-)-sulpiride) antagonists. The reaction was started by adding the cell homogenate (approximately 1.5 µg protein/sample), and incubation was carried out at 30°C for 20 min and stopped by placing samples in boiling water for 5 min. The cAMP in the supernatant was measured by radioimmunoassay using the reagents supplied by PerkinElmer.

Results

Development and Characterization of the Anti-D1R Polyclonal Antibody. As shown in Fig. 1A (lanes 1 and 2), the affinity-purified anti-D1R822 antibody (1.5 µg/ml) detected a single band of ~70 kDa in Western blot (WB) experiments with membrane preparations from rat striatum. When the anti-D1R822 antibody was preabsorbed with an excess of its specific immunizing peptide (80 µg/ml), the signal corresponding to the ~70-kDa species was lost (Fig. 1A, lanes 3 and 4), suggesting that the immunoreactive band is specific. It has been reported that the mature D1R in the striatum is a glycosylated protein with a molecular size of ~72 kDa and that deglycosylation results in the appearance of low molecular mass forms of ~60 and ~48 kDa (Amlaiky et al., 1987; Jarvie et al., 1989). The ~70-kDa band recognized by our antibody thus probably represents the fully glycosylated form of the D1R. The anti-D1R822 antibody was also tested by WB in different rat brain areas, characterized by specific D1R expression. The immunoblot reported in Fig. 1B shows that a major ~70-kDa band was present in membranes from the striatum (lane 1), hippocampus (lane 2), cerebellum (lane 3), and prefrontal cortex (lane 4). The intensity of this signal was stronger in the striatum, hippocampus, and prefrontal cortex, which express high levels of D1R, than in the cerebellum, where the D1R is poorly expressed (Missale et al., 1998). Moreover, no specific immunoreactivity was detected by the anti-D1R822 antibody in the anterior pituitary (lane 5), suggesting that this antibody does not cross-react with the D2R, which is highly concentrated in this region (Missale et al., 1998). To further evaluate the specificity of this antibody, striatal proteins were immunoprecipitated with a commercial anti-D1R antibody (anti-D1R H-109; Santa Cruz Biotechnology), and the resulting material was subjected to SDS-PAGE and blotted with the anti-D1R822 antibody. As shown in Fig. 1C, the ~70-kDa band, corresponding to the D1R, was detected by the anti-D1R822 antibody in both striatal proteins (lane 1) and in striatal proteins immunoprecipitated by the anti-D1R H-109 antibody (lane 3). This band was undetectable when the precipitating antibody was omitted (lane 2). Moreover, as reported in Fig. 1D, the anti-D1R822 antibody (6 µg/ml) immunoprecipitated a ~70-kDa species from striatal membranes that was recognized by the anti-D1R H-109 antibody (lane 2), further confirming the specificity of our antibody. Taken together, these data demonstrate the selectivity of the anti-D1R822 antibody for the D1R and suggest that it represents a useful tool in both IP and WB assays. This antibody was thus used in subsequent experiments.

**Fig. 1.** Characterization of the anti-D1R822 antibody. A, WB analysis in striatal membranes. Increasing amounts of striatal proteins were immunoblotted with either the anti-D1R822 antibody (lanes 1 and 2) or the anti-D1R822 antibody preabsorbed with an excess of its specific immunizing peptide (lanes 3 and 4). B, WB analysis in different rat brain areas. C and D, characterization of the anti-D1R822 antibody by IP. C, striatal proteins were immunoprecipitated with a commercial anti-D1R antibody (H-109; Santa Cruz Biotechnology), and the resulting proteins were immunoblotted with the anti-D1R822 antibody. A single specific band of approximately 70 kDa was detected in the immunoprecipitated material. D, striatal proteins were immunoprecipitated with the anti-D1R822 antibody, and the resulting proteins were immunoblotted with the commercial anti-D1R antibody. Each experiment was repeated four times. Representative blots are shown.
tor-specific antibodies, HEK 293 cells were cotransfected with HA-tagged D1R and GFP-tagged D3R, and proteins were immunoprecipitated with anti-HA or anti-GFP antibodies and revealed with anti-GFP or anti-HA antibodies, respectively. As reported in Fig. 2C, a ~70-kDa species, corresponding to HA-tagged D1R, was detectable in proteins immunoprecipitated with the anti-GFP antibody and revealed with the anti-HA antibody and two major bands between ~80 and ~100 kDa, corresponding to GFP-tagged D3R, were detected in proteins immunoprecipitated with the anti-HA antibody and revealed with the anti-GFP antibody.

**D1R and D3R Constitutively Interact in Living Cells.** Although communoprecipitation is a generally accepted method to document protein-protein interactions, in the case of membrane receptors, the interpretation of these experiments may be complicated by detergent solubilization that could promote artifactual aggregation. To assess whether D1R/D3R heterodimers could be detected in living cells we used the BRET method, which detects energy transfer from a luminescent donor to a fluorescent acceptor when they are folded polypeptides capable of binding selective dopaminergic ligands, we assessed their binding properties by saturation binding assays with [3H]SCH23390 (D1R) or [3H]raclopride (D3R). The obtained Kd values were as follows: Kd = 1.2 ± 0.1 and 1.0 ± 0.06 nM for D1R and D1R-Rluc, respectively, and Kd = 2.02 ± 0.1 and 1.5 ± 0.08 nM for D3R and D3R-GFP2, respectively. BRET2 signals were determined in HEK 293 cells simultaneously or individually expressing the D1R-Rluc and D3R-GFP2 constructs as described previously (Fiorentini et al., 2003). Cells expressing a fusion construct covalently linking Rluc to GFP2 (pRluc-GFP2) were used as a positive control. As shown in Fig. 3A, D1R-Rluc expressed in HEK 293 cells generated a small, nonspecific BRET2 signal; likewise, no BRET2 was observed in cells expressing the D3R-GFP2 construct. A significant BRET2 signal was observed in cells expressing the pRluc-GFP2 construct (Fig. 3A), confirming the importance of molecular proximity between the receptors.

**Fig. 3.** Detection of D1R and D3R interaction by BRET2 in transfected HEK 293 cells. The D1R fused to *R. reniformis* luciferase (D1R-Rluc) and the D3R fused to GFP2 (D3R-GFP2) were transfected either individually or simultaneously in HEK 293 cells. The DeepBlueC coelenterazine substrate was added at a final concentration of 5 μM. A, quantification of BRET2 data from a series of control experiments with single receptor constructs (D1R-Rluc, D3R-GFP2, and pRluc-GFP2) or with D1R-Rluc and D3R-GFP2 coexpressed in the same cells (D1R-Rluc/D3R-GFP2) or with cells individually expressing D1R-Rluc and D3R-GFP2 mixed together before the BRET2 experiment (D1R-Rluc + D3R-GFP2). Bars are the means ± S.E. of five experiments. ●, p < 0.001 versus D1R-Rluc, Student's t test. B, BRET2 titration analysis. Cells were transfected with D1R-Rluc in the presence of increasing concentrations of either D3R-GFP2 or ChemR23-GFP2. BRET2, total luminescence, and total fluorescence were determined. BRET2 ratio values are plotted as a function of the total fluorescence/total luminescence ratio. Data are representative of three experiments. The curves were fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism 4). ●, specific BRET2 signal generated in the presence of D3R-GFP2; ○, nonspecific signals generated in the presence of ChemR23-GFP2. C, BRET2 competition analysis. Experiments were carried out at the constant D1R-Rluc/D3R-GFP2 ratio of 3.4 ± 0.5 in the absence or presence of increasing concentrations of either untagged D1R (●), D3R (○), or D2R (■). The expression of untagged receptors was determined by radioreceptor binding. D, cells transfected with D1R-Rluc and D3R-GFP2 were exposed to either 1 μM SKF 81297 or 1 μM quinpirole or 10 μM dopamine in the absence or in the presence of dynamin-IK44A. Bars are the means ± S.E. of five independent experiments. ●, p < 0.005 versus untreated cells, Student's t test.
BRET² partners for signal detection. Coexpression of D1R-RLuc and D3R-GFP² yielded a significantly high BRET² signal that could be best explained with the formation of a D1R/D3R complex. Energy transfer was in fact undetectable when cells individually expressing D1R-RLuc and D3R-GFP² were mixed together before the BRET² analysis. The specificity of the BRET² signal was further confirmed in titration and competition experiments. As shown in Fig. 3B, increasing the amount of the D3R-GFP² acceptor in the presence of a constant concentration of the D1R-RLuc donor resulted in a hyperbolic increase of the BRET² signal as a function of increasing D3R-GFP²/D1R-RLuc ratio, indicating specificity of the interaction. The expression level of D1R-RLuc was determined by measuring total luminescence, and the expression levels of D3R-GFP² were monitored by measuring total fluorescence. BRET² signals were plotted as a function of the fluorescence/luminescence ratio. The chemokine ChemR23 receptor (Wittamer et al., 2003) was used as a negative control. As reported in Fig. 3B, increasing the concentration of ChemR23-GFP² in cells expressing the D1R-RLuc resulted in a nonspecific linear increase of the BRET² signal. Figure 3C shows the results of BRET² competition experiments carried out at the constant D1R-RLuc/D3R-GFP² ratio of 3.4 ± 0.5 in the presence of increasing concentrations of either untagged D1R, or untagged D3R or untagged D2R, or untagged ChemR23. BRET² signals were plotted as a function of receptor expression. Both D1R and D3R competed with their tagged counterparts in a concentration-dependent way, as shown by the decrease of the BRET² signal as a function of the concentration of the untagged competitor. Moreover the D2R, that has been shown to interact with both D1R (Rashid et al., 2007) and D3R (Scarselli et al., 2001) decreased the BRET² signal generated by D1R-RLuc and D3R-GFP² in a concentration-dependent manner. By contrast, the ChemR23 receptor does not interfere with the BRET² signal generation (Fig. 3C, insert). Taken together, these data suggest that D1R-Rluc and D3R-GFP² are true interacting partners. As shown in Fig. 3D, the BRET² signal recorded in cells cotransfected with D1R-RLuc and D3R-GFP² was insensitive to stimulation by the D1R agonist SKF 81297 (1 μM) and the D3R agonist quinpirole (1 μM). However, DA induced a slight but significant increase of BRET² signal. To evaluate whether this effect of DA might reflect the modulation of D1R/D3R interaction at the plasma membrane or internalization of the D1R/D3R complex, cells were cotransfected with the dynamin-IK44A dominant-negative mutant, which blocks agonist-induced GPCR internalization (Zhang et al., 1997). As shown in Fig. 3D, DA-mediated increase of BRET² signal but not the basal BRET² signal in cells coexpressing D1R-RLuc and D3R-GFP² was inhibited by dynamin-IK44A, suggesting that it could probably reflect D1R/D3R complex internalization. Taken together, these data demonstrate a physical proximity between D1R-RLuc and D3R-GFP² that is consistent with the formation of a constitutive heterodimeric complex and strongly support the results obtained by communoprecipitation in the striatum.

**D1R/D3R Heterodimerization Increases the Potency of DA in Stimulating Adenyl Cyclase through the D1R.** We investigated whether D1R/D3R heterodimerization affects D1R coupling to the AC signaling pathway. It is known in fact that D1R stimulates AC, whereas the D3R inhibits this effector in different cell systems (Missale et al., 1998). For this purpose, HEK 293 cell clones stably expressing the D1R, the D3R, and both D1R and D3R were generated. D1R and D3R expression levels in the different cell clones were determined by [³H]SCH23390 and [³H]sulpiride binding. D1R expression in the different clones was as follows: HEK-D1R, 525 ± 56 fmol/mg protein; HEK-D1R/D3R, 512 ± 46 fmol/mg protein. D3R expression was as follows: HEK-D3R, 610 ± 58 fmol/mg protein; HEK-D1R/D3R, 714 ± 67 fmol/mg protein. The HEK-D1R and HEK-D1R/D3R clones thus had the same level of D1R expression. Moreover, in HEK-D1R/D3R cells, the expression of D3R was in slight excess, thus ensuring that a relevant amount of D1R present was associated with D3R. The effects of DA on cAMP formation are reported in Fig. 4A. DA dose-dependently stimulated AC activity with an EC₅₀ value of 560 ± 52 nM and a maximal stimulation of 75 ± 5% at the dose of 50 μM in HEK-D1R cells. On the other hand, according to previous studies showing that the D3R only inhibits type V AC, which is poorly expressed in HEK 293 cells (Robinson and Caron, 1997), DA weakly inhibited cAMP formation in HEK-D3R cells (maximal inhibition, 20 ± 2%). In HEK-D1R/D3R cells, however, DA stimulated AC activity with higher potency.

![Fig. 4. Effects of DA on cAMP formation in cells expressing the D1R/D3R complex.](image-url)
than in HEK-D1R cells. The calculated EC$_{50}$ value for DA-stimulated cAMP formation in cells expressing the D1R/D3R complex was 39 ± 1.5 nM. The maximal extent of DA stimulation of AC in cells expressing the D1R/D3R complex was similar to that found in cells expressing only the D1R (85 ± 7% increase over basal) but was detectable at a 10-fold lower concentration (5 μM). To confirm the requirement of the concurrent activation of both D1R and D3R to potentiate the stimulatory effect of DA on cAMP formation, we used the D1R antagonist SCH23390 and D3R antagonist (-)-sulpiride. As reported in Fig. 4B, the increased potency of DA in stimulating AC in cells expressing the D1R/D3R complex was counteracted by both antagonists. In particular, inhibition of DA interaction with the D3R by (-)-sulpiride (1 μM) shifted the dose-response curve of DA to the right. The EC$_{50}$ value for DA-stimulated AC was in fact shifted from 39 ± 1.5 to 520 ± 47 nM, a value consistent with the potency of DA at the D1R expressed alone. The D1R antagonist SCH23390 (1 μM) completely abolished cAMP formation induced by DA in cells coexpressing the D1R and the D3R, suggesting that AC activation was completely sustained by the D1R. In these conditions, a weak D3R-dependent inhibition of AC activity was detected. The increased potency of DA in stimulating AC in HEK-D1R/D3R cells was correlated with the increased affinity of DA for the D1R, as determined by radioreceptor binding with $[^3]$H]SCH23390. In particular, the curves for DA displacement of $[^3]$H]SCH23390 in cells expressing D1R alone or in combination with the D3R, resolved by a two-site analysis (GraphPad Prism 4; GraphPad Software Inc., San Diego, CA), showed a high-affinity and a low-affinity site, probably corresponding to G protein-coupled and -uncoupled receptors, respectively. Interaction with the D3R increases the affinity of DA for the high-affinity site. The calculated $K_i$ values (mean ± S.E. of three independent experiments) for DA displacement of $[^3]$H]SCH23390 binding were 60 ± 4 nM (high-affinity site) and 4.4 ± 0.5 μM (low-affinity site) in HEK-D1R cells and 1.3 ± 0.3 nM (high-affinity site) and 4.0 ± 0.6 μM (low-affinity site) in HEK-D1/D3.

**Heterodimerization Influences Agonist-Mediated D1R and D3R Sequestration.** Because GPCR heterodimerization may affect the trafficking of interacting receptors (Angers et al., 2002), we investigated whether heteromeric assembly modifies agonist-induced cytoplasmic sequestration of D1R and D3R. This issue is particularly relevant because D1R and D3R are characterized by different adaptive properties (Oakley et al., 2000; Kim et al., 2001, 2005). For this purpose, we used immunofluorescence microscopy and receptor binding in transfected HEK 293 cells, which endogenously express adequate amounts of both GRKs and β-arrestin to allow DA receptor sequestration. As shown in Fig. 5A, in unstimulated HEK 293 cells expressing the D1R, the fluorescence distribution of D1R was confined to the plasma membrane (a). Exposure to 1 μM SKF 81297 for 1 h resulted in D1R sequestration into cytosolic compartments, as shown by the D1R immunofluorescence that was detectable also in the cytoplasm with a punctate appearance (b). The extent of D1R internalization was determined by $[^3]$H]SCH23390 binding in the purified heavy membrane fraction from HEK-D1R cells. As shown in Fig. 5B, a 1-h treatment with 1 μM SKF 81297 promoted a 30 ± 4% decrease of cell surface D1R. This effect was dose-dependent over the range of 10 nM to 10 μM with a maximum at 1 μM and was detectable within 10 min of treatment, reaching the maximum after a 30-min exposure. DA (1 μM) also induced a significant decrease of D1R at the cell surface (22 ± 3%). The effect of quinpirole (0.5 nM to 1 μM) or DA (1 μM) on D3R trafficking was studied in HEK 293 cells transiently transfected with D3R-GFP. Figure 5A shows the effect of a 1-h treatment with 1 μM quinpirole on D3R cellular localization. In line with previous data (Kim et al., 2001), this treatment did not significantly modify the membrane localization of D3R-GFP (c and d). Similar results were obtained by measuring cell surface D3R in binding studies with $[^3]$H]sulpiride in the HEK-D3R clone (Fig. 5B), suggesting that the D3R does not internalize in response to agonist stimulation in this heterologous system. The adaptive responses of the D1R/D3R complex to agonist stimulation were studied by immunofluorescence in the HEK-D1R cells transiently transfected with D3R-GFP. As shown in Fig. 6, D1R and D3R expressed in HEK 293 cells were mostly targeted to the plasma membrane (a and b) where they were colocalized (c). A 1-h stimulation with 1 μM quinpirole did not modify the membrane localization of the D1R/D3R complex, as shown by the fluorescence of both D1R (d) and D3R (e) that remained colocalized at the plasma membrane (f). Likewise, exposure of cotransfected cells to 1 μM SKF 81297, which induced internalization of D1R in individually transfected cells (see Fig. 4), did not modify the membrane retention of the D1R/D3R complex (g–i), indicating that association with the D3R may affect D1R function by impairing its desensitization. It is interesting that the paired stimulation of the two receptor components of the D1R/D3R heterodimer by a combination of SKF 81297 (1 μM) and quinpirole (1 μM) relieved the membrane retention of the D1R/D3R complex, enabling its internalization. In these conditions, D1R and D3R fluorescence was colocalized at cytoplasmic sites with a punctate appearance (l–n). Similar results were obtained with DA. As shown in o to q, 1-h stimulation with 1 μM DA induced cytoplasmic sequestration of both D1R and D3R. This effect, which was dose-dependent over the range of 100 nM to 10 μM, was...
detectable after a 5-min incubation and reached a maximum at 30 min (data not shown). These observations were confirmed by radioreceptor binding with [3H]SCH23390 and [3H]sulpiride in the HEK-D1R/D3R cell clone. As shown in Fig. 7A, treatment with either SKF 81297 (1 μM) or quinpirole (1 μM) did not affect the abundance of cell surface [3H]sulpiride and [3H]SCH23390 binding sites (Fig. 7B). These effects were prevented by either D1R- or D3R-selective antagonists. The D1R antagonist SCH 23390 (1 μM) abolished the loss of both cell surface [3H]sulpiride and [3H]SCH23390 binding sites in HEK-D1R/D3R cells exposed to 1 μM DA (Fig. 7B). Likewise, the D3R antagonist (-)sulpiride (1 μM) prevented DA-induced decrease of membrane [3H]sulpiride and [3H]SCH23390 binding. The observation that stimulation of both D1R and D3R induces the same extent of D1R and D3R internalization suggests that in our experimental conditions, a significantly high proportion of D1R and D3R is associated into the heterodimeric complex with respect to the corresponding homodimeric complexes.

These results point to the critical importance of the paired stimulation of both receptor components to induce D1R/D3R complex internalization. Moreover, as shown in Fig. 7B, DA-induced cytoplasmic sequestration of both [3H]sulpiride and [3H]SCH23390 binding sites was abolished by β-arrestin-1V53D, a dominant-negative β-arrestin mutant that prevents agonist-induced GPCR sequestration (Zhang et al., 1997). Because internalization may target GPCR to either a degradative pathway, leading to prolonged attenuation of cell signaling, or to a cell surface recycling pathway, facilitating receptor resensitization (Gainetdinov et al., 2004), we evaluated the time course of D1R/D3R recycling to the plasma membrane. Cells were treated with a combination of SKF 81297 (1 μM) and quinpirole (1 μM) for 60 min to promote sequestration of the receptor complex. Agonists were then removed, and the reappearance of D1R and D3R at the cell surface was monitored over time. As shown in Fig. 8A, in unstimulated cells, D1R and D3R-GFP were colocalized at the plasma membrane (a–c). Exposure of transfected cells to SKF 81297 (1 μM) and quinpirole (1 μM) for 60 min induced the cointernalization of D1R and D3R-GFP (d–f). Fifteen minutes after agonist removal, a significant proportion of D1R and D3R was detected back at the plasma membrane, where they were still colocalized (g–i). Figure 8B shows the time course of D1R and D3R recycling in HEK-D1R/D3R cells evaluated in binding studies with [3H]sulpiride and [3H]SCH23390. A significant amount of both [3H]sulpiride and [3H]SCH23390 binding sites returned to the cell surface within 15 min of treatment withdrawal. The density of [3H]sulpiride and [3H]SCH23390 binding sites measured 30 and 60 min after treatment withdrawal was indistinguishable from that detected in untreated cells.

**Discussion**

In this study, we reveal heterodimerization between the DA D1R and D3R in both the striatum and transfected cells. The evidence for the physical interaction of these receptor subtypes is derived from communoprecipitation, BRET$^2$.

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**Fig. 6.** Effects of agonist stimulation on receptor localization in HEK 293 cells coexpressing D1R and D3R-GFP. HEK 293 cells stably expressing the D1R were transfected with D3R-GFP and exposed to either 1 μM SKF 81297 or 1 μM quinpirole or a combination of 1 μM SKF 81297 and 1 μM quinpirole or 1 μM DA for 60 min at 37°C. Cell localization of D1R was evaluated by immunofluorescence with the rat monoclonal anti-D1R antibody and the Cy3-conjugated secondary antibody as described under Materials and Methods. a to c, colocalization of D1R and D3R-GFP at the plasma membrane; d to f, quinpirole administration does not modify the membrane colocalization of D1R and D3R-GFP; g to l, membrane colocalization of D1R and D3R-GFP in SKF 81297-treated cells; 1 to n, intracellular colocalization of D1R and D3R-GFP in cells exposed to a combination of SKF 81297 and quinpirole; o to q, intracellular colocalization of D1R and D3R-GFP in cells exposed to DA. Data are representative of five independent experiments.

**Fig. 7.** Quantitative analysis of agonist-induced D1R/D3R sequestration by [3H]SCH23390 and [3H]sulpiride binding. A, HEK-D1R/D3R cells were exposed to agonists for 60 min at 37°C. [3H]Sulpiride binding was carried out in intact cells and [3H]SCH23390 binding in the heavy membrane fraction. Data are expressed as the percentage loss of cell surface receptors. Bars are the means ± S.E. of five independent experiments; *, p < 0.001 versus either SKF 81297 or quinpirole. B, HEK-D1R/D3R cells were exposed to 1 μM DA for 60 min at 37°C in the absence or presence of either the D1R antagonist SCH23390 (1 μM) or the D3R antagonist (-)sulpiride (1 μM). HEK-D1R/D3R cells were also transfected with β-arrestin-1V53D and exposed to DA. [3H]Sulpiride binding was carried out in intact cells and [3H]SCH23390 binding in the heavy membrane fraction. Data are expressed as the percentage loss of cell surface receptors. Bars are the means ± S.E. of three independent experiments; *, p < 0.001 versus DA, Student’s t test.
and cointernalization experiments. As a result of heterodimerization, these receptors display functional properties that are remarkably different from those of D1R and D3R homo-oligomers. In particular, a unique characteristic of D1R/D3R heterodimerization is that it increases the affinity of DA for the D1R and the potency of DA in stimulating AC through the D1R, abolishes agonist-induced D1R internalization, and enables the cytoplasmic sequestration of the receptor complex in response to the paired stimulation of D1R and D3R.

Using a conventional biochemical approach, we have shown that the D3R was coimmunoprecipitated with the D1R from striatal proteins, suggesting that these receptors may be physically associated in this structure. The observation that D1R and D3R are coexpressed in specific neuronal populations of both limbic (Le Moine and Bloch, 1996; Ridray et al., 1998; Schwartz et al., 1998) and motor areas (Surmeier et al., 1996; Bordet et al., 2000; Guillin et al., 2001) supports this finding and provides the anatomical basis for D1R-D3R direct interactions. By using BRET2 in transfected HEK 293 cells, we further demonstrated that D1R and D3R colocalization reflects the existence of a physical proximity between these receptors that can be explained best by the formation of protein heterodimers. Tagged D1R and D3R generated, in fact, a significant and specific BRET² signal in cotransfected HEK 293 cells that was insensitive to stimulation with either D1R- or D3R-selective agonists. Costimulation of D1R and D3R by DA, however, increased the BRET² signal, an effect that could potentially reflect either the further clustering of nonheteromeric D1R and D3R or the occurrence of conformational changes at preformed D1R/D3R complexes, increasing the molecular proximity of BRET² partners or the clustering of complexes into endocytotic vesicles, also resulting in increased proximity of BRET² partners (Angers et al., 2002). The observation that mutant dynamin I-K44A, which prevents agonist-mediated GPCR internalization (Zhang et al., 1997), antagonized DA-induced increase of BRET² signal points to D1R/D3R complex internalization as the most likely event to explain this finding. However, it cannot be excluded that other mechanisms could contribute to the effect of DA in the BRET² assay. The existence of a functional cross-talk between D1R and D3R, involving the convergence of their signaling pathways, has been reported previously (Ridray et al., 1998; Schwartz et al., 1998). Our present data, showing that D1R and D3R are constitutively assembled into a heterodimeric complex, extend these observations and provide the molecular basis for the reported functional interactions between these receptors.

In transfected cells, the interaction between D1R and D3R finds an important functional implication in the modulation of D1R-mediated stimulation of cAMP formation. D1R and D3R primarily exert opposite effects on AC, being the D1R-stimulatory and the D3R-inhibitory (Missale et al., 1998). In HEK 293 cells, however, the D3R only marginally inhibits cAMP formation, because these cells poorly express AC type V, which is targeted by the D3R (Robinson and Caron, 1997). Nevertheless, coexpression of D1R and D3R potentiated DA stimulation of cAMP formation via the D1R. Whether this effect is detectable also in cells expressing AC type V, which is inhibited by the D3R, remains to be established. The increased potency of DA in stimulating AC in HEK-D1R/D3R cells was correlated with increased affinity of DA for the high-affinity site of D1R. Whether the interaction between D1R and D3R also modifies the affinity of selective compounds for D1R or D3R is still matter of investigation. One function of the D1R/D3R heteromorphic complex may therefore be to allow a stronger stimulatory coupling of the D1R to AC.

In animal models of l-DOPA-induced dyskinesias (LIDs) D1R-related cAMP signaling is enhanced (Aubert et al., 2005) and D3R expression is increased in striatal neurons containing the D1R (Bordet et al., 2000; Guillin et al., 2001; Bézard et al., 2003). Both dysfunctions have been causally linked to the development of LIDs. Our present data may provide a mechanism by which to converge D1R- and D3R-related alterations in the development of LIDs. It is possible, in fact, that D1R/D3R interaction in striatal neurons is increased in dyskinetic animals as a result of the increased expression of the D3R, leading to supersensitivity of D1R-mediated responses.

The interaction between D1R and D3R also influenced both D1R and D3R trafficking from the plasma membrane to intracellular compartments. Internalization, involving both GRK-mediated phosphorylation and arrestin binding, is a common adaptive response of GPCR to agonist stimulation (Gainetdinov et al., 2004). This mechanism not only terminates receptor signaling, but also promotes receptor resensitization and recycling to the plasma membrane. In this study, we demonstrated that D1R/D3R dimerization modifies agonist-mediated internalization of both D1R and D3R, a finding of relevance because D1R and D3R show different adaptive properties. The D1R undergoes agonist-induced cytoplasmic sequestration and rapidly recycles back to the plasma membrane fully resensitized (Oakley et al., 2000; Gainetdinov et al., 2004), whereas D3R desensitization involves GRK-mediated impairment of D3R binding to filamin (Kim et al., 2005) resulting in decreased G protein coupling with only marginal changes of membrane receptor density (Kim et al., 2001, 2005). Our data show that heterodimerization with the D3R abolished agonist-induced D1R cytoplasmic sequestration, suggesting that the adaptive responses of

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**Fig. 8.** Recycling of internalized D1R/D3R receptors at the plasma membrane. HEK 293 cells expressing D1R and D3R-GFP or HEK-D1R/D3R cells were exposed to SKF 81297 (1 μM) and quinpirole (1 μM) for 60 min at 37°C. Agonists were removed, and the reappearance of the D1R/D3R at the cell surface was monitored over time. A, immunofluorescence analysis of D1R/D3R recycling. a to c, colocalization of D1R and D3R-GFP at the cell surface in untreated cells; d to f, cytoplasmic colocalization of D1R and D3R-GFP in agonist-treated cells; g to i, reappearance and colocalization of D1R and D3R-GFP at the cell surface 15 min after agonist removal. Data are representative of three independent experiments. B, time course of D1R/D3R recycling evaluated by [3H]sulpiride binding in intact cells (○) and [3H]SCH23390 binding in the heavy membrane fraction (●). Points are the means ± S.E. of four independent experiments. *p < 0.001 versus time 0, Student’s t test.
D1R may differ from neuron to neuron or in different microdomains of the same neuron, depending on its interaction with other membrane proteins. On the other hand, D1R/D3R dimerization enabled cointernalization of both D1R and D3R in response to the paired stimulation of both receptor components within the heterodimer, suggesting that this interaction could represent a novel mechanism of D1R/D3R reciprocal regulation. Furthermore, our data point to an additional mechanism of D3R desensitization, occurring when this receptor is assembled with the D1R. Internalization of D1R/D3R complex probably occurs via the clathrin-coated vesicle-mediated endocytotic pathway involving β-arrestin binding because it was blocked by mutant β-arrestin-1V53D, which prevents GPCR internalization (Zhang et al., 1997). These data thus suggest that as a result of dimerization, the D3R is switched to the trafficking mechanisms typical of the D1R. In line with our observations, changes in the trafficking of a given receptor due to heterodimerization have been reported previously. In some cases, agonist occupancy of only one protomer within the complex is sufficient to induce internalization of the heterodimer (Angers et al., 2002). In other cases, costimulation of both protomers within the dimer is crucial to promote internalization. In particular, internalization of the D1R/N-methyl-D-aspartate receptor complex (Fiorentini et al., 2003) and recruitment of β-arrestin-1 by M2/M3 muscarinic heterodimer and by adrenergic α2/μ2 muscarinic M3 heterodimeric unit (Novi et al., 2005) have been reported to require the paired activation of the single receptors within the heterodimers. Different mechanisms could explain the finding that oligomerization with D1R enables D3R cytoplasmic sequestration. For example, dimerization with the D1R might enable the recruitment of the endocytic machinery to the D3R itself or might enable the D3R to access the endocytic effectors linked to the D1R. However, it is also possible that the novel D1R/D3R unit has different internalization characteristics compared with that of D1R and D3R. This last possibility is supported by the observation that SKF 81297 did not induce D1R cytoplasmic sequestration in the presence of the D3R. It has been suggested that in DA neurons, the function of D3 autoreceptors might be regulated by DA through modulation of filamin binding and G protein interaction to allow its fast desensitization and resensitization, a mechanism that may be crucial to provide continuous control of synaptic DA concentrations (Kim et al., 2005). On the other hand, our present data suggest that in neurons coexpressing D3R and D1R at the postynaptic level, the D3R might undergo internalization in response to DA as a result of heterodimerization with the D1R, allowing a sustained adaptive cell response to the strength of synaptic transmission. The internalized D1R and D3R rapidly recycle back to the plasma membrane, where they are still colocalized. Whether the intact heteromeric complex recycles back to the cell surface or it is dissociated after internalization and each receptor recycles independently to form again the complex at the plasma membrane cannot be established by our present data.

Both D1R and D3R have been implicated in several disorders, including schizophrenia and motor dysfunctions. In particular, both the symptoms of schizophrenia and the abnormal involuntary movements induced by L-DOPA in patients with Parkinson’s disease have been suggested to reflect imbalances in the relative abundance and function of D1R and D3R (Schwartz et al., 1998; Bordet et al., 2000; Bézard et al., 2003; Aubert et al., 2005). Our present data give a novel insight into how these receptors may function in an integrated way, thus providing a molecular mechanism by which to converge D1R- and D3R-related dysfunctions. The D1R/D3R heterodimer could therefore represent a potential and promising drug target for disorders related to the dopaminergic system.

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References


Address correspondence to: Dr. Cristina Missale, Section of Pharmacology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Viale Europa 11, 25124 Brescia, Italy. E-mail: cmissale@med.unibs.it

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**D1 and D3 Receptor Heterodimerization**


Address correspondence to: Dr. Cristina Missale, Section of Pharmacology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Viale Europa 11, 25124 Brescia, Italy. E-mail: cmissale@med.unibs.it