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RECIPROCAL REGULATION OF DOPAMINE D1 AND D3 RECEPTOR FUNCTION AND TRAFFICKING BY HETERODIMERIZATION

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Abbreviations: GFP, green fluorescent protein; Rluc, *Renilla* Luciferase; DA, dopamine; GPCR, G protein-coupled receptors; GRK, G protein-coupled receptor kinases; NMDAR, N-methyl-D-aspartate receptor; SDS-PAGE, Sodium Dodecyl Sulphate-polyAcrylamide Gel Electrophoresis; BRET, bioluminescence resonance energy transfer; D1R, D1 receptor, D3R, D3 receptor; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; HEK 293, human embryonic kidney 293; DMEM, Dulbecco's modified Eagle's Medium; EDTA, ethylendiaminetetraacetic acid; HRP, horseradish peroxidase; EGTA, ethyleneglycotetraacetic acid; IP, immunoprecipitation; WB, Western blot; AC, adenylyl cyclase; cAMP, cyclic AMP, LID, l-DOPA-induced dyskinesias

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Abstract.

Co-localization 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 co-immunoprecipitates with the D3R from striatal protein preparations suggesting that they are clustered together in this region. Using bioluminescence resonance energy transfer ($BRET^2$), we further suggest the existence of a physical interaction between D1R and D3R. Tagged D1R and D3R co-transfected in HEK293 cells generated a significant $BRET^2$ 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 co-expressing D1R and D3R dopamine stimulated AC with higher potency and displaced [^{3}H]SCH23390 binding with higher affinity than in cells expressing the D1R. In HEK293 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 beta-arrestin binding since it was blocked by mutant beta-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.

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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, belonging to the G protein-coupled receptor (GPCR) family, that are divided into D1-like (D1 and D5) and D2-like (D2, D3, D4) subtypes. Each receptor displays unique properties, including affinity for DA and specificity for G-protein coupling and signalling and shows 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, particularly in the shell of the nucleus accumbens and islands of Calleja (Sokoloff et al., 1990; Levesque et al., 1992) and lower expression in other brain regions (Sokoloff et al., 1990; Levesque et al., 1992; Schwartz et al., 1998). Both D1R and D3R have been implicated in the regulation of rewarding mechanisms and motivated behaviour and in the modulation of emotional and cognitive processes (Schwartz et al., 1998; Xu et al., 1997; Karasinska et al., 2000; Karasinska et al., 2005). Moreover, both alterations of D1R function (Aubert et al., 2005) and overexpression of D3R in the dorsal striatum have been related to the development of motor dysfunctions (Bordet et al., 2000; Guillin et al., 2001; Bezard et al., 2003).

Biochemical and behavioural evidence suggests that D1R and D3R may functionally interact. For example, D1R stimulation induces D3R mRNA expression in rat striatum and medulloblastoma cells (Bordet et al., 2000; Levavi-Sivan et al., 1998) and co-activation 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 behavioural sensitivity to stimulation of D1R and D2R (Xu et al., 1997) and

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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 CREB phosphorylation (Karasinska et al., 2000; Karasinska et al., 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 co-localized 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., 1996) and that L-DOPA administration to hemiparkinsonian rats induces the overexpression of D3R in striatonigral neurons that constitutively express the D1R (Bordet et al., 2000; Guillin et al., 2001). Interaction between D1R and D3R in single neurons might involve either the convergence of their signalling 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, signalling 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 NMDAR (Lee et al., 2002; Fiorentini et al., 2003; Scott et al., 2006) and that the formation of these novel signalling 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 co-transfected cells and

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that this interaction influences D1R coupling to adenylyl cyclase and the adaptive responses of both D1R and D3R to agonist stimulation.

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MATERIALS AND METHODS

Materials. Human embryonic kidney cells (HEK 293) were provided by Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (Braunschweg, Germany). Tissue culture media and foetal bovine serum were purchased from Euroclone Celbio (Milano, Italy). SKF 81297, quinpirole and SCH 23390 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-HA antibody was from Sigma (St. Louis MO, USA) and the anti-GFP antibody was from Invitrogen. The Cy3-labeled secondary antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). [^{3}H]sulpiride (78.2 Ci/mmol) [^{3}H]SCH23390 (86 Ci/mmol) and [^{3}H]raclopride (62.2 Ci/mmol) were from PerkinElmer Life Sciences (Waltham, Massachusetts, USA). Human D3R-GFP, human D3R, human D1R, beta-arrestin-1V53D and dynamin-IK44A were kindly provided by Dr. Marc Caron (Duke University, Durham, NC, USA); the ChemR23 chemokine receptor was kindly provided by Dr. Silvano Sozzani (University of Brescia).

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

Protein preparation, immunoprecipitation (IP) and Western Blot (WB). The rat striatum was homogenized with a glass-glass homogenizer in ice-cold 10mM Tris-HCl containing

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5mM ethylendiamine tetraacetic acid (EDTA), and a complete set of protease inhibitors (Roche, Milano, Italy) (pH 7.4) and centrifuged at 700 x g at 4°C for 10 min. The resulting 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 centrifuged at 80 x g for 10 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 30,000 x 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, Milano, Italy). To detect the D1R, 60 μg of protein preparations were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and blotted for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% non fat 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 Na_2HPO_4, 0.5% NP-40, 0.1% SDS (buffer A). Protein A-agarose beads were added and incubation was continued for 2h 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 TBS containing 0.1% Tween 20 and 5% non fat 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

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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 ($BRET^2$) fusion constructs. The preparation of the D1R-luciferase construct (D1R-Rluc) was previously described (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 the native Pfu DNA polymerase (Stratagene, Milano, Italy) to generate a stop codon-free fragment. This fragment was cloned in-frame into the $pGFP^2$-N2(h) vector containing the green fluorescent protein (GFP^2) (PerkinElmer Life Science) to generate the plasmid D3R-GFP^2. The coding sequence of ChemR23 receptor was amplified out of its original vector using primers containing unique BamHI and EcoRI sites and the native Pfu DNA polymerase (Stratagene, Milano, Italy) to generate a stop codon-free fragment that was cloned into the $pGFP^2$ vector to generate the plasmid ChemR23-GFP^2.

Cell culture, transfection and $BRET^2$ assay. HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% foetal bovine serum, 2 mM glutamine, 0,1 mM nonessential aminoacids, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Semiconfluent cells were co-transfected with D1R-Rluc (0.2 μg) and increasing concentrations of either D3R-GFP^2 (0.2-2 μg) or ChemR23-GFP^2 (0.2-2 μg) using the LipofectAMINE 2000 reagent (Invitrogen, Milano, Italy) 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^2 (0.5 μg) in the absence or in the

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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 post-transfection, cells were harvested, centrifuged and resuspended in PBS containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$, and 1 mg/ml D-glucose. Approximately 15000 cells/well were distributed in a 96-well microplate (white Optiplate, PerkinElmer Life Science). DeepBlueC™ coelenterazine (PerkinElmer Life Science) was added at the final concentration of 5 μM and BRET^2 signals were determined using a Fusion™ universal microplate analyzer (PerkinElmer Life Science), which allows sequential integration of signals detected at 390/400 and 505/510 nm. To define the D1R-Rluc/D3R-GFP^2 expression ratio in each sample HEK 293 cells transfected with increasing amounts of either D1R-Rluc or D3R-GFP^2 or ChemR23-GFP^2 were evaluated for total luminescence or total fluorescence and for D1R-Rluc or D3R-GFP^2 or ChemR23-GFP^2 protein level expression. D1R-Rluc and D3R-GFP^2 levels were determined by radioreceptor binding with [^{3}H]SCH23390 and [^{3}H]raclopide, respectively. ChemR23-GFP^2 levels were determined by flow cytometry. Briefly cells were labelled using an anti-ChemR23 monoclonal antibody (IgG3, R&D System Inc. Minneapolis, Mn, USA) or an isotype control (mouse IgG3, Biolegend, San Diego, CA) followed by a goat anti-mouse-PE secondary antibody (Invitrogen). Samples were acquired on a Pas II (Partec GmbH, Germany) and analyzed using FlowJo version 7.2 (Tree Star, Inc, USA). Luminescence was plotted against D1R-RLuc expression levels and fluorescence was plotted against D3R-GFP^2 or ChemR23-GFP^2 expression levels. Since 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 co-transfected with D1R-Rluc and D3R-GFP^2 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, 1μM quinpirole or 10 μM dopamine for 10 min at 37° C. DeepBlueC™

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coelenterazine (5μM) was added and $BRET^2$ signals were determined as previously described. Untransfected cells and cells individually transfected with D1R-Rluc or D3R-GFP^2 were used to define the non-specific signals; cells transfected with the p-Rluc-GFP^2 control vector (PerkinElmer Life Science) were used as a positive controls. The $BRET^2$ signal was calculated as [(emission at 505/510)–(emission at 390/400) x Cf]/(emission at 390/400), where Cf corresponds to (emission at 505/510)/(emission at 390/400) for the D1R-Rluc expressed alone in the same experiment.

Generation of cell clones stably expressing the D1R, the D3R and both D1R and D3R. HEK293 cells were transfected with the D1R cDNA using the lipofectAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen-Life Technology, Milano Italy). Cell clones stably expressing D1R (HEK-D1R) were isolated by zeocin selection (100 μg/ml). HEK293 cells were transfected with the D3R cDNA and cultured in the presence of G418 (800 μg/ml) to select clones expressing the D3R (HEK-D3R). HEK-D1R cells, cultured in the standard medium containing zeocin (100 μg/ml) were transfected with the D3R cDNA and cell clones stably expressing D1R and D3R (HEK-D1R/D3R) were isolated by zeocin (100 μg/ml) and G418 (800 μg/ml) selection. HEK-D1R cells were maintained in culture in the presence of zeocin (100 μg/ml), HEK-D3R cells were cultured in the presence of G418 (800 μg/ml) and HEK-D1R/D3R cells were cultured in the presence of both zeocin (100 μg/ml) and G418 (800 μg/ml). Cell clones expressing the D1R, the D3R or both D1R and D3R were characterized for receptor levels in binding studies with [^{3}H]SCH23390 and [^{3}H]sulpiride.

Receptor sequestration and recycling. HEK293 and HEK-D1R cells were transiently transfected with D3R-GFP (kindly provided by Dr. Marc Caron, Duke University, USA) in the absence or in the presence of beta-arrestin-1V53D using the LipofectAMINE 2000

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reagent. Cells expressing D1R or D3R-GFP or both D1R and D3R-GFP were incubated for 5-60 min at 37°C with 1) the D1R agonist SKF 81297 (10 nM-10 μM); 2) the D3R agonist quinpirole (0.5 nM -1 μM); 3) a combination of 1 μM SKF 81297 and 1 μM quinpirole; 4) DA (100 nM- 10 μM); 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 5-60 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 (BSA) 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 immunolabelled cells were recorded with a fluorescence microscope (IX51; Olympus, Tokyo, Japan) at a 100x magnification. Non-transfected cells and omission of the primary antibody were used as negative controls.

[^{3}H]sulpiride binding in intact cells. Sequestration of D3R was measured according to Kim et al. (2001) exploiting the hydrophilic properties of [^{3}H]sulpiride. HEK-D3R and HEK-D1R/D3R cells were plated at the density of 2 x 10^5 cells/well in 24-well plates, allowed to recover for 24 hours and stimulated with agonists as previously described. Incubation was

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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 [^{3}H]sulpiride at the final concentration of 2.2 nM. The non specific 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 analyser.

Membrane preparation and radioreceptor binding. Transfected HEK293 cells were rinsed, harvested and centrifuged at 100 x 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 x g for 10 min. The supernatant was centrifuged at 30,000 x 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 $MgCl_2$, 1 mM EGTA and the protease inhibitors (pH 7.8), layered on a 35% sucrose cushion and centrifuged at 150,000 x g for 90 min to separate the light vesicular and heavy membrane fractions as previously described (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 $CaCl_2$, 5 mM $MgCl_2$, 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, Milano, Italy). Aliquots of membrane suspension (50 μg of protein/sample) were incubated at room temperature for 90 min with a saturating concentration (4 nM) of [^{3}H]SCH23390. The non-specific binding was defined with 1 μM d-butaclamol. To define the Kd and Bmax of D1R and D3R and D1R-RLuc and D3R-GFP^2 in HEK293 cells aliquots of total cell membranes (50 μg protein/sample) were incubated with increasing concentrations of [^{3}H]SCH23390 (0.05 nM - 2.5 nM) or increasing concentrations of [^{3}H]raclopride (0.5 nM - 7.5 nM) for 30 min at 37°C.

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The non specific binding was defined with 1μM d-butaclamol in the case of [3H]SCH 23390 and with 1 μM (-)sulpiride in the case of [^{3}H]raclopride. The reactions were stopped by rapid filtration under reduced pressure through Whatman GF/C filters.

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 $MgSO_4$, 0.5 mM 3-isobutyl-methylxantine (IBMX), 0.6 mM EGTA, 0.02% ascorbic acid (pH 7.4), 2 mM ATP, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase and various concentrations of DA (10 nM - 10 μM) in the absence and in the presence of selective D1R (1 μM SCH23390) or D3R (1 μM (-)sulpiride) antagonists. The reaction was started by adding the cell homogenate (about 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 cyclic AMP (cAMP) in the supernatant was measured by radioimmunoassay using the reagents supplied by Perkin Elmer.

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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 WB experiments with membrane preparations from rat striatum. When the anti-D1R822 antibody was pre-absorbed with an excess of its specific immunizing peptide (80μg/ml), the signal corresponding to the ~70 kDa species was lost (Fig. 1A, lanes 3, 4), suggesting that the immunoreactive band is specific. It has been reported that the mature D1R in the striatum is a glycosilated protein with a molecular size of ~72 kDa and that de-glycosilation results in the appearance of low molecular weight forms of ~60 kDa and ~48 kDa (Amlaiky et al., 1987; Jarvie et al., 1989). The ~70 kDa band recognized by our antibody thus likely represents the fully glycosilated 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, that 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, that is highly concentrated in this region (Missale et al., 1998). To further evaluate the specificity of this antibody, striatal proteins were immunoprecipitated by 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).

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This band was undetectable when the precipitating antibody was omitted (lane 2). Moreover, as reported in Fig. 1D, the anti-D1R822 antibody (6μg/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 support 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.

D1R and D3R co-immunoprecipitate from striatal membranes and transfected cells.

Co-IP studies were performed to determine whether D1R and D3R may directly interact in rat striatum. As shown in Fig. 2A, incubation of striatal proteins with the anti-D3R antibody immunoprecipitated a ~70 kDa species that was recognized by the anti-D1R antibody (lane 2) and was absent when the immunoprecipitating antibody was omitted (lane 1). Moreover, according to Nimchinsky et al. (1997), two major bands between ~60 and ~75 kDa, that were detected by the anti-D3R antibody, were present in striatal proteins immunoprecipitated with the anti-D1R antibody (fig. 2B, lane 2). These species were undetectable when the immunoprecipitating antibody was omitted (lane 1). Taken together, these data indicate that a significant proportion of striatal D1R and D3R might physically interact. To investigate whether D1R and D3R are assembled into a complex also in transfected cell systems and to exclude the possibility of artefacts generated by the receptor-specific antibodies, HEK293 cells were co-transfected with haemoagglutinin (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

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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 co-immunoprecipitation 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 asses whether D1R/D3R heterodimers could be detected in living cells we used the bioluminescence resonance energy transfer ($BRET^2$) method, that detects energy transfer from a luminescent donor to a fluorescent acceptor when they are less than 50-80 Å apart. For this purpose the D1R was fused on its C-terminus with the *Renilla* Luciferase (D1R-Rluc) and the D3R with the GFP^2 (D3R-GFP^2). To ensure that the expressed fusion proteins were properly folded polypeptides capable of binding selective dopaminergic ligands, we assessed their binding properties by saturation binding assays with [^{3}H]SCH23390 (D1R) or [^{3}H]raclopride (D3R). The obtained Kd values were: Kd = 1.2 ± 0.1 nM and Kd = 1.0 ± 0.06 nM for D1R and D1R-Rluc, respectively and Kd = 2.02 ± 0.1 nM and Kd = 1.5 ± 0.08 nM for D3R and D3R-GFP^2, respectively. $BRET^2$ signals were determined in HEK293 cells simultaneously or individually expressing the D1R-Rluc and D3R-GFP^2 constructs as previously described (Fiorentini et al., 2003). Cells expressing a fusion construct covalently linking Rluc to GFP^2 (pRluc-GFP^2) were used as a positive control. As shown in Fig. 3A, D1R-Rluc expressed in HEK293 cells generated a small, non-specific $BRET^2$ signal; similarly, no $BRET^2$ was observed in cells expressing the D3R-GFP^2 construct. A significant $BRET^2$ signal was observed in cells expressing the pRluc-GFP^2 construct (Fig. 3A), confirming the importance of molecular proximity between the $BRET^2$ partners for signal detection. Co-expression of D1R-Rluc and D3R-GFP^2 yielded a

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significantly high $BRET^2$ 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-GFP^2 were mixed together before the $BRET^2$ analysis. The specificity of the $BRET^2$ signal was further confirmed in titration and competition experiments. As shown in fig. 3B, increasing the amount of the D3R-GFP^2 acceptor in the presence of a constant concentration of the D1R-Rluc donor resulted in a hyperbolic increase of the $BRET^2$ signal as a function of increasing D3R-GFP^2/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-GFP^2 were monitored by measuring total fluorescence. $BRET^2$ 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-GFP^2 in cells expressing the D1R-RLuc resulted in a non specific linear increase of the $BRET^2$ signal. Fig. 3C shows the results of $BRET^2$ competition experiments carried out at the constant D1R-Rluc/D3R-GFP^2 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. $BRET^2$ 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 $BRET^2$ 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 $BRET^2$ signal generated by D1R-RLuc and D3R-GFP^2 in a concentration dependent manner. By contrast the ChemR23 receptor does not interfere with the $BRET^2$ signal generation (fig. 3C, insert). Taken together these data suggest that D1R-Rluc and D3R-GFP^2 are true interacting partners. As shown in Fig. 3D, the $BRET^2$ signal recorded in cells co-transfected with D1R-Rluc and D3R-GFP^2 was insensitive

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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 $BRET^2$ signal. To evaluate whether this effect of DA might reflect modulation of D1R-D3R interaction at the plasma membrane or internalization of the D1R/D3R complex, cells were co-transfected with the dynamin-IK44A dominant negative mutant, that blocks agonist-induced GPCR internalization (Zhang et al., 1997). As shown in Fig. 3D, DA-mediated increase of $BRET^2$ signal, but not the basal $BRET^2$ signal in cells co-expressing D1R-RLuc and D3R-GFP^2 was inhibited by dynamin-IK44A suggesting that it could likely reflect D1R/D3R complex internalization. Taken together, these data demonstrate a physical proximity between D1R-Rluc and D3R-GFP^2 that is consistent with the formation of a constitutive heterodimeric complex and strongly support the results obtained by co-immunoprecipitation in the striatum.

D1R/D3R heterodimerization increases the potency of DA in stimulating adenylyl cyclase through the D1R.

We investigated whether D1R/D3R heterodimerization affects D1R coupling to the adenylyl cyclase (AC) signalling pathway. It is known in fact that D1R stimulates AC while the D3R inhibits this effector in different cell systems (Missale et al., 1998). For this purpose HEK293 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 [^{3}H]SCH23390 and [^{3}H]sulpiride binding. D1R expression in the different clones was: HEK-D1R, 525 ± 56 fmol/mg protein; HEK-D1R/D3R, 512 ± 46 fmol/mg protein. D3R expression was: 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 the a relevant amount of D1R present were associated with D3R. The effects of DA on

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cyclic AMP (cAMP) formation are reported in fig 4A. DA dose-dependently stimulated AC activity with an EC-50 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, that is poorly expressed in HEK293 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 than in HEK-D1R cells. The calculated EC-50 for DA-stimulated cAMP formation in cells expressing the D1R/D3R complex was in fact 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 nM 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 co-expressing 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 Prism4) showed a

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high affinity and a low affinity site likely 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 Ki (mean ± SE 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.

Since 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 since D1R and D3R are characterized by different adaptive properties (Kim et al., 2001; Oakley et al., 2000; Kim et al., 2005). For this purpose we used immunofluorescence microscopy and receptor binding in transfected HEK293 cells, that endogenously express adequate amounts of both GRKs and beta-arrestin to allow DA receptor sequestration. As shown in Fig. 5A, in unstimulated HEK293 cells expressing the D1R the fluorescence distribution of D1R was confined to the plasma membrane (panel a). Exposure to 1μM SKF 81297 for 1h resulted in D1R sequestration into cytosolic compartments, as shown by the D1R immunofluorescence that was detectable also in the cytoplasm with a punctate appearance (panel 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 1h 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 – 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 (1μM) also induced a significant decrease of D1R at the cell surface (22 ± 3%). The effect of quinpirole (0.5 nM –

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1 μM) or DA (1 μM) on D3R trafficking was studied in HEK293 cells transiently transfected with D3R-GFP. Fig. 5A shows the effect of a 1h 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 (panels 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 HEK293 cells were mostly targeted to the plasma membrane (panels a and b) where they were co-localized (panel c). A 1h stimulation with 1μM quinpirole did not modify the membrane localization of the D1R/D3R complex as shown by the fluorescence of both D1R (panel d) and D3R (panel e) that remained co-localized at the plasma membrane (panel f). Similarly, exposure of co-transfected cells to 1μM SKF 81297, that induced internalization of D1R in individually-transfected cells (see Fig. 4), did not modify the membrane retention of the D1R/D3R complex (panels g-i), indicating that association with the D3R may affect D1R function by impairing its desensitization. Interestingly, 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 co-localized at cytoplasmic sites with a punctate appearance (panels l-n). Similar results were obtained with DA. As shown in panels o-q, 1h stimulation with 1 μM DA induced cytoplasmic sequestration of both D1R and D3R. This effect, that was dose-dependent over the range of 100 nM-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 [^{3}H]SCH23390 and

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[^{3}H]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 [^{3}H]sulpiride and [^{3}H]SCH23390 binding sites, confirming that in these conditions the D1R/D3R complex is retained at the plasma membrane. By contrast, the coincident stimulation of both D1R and D3R with a combination of SKF 81297 and quinpirole resulted in a 28% ± 3% reduction of cell surface [^{3}H]sulpiride binding sites and 30% ± 2% loss of membrane [^{3}H]SCH23390 binding sites. On the same line, DA treatment (1 μM) induced a 30% ± 4% decrease of membrane [^{3}H]sulpiride binding and a 25% ± 3% decrease of membrane [^{3}H]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 [^{3}H]sulpiride and [^{3}H]SCH23390 binding sites in HEK-D1R/D3R cells exposed to 1μM DA (Fig 7B). Similarly the D3R antagonist (-)sulpiride (1 μM) prevented DA-induced decrease of membrane [^{3}H]sulpiride and [^{3}H]SCH23390 binding. The observation that stimulation of both D1R and D3R induces the same extent of D1R and D3R internalization suggest that in our experimental conditions a significantly high proportion of D1R and D3R are associated into the heterodimeric complex 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 [^{3}H]sulpiride and [^{3}H]SCH23390 binding sites was abolished by beta-arrestin-1V53D, a dominant negative beta-arrestin mutant that prevents agonist-induced GPCR sequestration (Zhang et al., 1997). Since internalization may target GPCR to either a degradative pathway, leading to prolonged attenuation of cell signalling, 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

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were treated with a combination of SKF 81297 (1μM) and quinpirole (1μM) 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 co-localized at the plasma membrane (panels a-c). Exposure of transfected cells to SKF 81297 (1 μM) and quinpirole (1 μM) for 60 min induced the co-internalization of D1R and D3R-GFP (panels d-f). Fifteen min after agonist removal a significant proportion of D1R and D3R were detected back at the plasma membrane where they were still co-localized (panels g-i). Fig. 8B shows the time-course of D1R and D3R recycling in HEK-D1R/D3R cells evaluated in binding studies with [^{3}H]sulpiride and [^{3}H]SCH23390. A significant amount of both [^{3}H]sulpiride and [^{3}H]SCH23390 binding sites returned to the cell surface within 15 min of treatment withdrawal. The density of [^{3}H]sulpiride and [^{3}H]SCH23390 binding sites measured 30 and 60 min after treatment withdrawal was indistinguishable to that detected in untreated cells.

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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 co-immunoprecipitation, $BRET^2$ and co-internalization experiments. As a result of heterodimerization these receptors display functional properties that are remarkably different from those of D1R and D3R homooligomers. 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 classical biochemical approach we have shown that the D3R was co-immunoprecipitated with the D1R from striatal proteins, suggesting that these receptors may be physically associated in this structure. The observation that D1R and D3R are co-expressed in specific neuronal populations of both limbic (Schwartz et al., 1998; Ridray et al., 1998; Le Moine and Bloch, 1996) and motor areas (Bordet et al., 2000; Guillin et al., 2001; Surmeier et al., 1996) supports this finding and provides the anatomical basis for D1R-D3R direct interactions. By using $BRET^2$ in transfected HEK293 cells we further demonstrated that D1R and D3R co-clustering 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 $BRET^2$ signal in co-transfected HEK293 cells that was insensitive to stimulation with either D1R- or D3R-selective agonists. Co-stimulation of D1R and D3R by DA, however, increased the $BRET^2$ signal, an effect that could potentially reflect either the further clustering of non-heteromeric D1R and D3R or the occurrence of conformational changes at preformed D1R/D3R complexes, increasing the molecular proximity of $BRET^2$ partners, or the clustering of complexes into endocytotic

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vesicles also resulting in increased proximity of $BRET^2$ partners (Angers et al., 2002). The observation that mutant dynamin I-K44A, that prevents agonist-mediated GPCR internalization (Zhang et al., 1997), antagonized DA-induced increase of $BRET^2$ signal points to D1R/D3R complex internalization as the most like event to explain this finding. However, it cannot be excluded that also other mechanisms could contribute to the effect of DA in the $BRET^2$ assay. The existence of a functional cross-talk between D1R and D3R, involving the convergence of their signalling pathways, has been previously reported (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 HEK293 cells, however, the D3R only marginally inhibits cAMP formation since these cells poorly express AC type V which is targeted by the D3R (Robinson and Caron, 1997). Nevertheless, co-expression 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, that 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 heteromeric complex may therefore be to allow a stronger stimulatory coupling of the D1R to AC. In animal models of l-DOPA-induced dyskinesias (LID) D1R-related cAMP signalling is enhanced (Aubert et al., 2005) and D3R expression is increased in striatal neurons containing the D1R (Guillin et al., 2001; Bordet et

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al., 2000; Bezard et al., 2003). Both dysfunctions have been causally linked to the development of LID. Our present data may provide a mechanism by which to converge D1R and D3R-related alterations in the development of LID. It is possible, in fact, that D1R/D3R interaction in striatal neurons is increased in dyskinetic animals due to 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 signalling, 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 since D1R and D3R show different adaptive properties. The D1R undergoes in fact agonist-induced cytoplasmic sequestration and rapidly recycles back to the plasma membrane fully resensitized (Gainetdinov et al., 2004; Oakley et al., 2000), while 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., 2005; Kim et al., 2001). Our data show that heterodimerization with the D3R abolished agonist-induced D1R cytoplasmic sequestration, suggesting that the adaptive responses of 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 co-internalization 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

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the D1R. Internalization of D1R/D3R complex likely occurs via the clathrin-coated vesicle-mediated endocytotic pathway involving beta-arrestin binding since it was blocked by mutant beta-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 previously reported. 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, co-stimulation of both protomers within the dimer is crucial to promote internalization. In particular, internalization of the D1R/NMDAR complex (Fiorentini et al., 2003) and recruitment of beta-arrestin-1 by M2/M3 muscarinic heterodimer and by adrenergic α2/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 endocytotic machinery to the D3R itself or might enable the D3R to access the endocytotic 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 co-expressing D3R and D1R at the postsynaptic level, the D3R might undergo internalization in response to DA as a result of heterodimerization with the D1R, allowing a

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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 co-localized. 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 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; Bezard et al., 2003; Bordet et al., 2000; 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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FOOTNOTES

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FIGURE LEGENDS

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 pre-absorbed 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 Inc.) and the resulting proteins were immunoblotted with the anti-D1R822 antibody. A single specific band of about 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.

Fig. 2. Co-immunoprecipitation of D1R and D3R in rat striatum and transfected HEK 293 cells. A. Representative co-IP of D1R from striatal proteins by the anti-D3R antibody (lane 2) but not by omission of the precipitating antibody (lane 1). B. Representative co-IP of D3R from striatal proteins by the anti-D1R antibody (lane 2) but not by omission of the precipitating antibody (lane 1). Sixty micrograms of striatal proteins were used in each IP that was repeated four times. C, D. HEK 293 cells were co-transfected with HA-tagged D1R and GFP-tagged D3R and total proteins were either IP with the anti-GFP antibody and revealed with the anti-HA antibody (C) or IP with the anti-HA antibody and revealed with the anti-GFP antibody (D). Data are representative of three experiments.

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Fig. 3. Detection of D1R and D3R interaction by $BRET^2$ in transfected HEK293 cells. The D1R fused to *Renilla* luciferase (D1R-Rluc) and the D3R fused to GFP^2 (D3R-GFP^2) 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 $BRET^2$ data from a series of control experiments with single receptor constructs (D1R-Rluc, D3R-GFP^2, pRluc-GFP^2) or with D1R-Rluc and D3R-GFP^2 co-expressed in the same cells (D1R-Rluc/D3R-GFP^2) or with cells individually expressing D1R-Rluc and D3R-GFP^2 mixed together before the $BRET^2$ experiment (D1R-Rluc + D3R-GFP^2). Bars are the means ± SE of five experiments. * $p< 0.001$ vs. D1R-Rluc, Student's t test. B. $BRET^2$ titration analysis. Cells were transfected with D1R-Rluc in the presence of increasing concentrations of either D3R-GFP^2 or ChemR23-GFP^2. $BRET^2$, total luminescence and total fluorescence were determined. $BRET^2$ 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 non linear regression equation assuming a single binding site (GraphPad Prism4). Filled circles: specific $BRET^2$ signal generated in the presence of D3R-GFP^2; open circles: non specific signals generated in the presence of ChemR23-GFP^2. C. $BRET^2$ competition analysis. Experiments were carried out at the constant D1R-Rluc/D3R-GFP^2 ratio of 3.4 ± 0.5 in the absence or in the presence of increasing concentrations of either untagged D1R (filled circles), D3R (open circles), D2R (filled squares). The expression of untagged receptors was determined by radioreceptor binding. Data are representative of three experiments. Insert: experiments were carried out at the constant D1R-Rluc/D3R-GFP^2 ratio of 3.4 ± 0.5 in the absence or in the presence of increasing concentrations of untagged ChemR23. The expression of ChemR23 in each sample was determined by immunoflorescence and FACS analysis and is expressed as Medial Fluorescence Intensity (MFI). Data are representative of three experiments. D. Cells transfected with D1R-Rluc and D3R-GFP^2 were exposed to either 1μM SKF 81297 or 1μM

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quinpirole or 10 μM dopamine in the absence or in the presence of dynamin-IK44A. Bars are the means ± SE of five independent experiments. * p< 0.005 vs. untreated cells, Student's t test.

Fig. 4. Effects of DA on cAMP formation in cells expressing the D1R/D3R complex. HEK-D1R cells stably expressing the D1R (525 ± 56 fmol/mg protein) and HEK-D1R/D3R expressing both D1R (512 ± 46 fmol/mg protein) and D3R (714 ± 67 fmol/mg protein) were used. A. DA stimulated cAMP formation with an EC-50 value of 560 ± 52 nM in HEK-D1R cells (filled circles) and with an EC-50 of 39 ± 1.5 nM* in HEK-D1R/D3R (open circles); *p < 0.001 vs. EC-50 in HEK-D1R cells, Student's t test. Data are expressed as percent increase of cAMP over basal and represent the mean ± SE of five independent experiments.

B. Effects of D1R and D3R antagonists on DA-induced cAMP formation. HEK-D1R/D3R cell homogenates were treated with DA in the absence (open circles) or in the presence of either the D3R antagonist (-)sulpiride (open squares) or the D1R antagonist SCH23390 (filled squares). The calculated EC-50 values for DA-stimulated cAMP formation were 39 ± 1.5 nM and 520 ± 47 nM* in the absence or in the presence of (-)sulpiride, respectively(* p < 0.001, Student's t test vs. DA). In the presence of SCH23390 DA lost the capability of stimulating AC and slightly inhibited cAMP formation. Data are expressed as percent increase of cAMP over basal and represent the mean ± SE of three independent experiments.

Fig. 5. Effects of agonist stimulation on D1R and D3R localization in transfected HEK 293 cells. HEK 293 cells were individually transfected with D1R or D3R-GFP cDNAs and left untreated or exposed to either 1μM SKF 81297 or 1μM quinpirole or 1 μM dopamine for 60 min at 37°C. A. Immunofluorescence analysis of D1R localization in untreated cells (panel a) and in SKF 81297-treated cells (panel b) and of D3R localization in untreated cells (panel c)

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and quinpirole-treated cells (panel d). B. D1R and D3R receptor sequestration measured by radioreceptor binding in HEK293 cell clones stably expressing either the D1R (525 ± 56 fmol/mg protein) or the D3R (610 ± 58 fmol/mg protein). D1R internalization was evaluated by [^{3}H]SCH23390 binding in the heavy membrane fraction and D3R internalization was evaluated by measuring cell surface [^{3}H]sulpiride binding in intact cells. Bars are the means ± SE of three independent experiments * $p < 0.001$ vs. untreated cells, Student's t test.

Fig. 6. Effects of agonist stimulation on receptor localization in HEK 293 cells co-expressing D1R and D3R-GFP. HEK293 cells stably expressing the D1R were transfected with D3R-GFP and exposed to either 1μM 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 in Materials and Methods. Panels a-c, co-localization of D1R and D3R-GFP at the plasma membrane; panels d-f, quinpirole administration does not modify the membrane co-localization of D1R and D3R-GFP; panels g-i, membrane co-localization of D1R and D3R-GFP in SKF 81297-treated cells; panels l-n, intracellular co-localization of D1R and D3R-GFP in cells exposed to a combination of SKF 81297 and quinpirole; panels o-q, intracellular co-localization 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 [^{3}H]SCH23390 and [^{3}H]sulpiride binding. A. HEK-D1R/D3R cells were exposed to agonists for 60 min at 37°C. [^{3}H]sulpiride binding was carried out in intact cells and [^{3}H]SCH23390 binding in the heavy membrane fraction. Data are expressed as percent loss of cell surface receptors. Bars are the means ± SE of five independent experiments * $p < 0.001$ vs. either SKF 81297 or

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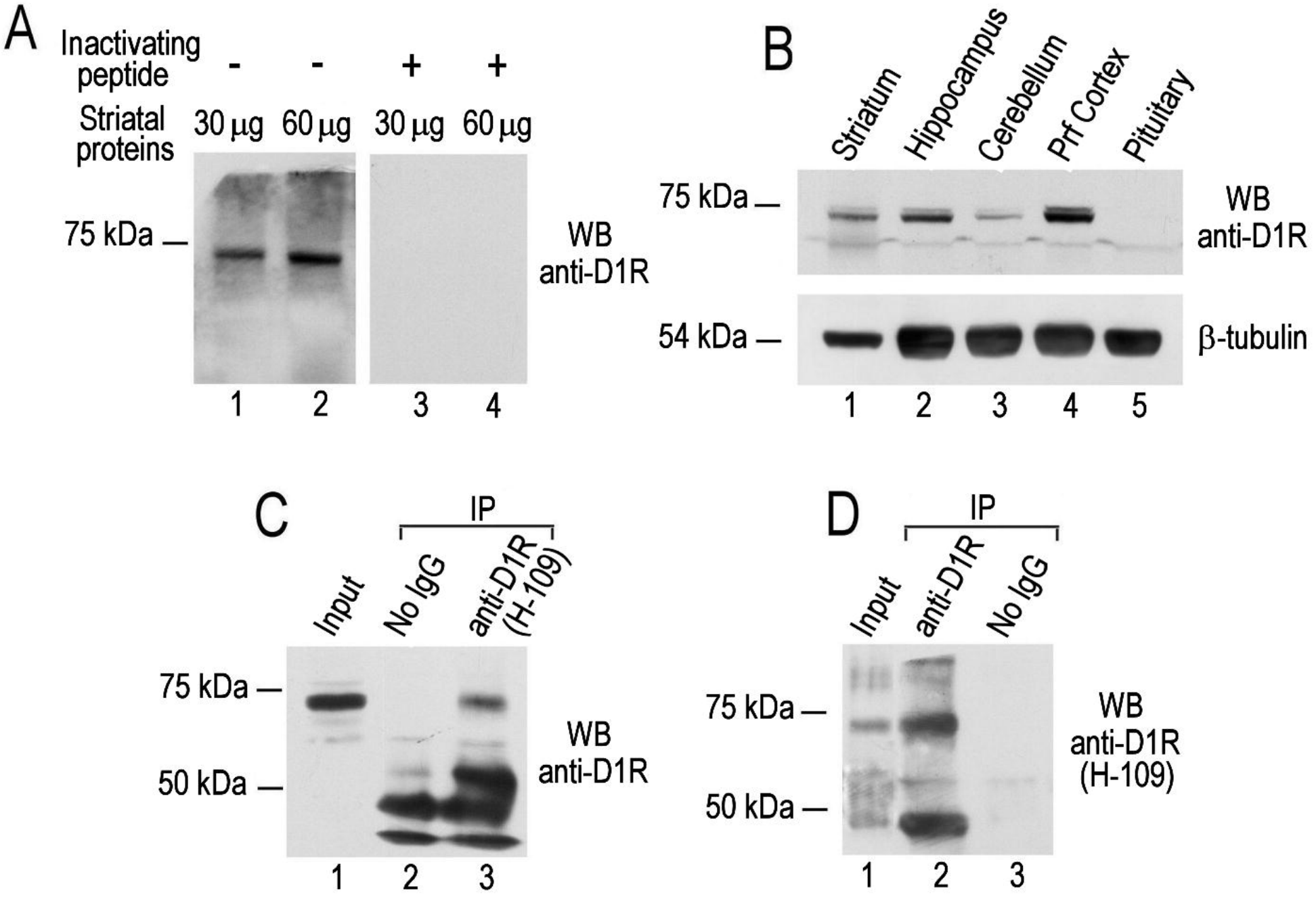
quinpirole. B. HEK-D1R/D3R cells were exposed to 1 μM DA for 60 min at 37°C in the absence or in the presence of either the D1R antagonist SCH23390 (1 μM) or the D3R antagonist (-)sulpiride (1 μM); HEK-D1R/D3R cells were also transfected with beta-arrestin-1-V53D and exposed to DA. [^{3}H]sulpiride binding was carried out in intact cells and [^{3}H]SCH23390 binding in the heavy membrane fraction. Data are expressed as percent loss of cell surface receptors. Bars are the means ± SE of three independent experiments. * $p < 0.001$ vs. DA, Student's t test.

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. Panel a-c, co-localization of D1R and D3R-GFP at the cell surface in untreated cells; panels d-f, cytoplasmic co-localization of D1R and D3R-GFP in agonist-treated cells; panels g-i, reappearance and co-localization 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 [^{3}H]sulpiride binding in intact cells (filled circles) and [^{3}H]SCH23390 binding in the heavy membrane fraction (open circles). Points are the means ± SE of four independent experiments. * $p < 0.001$ vs. time 0, Student's t test.

Fig. 1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WB anti-D1R
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B
Striatum
Hippocampus
Cerebellum
Prf Cortex
Pituitary
75 kDa
WB anti-D1R
54 kDa
β-tubulin
1 2 3 4 5
C
IP
Input
No IgG
anti-D1R (H-109)
75 kDa
50 kDa
WB anti-D1R
1 2 3
D
IP
Input
anti-D1R
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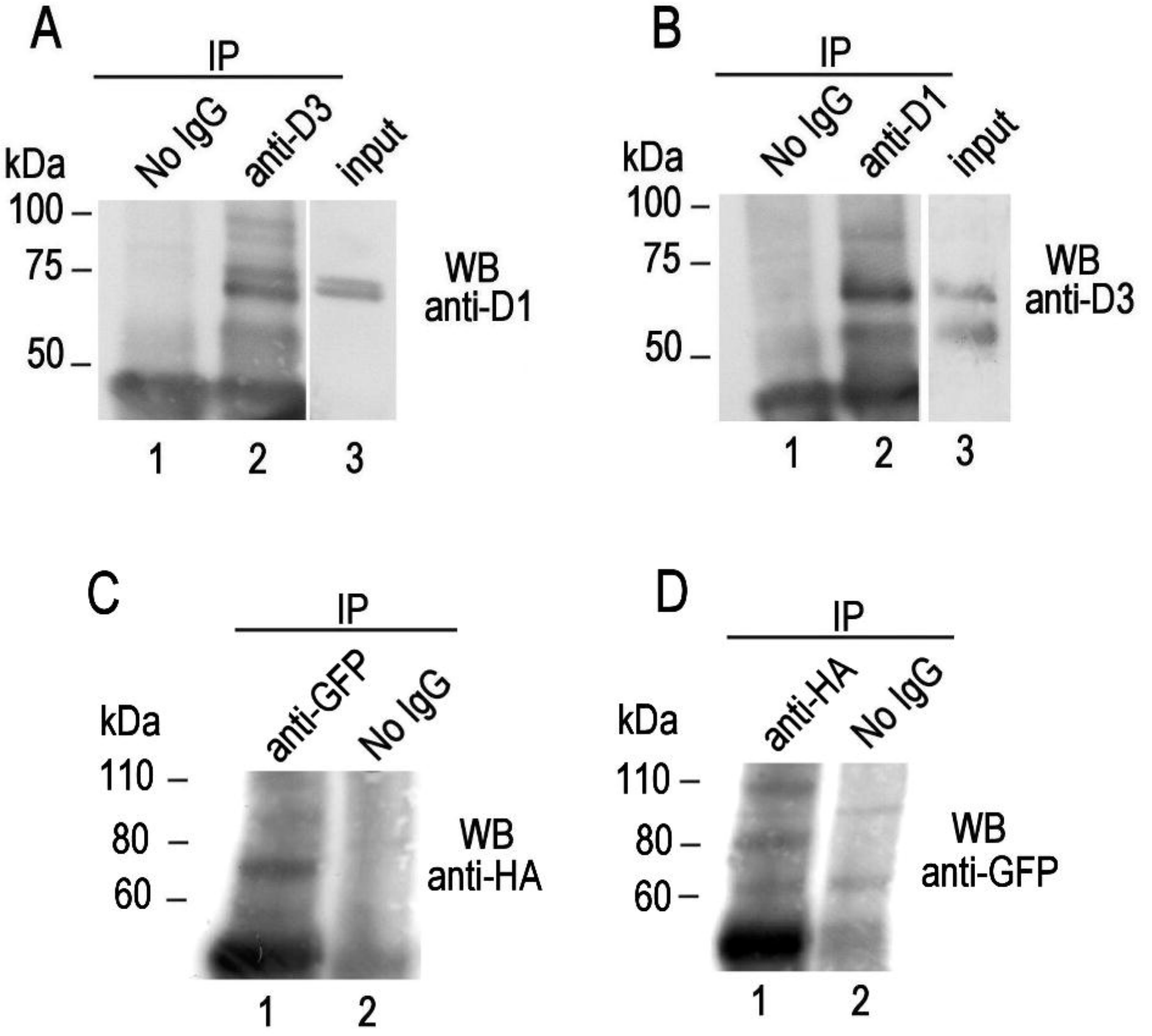
75 kDa
50 kDa
WB anti-D1R (H-109)
1 2 3

Fig. 2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No IgG
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kDa
100
75
50
WB
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1
2
3
B
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anti-D1
input
kDa
100
75
50
WB
anti-D3
1
2
3
C
IP
anti-GFP
No IgG
kDa
110
80
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WB
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80
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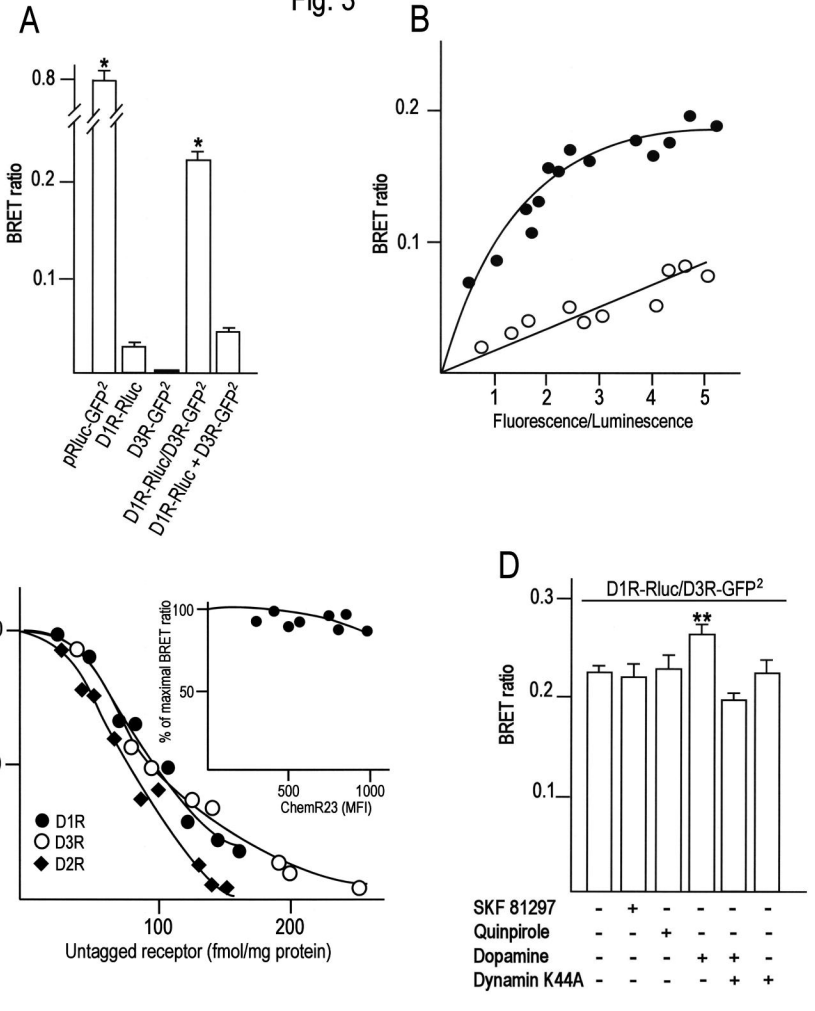
anti-GFP
1
2

Fig. 3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0.2
0.1
*
*
pRluc-GFP2
D1R-Rluc
D3R-GFP2
D1R-Rluc/D3R-GFP2
D1R-Rluc + D3R-GFP2
B
BRET ratio
0.2
0.1
1
2
3
4
5
Fluorescence/Luminescence
C
% of maximal BRET ratio
100
50
D1R
D3R
D2R
100
200
Untagged receptor (fmol/mg protein)
% of maximal BRET ratio
100
50
500
1000
ChemR23 (MFI)
D
D1R-Rluc/D3R-GFP2
BRET ratio
0.3
0.2
0.1
**
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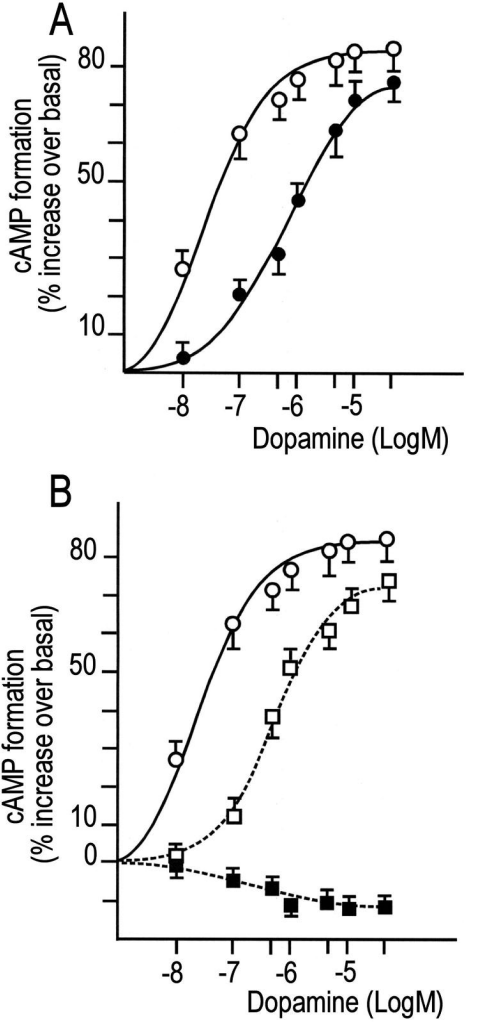
Quinpirole - - + - - -
Dopamine - - - + + -
Dynamin K44A - - - - + +

Fig. 4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10
-8
-7
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-5
Dopamine (LogM)
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(% increase over basal)
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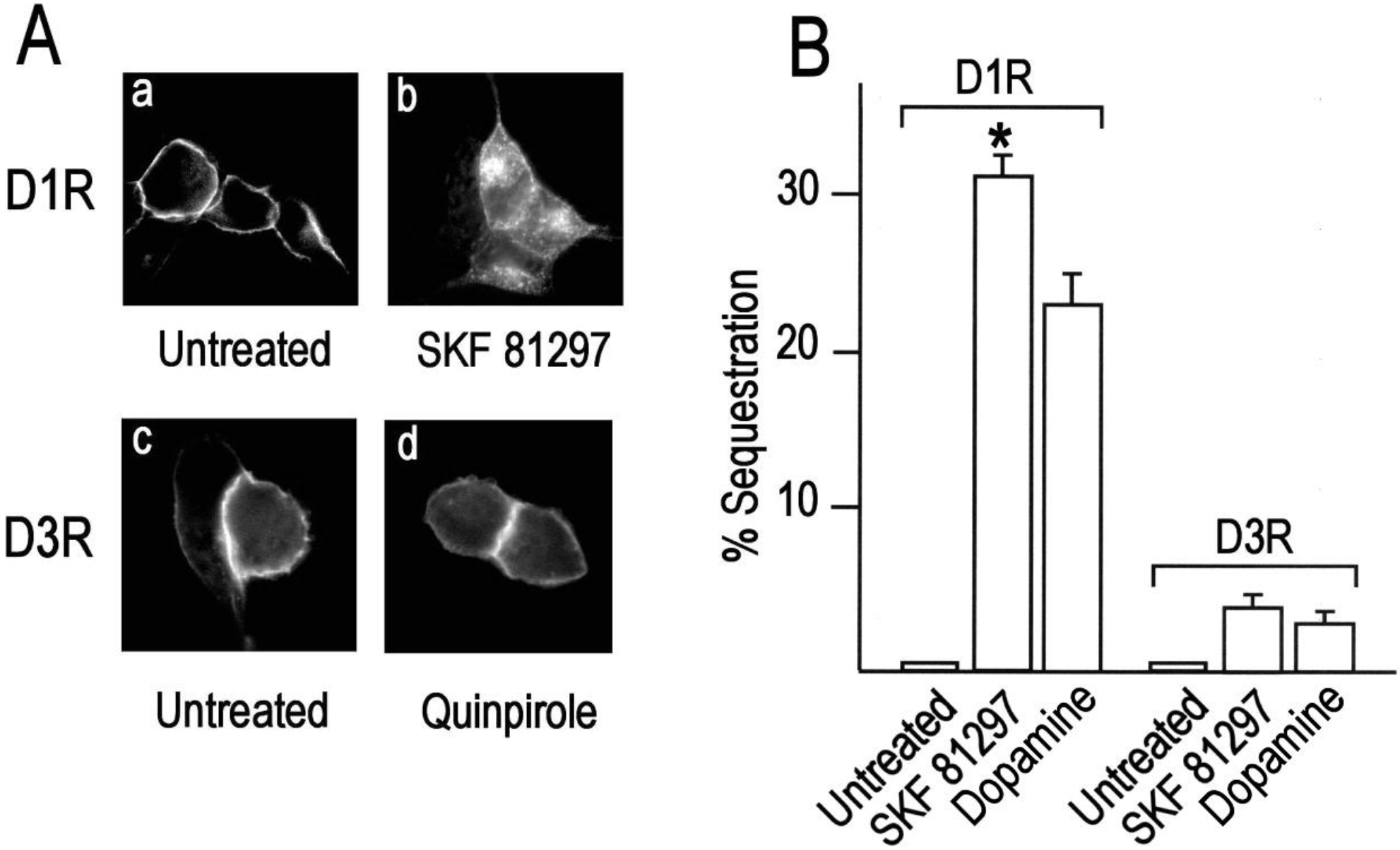
50
10
0
-8
-7
-6
-5
Dopamine (LogM)

Fig. 5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b
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c
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B
D1R
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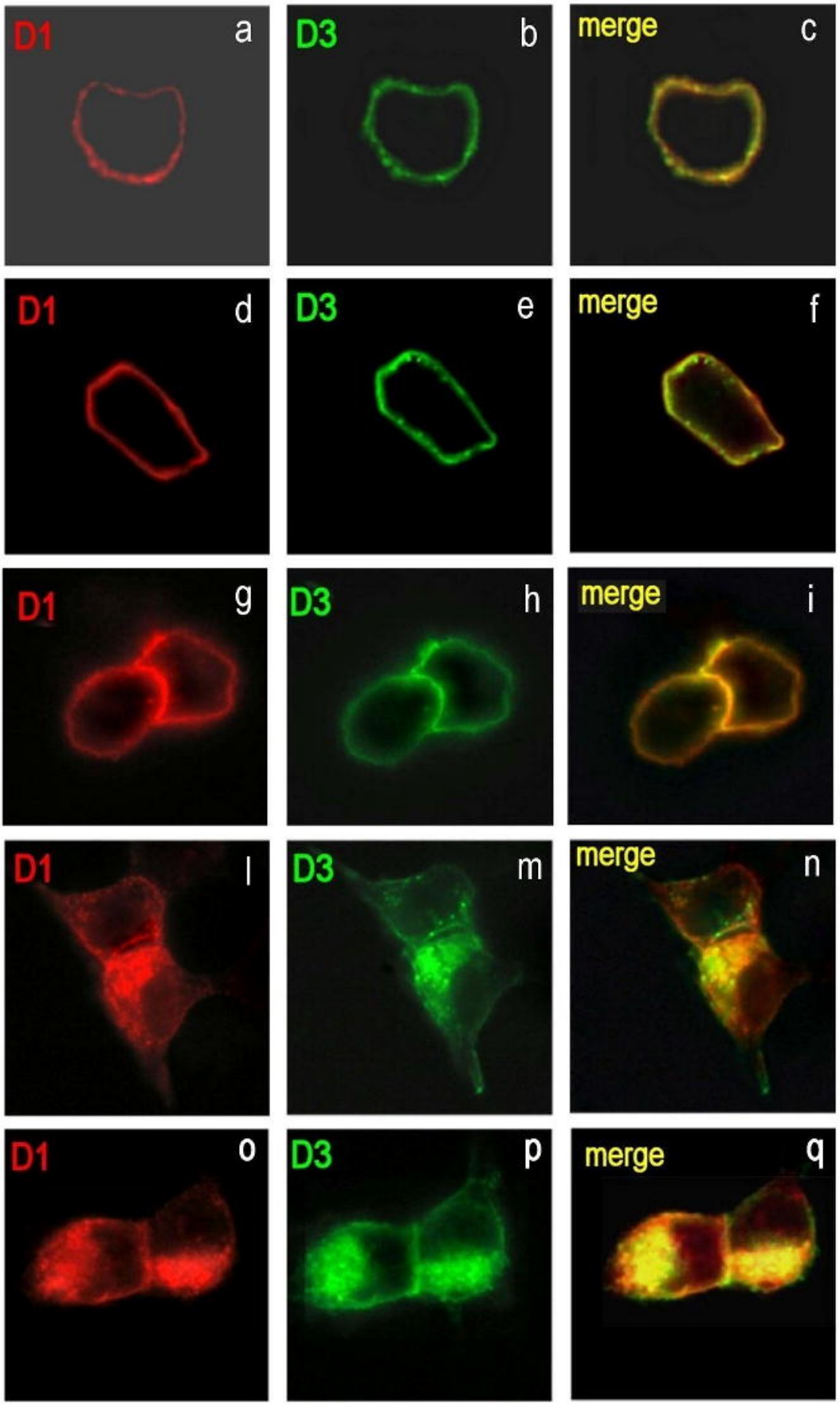
10
% Sequestration
D3R
Untreated
SKF 81297
Dopamine
Untreated
SKF 81297
Dopamine

Fig. 6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Untreated
D1
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D3
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merge
f
Quinpirole
D1
g
D3
h
merge
i
SKF 81297
D1
l
D3
m
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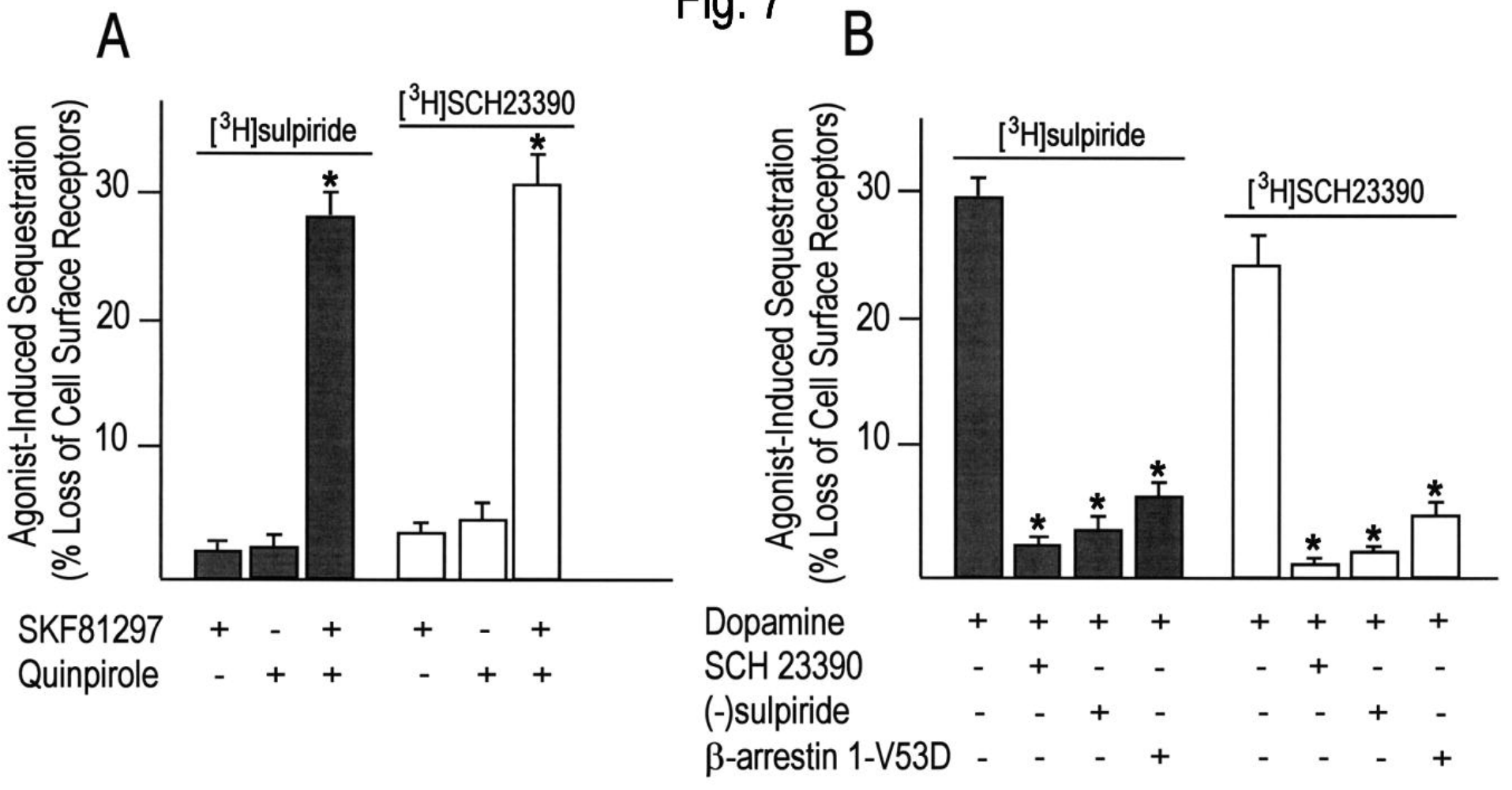
n
SKF 81297
+
quinpirole
D1
o
D3
p
merge
q
Dopamine

Fig. 7


A
[3H]sulpiride
[3H]SCH23390
Agonist-Induced Sequestration
(% Loss of Cell Surface Receptors)
30
20
10
SKF81297 + - + + - +
Quinpirole - + + - + +
B
[3H]sulpiride
[3H]SCH23390
Agonist-Induced Sequestration
(% Loss of Cell Surface Receptors)
30
20
10
Dopamine + + + + + + + +
SCH 23390 - + - - - + - -
(-)sulpiride - - + - - - + -
β-arrestin 1-V53D - - - + - - - +

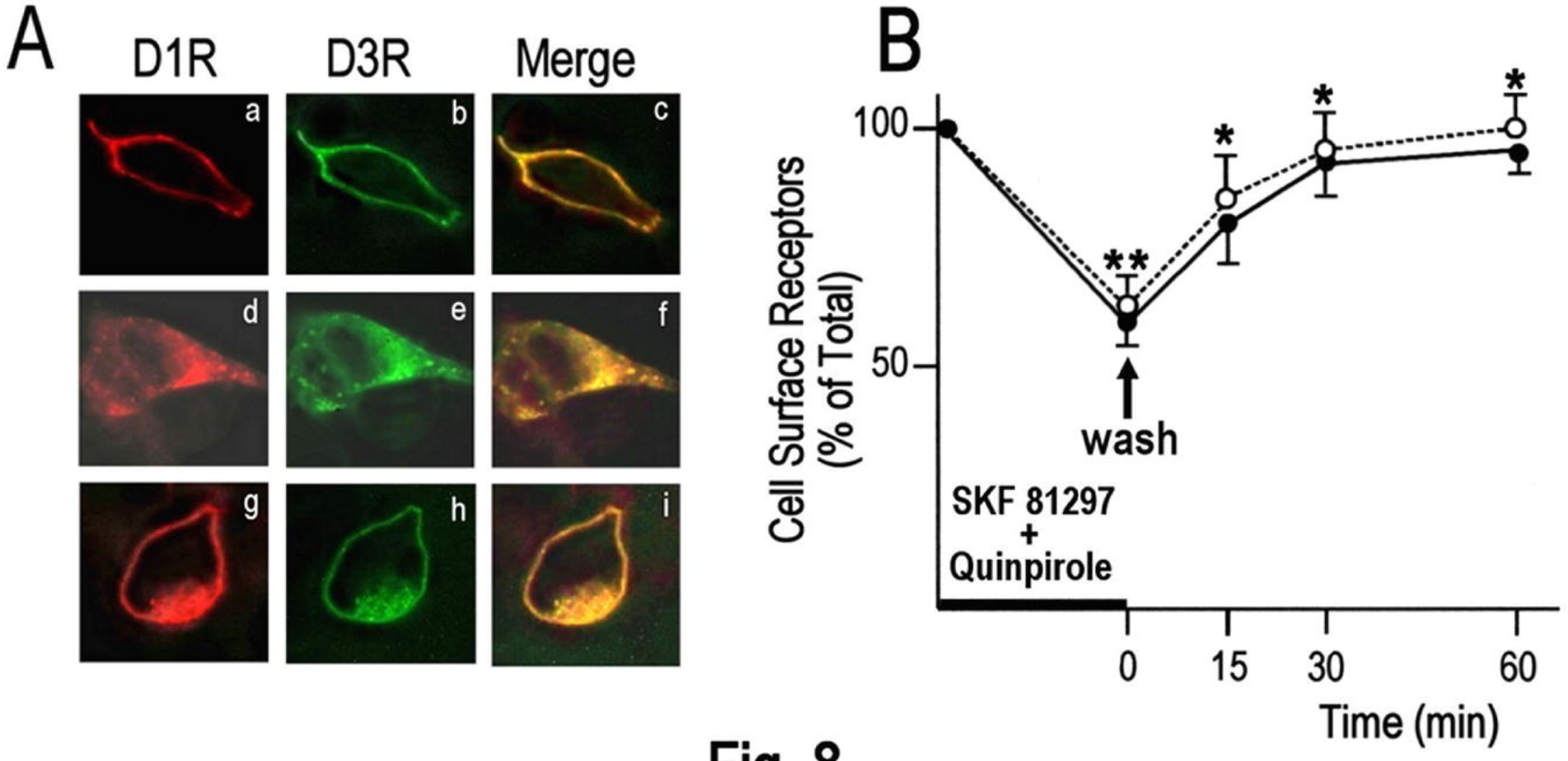

A
D1R
D3R
Merge
a
b
c
d
e
f
g
h
i
B
100
50
Cell Surface Receptors
(% of Total)
**
*
*
*
wash
SKF 81297
+
Quinpirole
0
15
30
60
Time (min)

Fig. 8