

# Identification of Dopamine D<sub>1</sub>–D<sub>3</sub> Receptor Heteromers

## INDICATIONS FOR A ROLE OF SYNERGISTIC D<sub>1</sub>–D<sub>3</sub> RECEPTOR INTERACTIONS IN THE STRIATUM\*

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The function of dopamine D<sub>3</sub> receptors present in the striatum has remained elusive. In the present study evidence is provided for the existence of dopamine D<sub>1</sub>–D<sub>3</sub> receptor heteromers and for an intramembrane D<sub>1</sub>–D<sub>3</sub> receptor cross-talk in living cells and in the striatum. The formation of D<sub>1</sub>–D<sub>3</sub> receptor heteromers was demonstrated by fluorescence resonance energy transfer and bioluminescence resonance energy transfer techniques in transfected mammalian cells. In membrane preparations from these cells, a synergistic D<sub>1</sub>–D<sub>3</sub> intramembrane receptor-receptor interaction was observed, by which D<sub>3</sub> receptor stimulation enhances D<sub>1</sub> receptor agonist affinity, indicating that the D<sub>1</sub>–D<sub>3</sub> intramembrane receptor-receptor interaction is a biochemical characteristic of the D<sub>1</sub>–D<sub>3</sub> receptor heteromer. The same biochemical characteristic was also observed in membrane preparations from brain striatum, demonstrating the striatal co-localization and heteromerization of D<sub>1</sub> and D<sub>3</sub> receptors. According to the synergistic D<sub>1</sub>–D<sub>3</sub> intramembrane receptor-receptor interaction, experiments in reserpinized mice showed that D<sub>3</sub> receptor stimulation potentiates D<sub>1</sub> receptor-mediated behavioral effects by a different mechanism than D<sub>2</sub> receptor stimulation. The present study shows that a main functional significance of the D<sub>3</sub> receptor is to obtain a stronger dopaminergic response in the striatal neurons that co-express the two receptors.

Dopamine receptors are grouped into two classes, D<sub>1</sub>-like receptors, which include D<sub>1</sub> and D<sub>5</sub> receptors, and D<sub>2</sub>-like receptors, which include D<sub>2</sub> (with two isoforms, D<sub>2S</sub> and D<sub>2L</sub>),

and D<sub>3</sub> and D<sub>4</sub> receptors (1, 2). The striatum receives the densest dopamine innervation and contains the highest density of dopamine receptors in the brain (3). The localization and function of the different subtypes of striatal dopamine receptors has been a matter of considerable debate, particularly that of the postsynaptic D<sub>1</sub> and D<sub>2</sub> receptors. It is widely accepted that D<sub>1</sub> and D<sub>2</sub> receptor subtypes are largely segregated in the two most populated types of striatal neurons, the  $\gamma$ -aminobutyric-acidergic (GABAergic)<sup>2</sup> dynorphinergic neuron, which also expresses substance P (SP), and the GABAergic enkephalinergic neuron (4, 5). In fact, results obtained with *in vivo* techniques indicate that dopamine exerts differential effects on the two types of GABAergic efferent neurons, by acting on stimulatory D<sub>1</sub> receptors localized in the GABAergic SP-dynorphinergic neurons and inhibitory D<sub>2</sub> receptors localized in the GABAergic enkephalinergic neurons (6–8). However, functional D<sub>1</sub>-like and D<sub>2</sub>-like receptors, as well as significant levels of D<sub>1</sub> and D<sub>2</sub> receptor mRNA expression, were detected in acutely dissociated striatonigral neurons (9). A more detailed and extensive analysis of the mRNA expression of the different receptor subtypes indicated that there is a limited subset of striatal neurons (~15% of all GABAergic efferent neurons) with a mixed phenotype of GABAergic SP-dynorphinergic and GABAergic enkephalinergic neurons, with D<sub>1</sub> and D<sub>2</sub> receptors (10). This co-expression of D<sub>1</sub> and D<sub>2</sub> receptors has been confirmed in neostriatal neurons at the confocal microscopy level (11, 12). George and coworkers (12, 13) have also found evidence for D<sub>1</sub>–D<sub>2</sub> receptor heteromerization (by co-immunoprecipitation) and for the generation of a unique pharmacology of the D<sub>1</sub>–D<sub>2</sub> receptor heteromer, with binding to selective ligands

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<sup>2</sup> The abbreviations used are: GABAergic,  $\gamma$ -aminobutyric-acidergic; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HEK, human embryonic kidney; PBS, phosphate-buffered saline; RLuc, *Renilla luciferase*; SP, substance P; i.p., intraperitoneally; SP, Substance P; D3KO, homozygous D<sub>3</sub> knockout mice; WT, wild type; GFP<sup>2</sup>, green fluorescence protein<sup>2</sup>; YFP, yellow fluorescent protein; EYFP, enhanced YFP; R(+)-7-OH-DPAT, 7-hydroxy-N,N-di-n-propyl-2-aminotetralin; ANOVA, analysis of variance; ROI, regions of interest.

and with selective coupling to G<sub>q/11</sub> and phospholipase C-mediated calcium signaling.

In contrast to the few GABAergic neurons expressing D<sub>1</sub> and D<sub>2</sub> receptors, the study of Surmeier *et al.* (10) showed that at least half of the GABAergic SP-dynorphinergic neurons and very few GABAergic enkephalinergic neurons express D<sub>3</sub> receptors, which have a very similar pharmacology to the D<sub>2</sub> receptors. Therefore, the D<sub>3</sub> receptors could in fact mediate most of the reported D<sub>1</sub>-D<sub>2</sub>-like receptor interactions demonstrated at the striatal neuronal level (reviewed in ref. 14). In the striatum, D<sub>3</sub> receptors are more concentrated in the shell of the nucleus accumbens and islands of Calleja, where they are also predominantly localized in GABAergic SP-dynorphinergic neurons, and pharmacological studies suggest that D<sub>1</sub>-D<sub>3</sub> receptor interactions could occur at the single cell level (15).

The evidence for striatal co-localization of D<sub>1</sub> and D<sub>3</sub> receptors gives a frame for the possible existence of direct D<sub>1</sub>-D<sub>3</sub> receptor intermolecular interactions. In the present study we demonstrate that D<sub>1</sub> and D<sub>3</sub> receptors can, in fact, interact at the molecular level, with the ability to form D<sub>1</sub>-D<sub>3</sub> receptor heteromers in transfected cells and in the striatum. These receptor heteromers show a synergistic D<sub>1</sub>-D<sub>3</sub> intramembrane receptor-receptor interaction that is most probably involved in the ability of D<sub>3</sub> receptor to potentiate the effects of D<sub>1</sub> receptor stimulation at the neuronal and behavioral level.

## EXPERIMENTAL PROCEDURES

**Animals**—Male Swiss Webster mice (Charles River Laboratories, Inc., Wilmington, MA) and homozygous D<sub>3</sub> knockout (D3KO) mice and their wild-type (WT) littermates (kindly donated by J. Drago from the Department of Medicine, Monash University, Australia) (8–12 weeks old) were used. D3KO mice used in these experiments were 5th through 8th generations of congenic C57BL/6J mice and generated by a backcrossing strategy. Genotypes of the D3KO and WT mice were identified by PCR with two pairs of primers flanking either exon 3 of the WT D<sub>3</sub> receptor or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (16). Animals were housed four per cage with food and water available *ad libitum* in temperature- and humidity-controlled rooms and were maintained on a 12-h light/dark cycle. They were experimentally naive at the start of the study and were used only once for locomotor activity experiments. Animals were maintained in accordance with guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse and the European Community Council Directive 86/609/EEC. The rationale, design, and methods of the experiments with D3KO mice and their WT littermates were approved by the Ethical Committee for Animal Research, University of Catania.

**Cell Culture and Transfection**—Human embryonic kidney (HEK)-293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum at 37 °C and in an atmosphere of 5% CO<sub>2</sub>. HEK-293T cells were transiently transfected with different amounts of cDNA encoding the indicated proteins by calcium phosphate precipitation (17) except for confocal FRET acceptor photobleaching exper-

iments in which the transfection reagent, FuGENE-6<sup>TM</sup> (Roche Molecular Biochemicals, Indianapolis, IN) was utilized following the protocol given by the manufacturer. In all cases, to maintain the total amount of DNA constant (10 μg) in co-transfections, the empty vector, pcDNA3.1, was used to equilibrate the amount of total DNA transfected. Cells were used 48 h after transfection. For confocal microscopy FRET experiments cells were fixed with a 3.5% paraformaldehyde in PBS for 15 min at room temperature, washed in PBS, and mounted onto slides using Mowiol coverslip mounting solution.

**Expression Vectors**—The human cDNA for the D<sub>3</sub> receptor without its stop codon was amplified using sense and antisense primers harboring unique EcoRI and KpnI sites. The fragment was then subcloned to be in-frame with either *Renilla* luciferase (*Rluc*) or green fluorescence protein<sup>2</sup> (GFP<sup>2</sup>) into the EcoRI and KpnI restriction site of a *Rluc*-expressing vector (p*Rluc*-N1, PerkinElmer Life Sciences) or a variant of GFP (pGFYP<sup>2</sup>-N3, Clontech, Heidelberg, Germany), respectively, to give the two plasmids, pD<sub>3</sub>-*Rluc* and pD<sub>3</sub>-GFP<sup>2</sup>, that express D<sub>3</sub> receptors fused to *Rluc* or GFP<sup>2</sup> on the C-terminal ends of the receptor (D<sub>3</sub>-*Rluc* and D<sub>3</sub>-GFP<sup>2</sup>). The human D<sub>1</sub> receptor was cloned in the enhanced yellow variant of GFP pEYFP-N1 (Clontech) vectors in a similar fashion and subcloned into the EcoRI and KpnI site to be in-frame with the GFP fluorescent protein variant enhanced yellow fluorescent protein (EYFP) (D<sub>1</sub>-YFP). The positive control vector used for the FRET experiments, pGFP<sup>2</sup>-EYFP (encoding for a fusion protein of GFP<sup>2</sup> and EYFP linked by six amino acids), was a gift from R. Pepperkok (EMBL, Heidelberg, Germany).

**cAMP Determination**—The accumulation of cAMP was measured in transfected HEK-293T cells (2 × 10<sup>6</sup> cells/sample) stimulated with different concentrations of the D<sub>1</sub> receptor agonist SKF 38393 (Tocris, Ellisville, MO) the D<sub>2-3</sub> receptor agonist quinpirole (Sigma) and/or forskolin (Sigma) for 15 min prior to the determination of cAMP levels by a [<sup>3</sup>H]cAMP assay system (Amersham Biosciences) as described by the manufacturer.

**FRET Experiments**—For FRET-based acceptor photobleaching experiments analyzed by confocal microscopy, the protocol described elsewhere was used (18). Confocal laser scanning microscopy was performed using a Leica SP2 microscope (Leica Microsystems, Mannheim, Germany) equipped with an acousto-optical beamsplitter, a 100-milliwatt argon laser for excitation at 514 nm, and a 20-milliwatt blue diode laser for excitation at 405 nm, and images were acquired using the acceptor photobleaching element of the Leica software. For FRET experiments analyzed by fluorometry, 48 h after transfection, cells were rapidly washed twice in PBS, detached, and resuspended in the same buffer. To control the number of cells, the protein concentration of the samples was determined using a Bradford assay kit (Bio-Rad) using bovine serum albumin dilutions as standards. FRET experiments were performed as described previously (18). The contribution of the GFP variants, GFP<sup>2</sup> and YFP proteins alone, to the two detection channels (spectral signature (19)) was measured in experiments with cells expressing only one of these proteins and normalized to the sum of the signal obtained in the two detection channels.

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The spectral signatures of the different receptors fused to either GFP<sup>2</sup> or YFP did not significantly vary from the determined spectral signatures of the fluorescent proteins alone. FRET quantitation was performed as described previously (18).

**BRET Experiments**—Forty-eight hours after transfection, cells were rapidly washed twice in PBS, detached, and resuspended in the same buffer. To control the number of cells, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. To quantify D<sub>1</sub>-YFP expression, cells (20 μg of protein) were distributed in 96-well microplates (black plates with a transparent bottom), and fluorescence was read in a Fluostar Optima fluorometer (BMG Labtechnologies, Offenburg, Germany) using a 10 nm bandwidth excitation filter at 400 nm for D<sub>2</sub>R-GFP<sup>2</sup> reading or 485 nm for YFP reading. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing D<sub>3</sub>-*Rluc* alone. For BRET measurements, the protocol previously described was used (18). To quantify D<sub>3</sub>-*Rluc* expression luminescence readings were performed after 10 min of adding 5 μM coelentrazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] – Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the *Rluc* construct expressed alone in the same experiment.

**Membrane Preparation**—Transfected HEK cells were washed twice with ice-cold PBS and sonicated for three 30-s periods in 10 volumes of 20 mM Tris-HCl buffer containing 100 mM NaCl, 7 mM MgCl<sub>2</sub>, and 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 1,500 × *g* for 10 min at 4 °C. The supernatant was centrifuged at 105,000 × *g*, 40 min, 4 °C. Membrane suspensions from striatal tissue were obtained by tissue disruption with a Polytron homogenizer (PTA 20 TS rotor, setting 3, Kinematica, Basel, Switzerland) for three 5-s periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, and the homogenates were processed as described above for cell membranes. Membranes were stored at –80 °C and were washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Protein was quantified by the bicinchoninic acid method (Pierce) using bovine serum albumin dilutions as standard.

**Radioligand Binding Experiments**—Membrane suspensions (0.5 mg of protein/ml) were incubated 1 h at 25 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl<sub>2</sub> with 2.5 nM of the D<sub>1</sub> receptor antagonist [<sup>3</sup>H]R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine ([<sup>3</sup>H]SCH 23390, PerkinElmer Life Sciences), 1.7 nM of the D<sub>3</sub> receptor agonist [<sup>3</sup>H]7-hydroxy-*N,N*-di-*n*-propyl-2-aminotetralin ([<sup>3</sup>H]R(+)-7-OH-DPAT, Amersham Biosciences), or 5.0 nM of the D<sub>2-3</sub> receptor antagonist [<sup>3</sup>H]3,5-dichloro-*N*-[[*(2S)*-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide ([<sup>3</sup>H]raclopride, PerkinElmer Life Sciences) and increasing concentrations of the partial D<sub>1</sub> receptor agonist (±)-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol hydrobromide (SKF 38393, triplicates of 13 different competitor concentrations from 0.1 nM to 50 μM), the full D<sub>1</sub> receptor agonist 6-chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide (SKF 81297, triplicates of 12 different competitor concentrations from 0.001

nM to 10 μM, Tocris) or the D<sub>3</sub> receptor agonist R(+)-7-OH-DPAT (triplicates of 13 different competitor concentrations from 0.01 nM to 10 μM; Sigma), in the absence or the presence of 10 nM of the D<sub>3</sub> receptor agonist R(+)-7-OH-DPAT, 100 nM of the D<sub>1</sub> agonist SKF 38393 or 100 nM of the D<sub>1</sub> agonist SKF 81297. Nonspecific binding was determined in the presence of 10 μM SCH 23390 (Tocris) for the D<sub>1</sub> receptor or 1 μM of the D<sub>3</sub> receptor antagonist GR 103691 (Tocris) for the D<sub>3</sub> receptor and confirmed that the value was the same as calculated by extrapolation of the displacement curves. In some experiments the D<sub>3</sub> receptor antagonist (*E*)-*N*-(4-[1,2,3,4-tetrahydroisoquinolin-2-yl]butyl)-3-phenylacrylamide (ST 198, 1 μM, prepared and analyzed by two of us (H.S. and O.S.) at the Johann Wolfgang Goethe University, Germany) was added instead R(+)-7-OH-DPAT to show its lack of modulation of D<sub>1</sub> receptor binding. In all cases, free and membrane-bound ligand were separated by rapid filtration of 500-μl aliquots in a cell harvester (Brandel, Gaithersburg, MD) through Whatman GE/C filters (Brandel) embedded in 0.3% polyethyleneimine, which were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation mixture (National Diagnostics, Atlanta, GA) overnight at room temperature, and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer Life Sciences) with an efficiency of 62% (20). Binding data from competition experiments were analyzed by nonlinear regression to the previously described equations (20, 21), using the commercial Graft curve-fitting software (Erithacus Software, Surrey, UK). Goodness of fit was tested according to reduced χ<sup>2</sup> value given by the nonlinear regression program and biphasic over monophasic competition curves were selected according to a *p* < 0.05 using an *F* test (22). The equilibrium dissociation constants for the high affinity (*K*<sub>DH</sub>) and low affinity (*K*<sub>DL</sub>) binding sites were determined from IC<sub>50</sub> values using the Cheng-Prusoff relation (23). Differences in *K*<sub>DH</sub> and *K*<sub>DL</sub> in the presence and absence of R(+)-7-OH-DPAT were tested for significance (two-tailed, *p* < 0.05) using Student's *t* test for paired samples.

**Locomotor Activity**—Activity chambers with 25.40 × 25.40 × 40.64 (high) cm open fields (Coulbourn Instruments, Allentown, PA) were used for the experiments with Swiss Webster mice and 41 × 41 × 33 (high) cm activity cages (7433 cage, Ugo Basile Instruments, Italy) were used for the experiments with D3KO mice and their WT littermates. Reserpine was purchased from Sigma and the D<sub>2-3</sub> receptor agonist quinpirole, the D<sub>3</sub> receptor agonist (4*a*R,10*b*R)-3,4*a*,4,10*b*-tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride (PD 128907) and the D<sub>2</sub> receptor antagonist 3-[4-(4-chlorophenyl)-4-hydroxyperidin-1-yl]methyl-1H-indole (L741626) were from Tocris. Reserpine was dissolved in a drop of glacial acetic acid, which was made up to volume with 5.5% glucose and was administered subcutaneously 20 h prior to the start of the locomotor activity recording. ST 198 was dissolved in sterile water and administered *per os* (due to the low pH of the solution). All the other drugs were dissolved in sterile saline and administered intraperitoneally (i.p.). The volume of administration was 10 ml/kg for all drugs. L741626 and ST 198 were administered 15 min prior to the locomotor activity recording

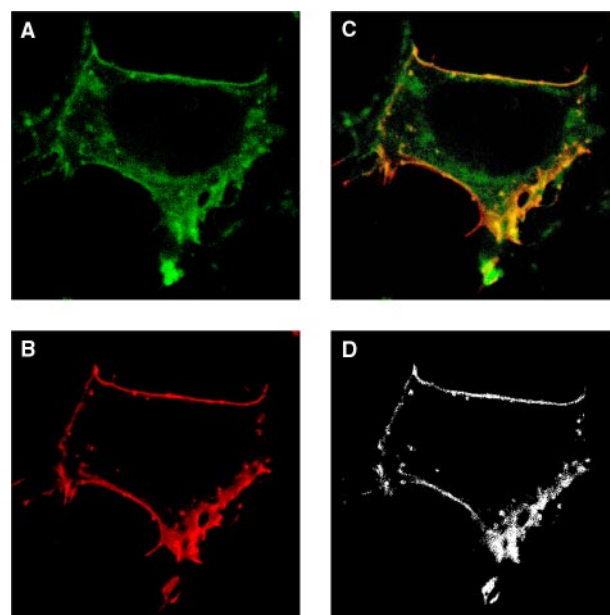
and SKF 38393, quinpirole, and PD 128907 were administered immediately before the locomotor activity recording. Locomotor activity was recorded immediately after the animals were introduced in the open field. All values (in centimeters of ambulation) registered per 10 min were transformed (square root (24)), and the average of the results obtained during the 10-min periods of the first hour of recording were used for calculations. Differences between genotypes and differently treated groups of animals were analyzed by two-way and one-way ANOVA followed by post-hoc Newman-Keuls test.

## RESULTS

**Heteromerization of  $D_1$  and  $D_3$  Receptors in Living Cells**—The formation of  $D_1$ - $D_3$  receptor heteromers was demonstrated by FRET and BRET techniques in cells transfected with fusion proteins consisting of each receptor and either a fluorescent protein (GFP<sup>2</sup> and YFP) or *Renilla* luciferase (*Rluc*). Expression of fusion proteins was assessed by Western blot and immunocytochemistry (data not shown). The functionality of the receptor-*Rluc*, -GFP<sup>2</sup>, or -YFP constructs was assessed by the determination of cAMP levels produced in transfected cells in response to agonists. According to the positive coupling of  $D_1$  receptor to the adenylyl cyclase, the  $D_1$  receptor agonist SKF 38393 properly induced cAMP accumulation in cells transfected with  $D_1$ -YFP similar to the wild-type untagged receptor. On the other hand, in agreement with the inhibitory role of  $D_3$  receptor on adenylyl cyclase activity, the  $D_{2-3}$  receptor agonist quinpirole was able to reduce forskolin-induced cAMP levels in cells transfected with either  $D_3$ -*Rluc* or  $D_3$ -GFP<sup>2</sup> similar to the wild-type untagged receptor (data not shown). The functional receptor fusion proteins were co-expressed in cells and the degree of co-localization was assessed. In HEK-293 cells cotransfected with the vectors encoding for both  $D_3$ -GFP<sup>2</sup> and  $D_1$ -YFP both receptors were expressed, and highly co-localized, at the membrane level (Fig. 1).

By using the FRET approach with the  $D_3$ -GFP<sup>2</sup> and  $D_1$ -YFP pair, and using the acceptor photobleaching technique and confocal microscopy analysis on cells expressing both receptors, it was possible to demonstrate the heteromerization between  $D_1$  and  $D_3$  receptors (Fig. 2). FRET was measured at the membrane level in different areas of the bottom section of a given cell and FRET efficiency was determined to be in the range of 14–23% (Fig. 2). To calculate the average of FRET efficiency in a co-transfected cell population, FRET was measured by fluorometric techniques determining the sensitized emission of YFP-tagged acceptor in live cells. The FRET efficiency was determined to be 34.8% ( $\pm 7.8$  S.D.) (Fig. 3). The low FRET efficiency found for a control constituted by mixing transfected  $D_3$ -GFP<sup>2</sup>-expressing cells with  $D_1$ -YFP-expressing cells proved the specificity in the energy transfer between  $D_3$ -GFP<sup>2</sup> and  $D_1$ -YFP when co-expressed in the same cell (Fig. 3).

In BRET experiments we studied  $D_1$ - $D_3$  receptor heteromerization by constructing a BRET saturation curve in living cells co-transfected with a constant amount of the  $D_3$ -*Rluc* construct while increasing concentrations of the  $D_1$ -YFP plasmid. A positive BRET signal for the transfer of energy between  $D_3$ -*Rluc* and  $D_1$ -YFP was obtained (Fig. 4). The BRET signal

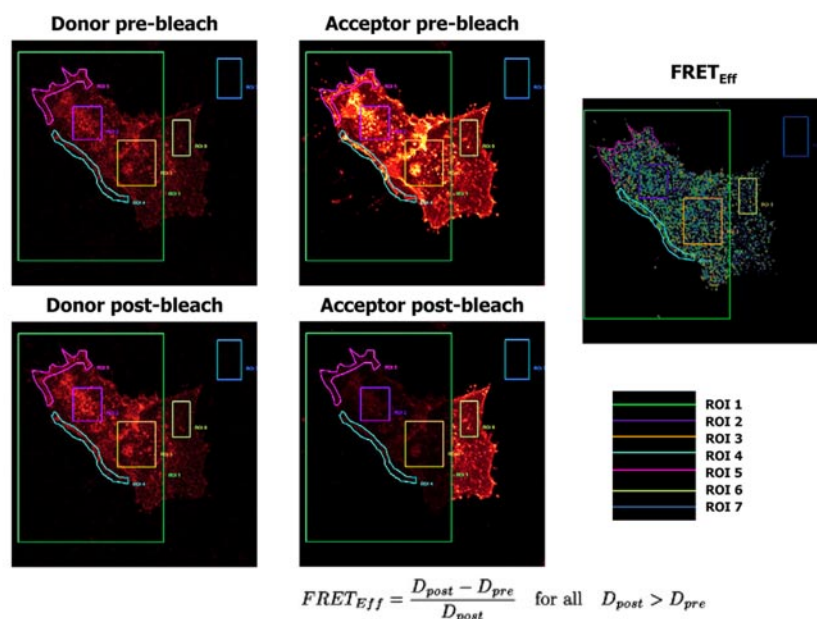


**FIGURE 1. Colocalization of  $D_1$  and  $D_3$  receptors on the plasma membrane of transiently transfected HEK-293 cells.** HEK-293 cells transiently transfected with  $D_3$ -GFP<sup>2</sup> and  $D_1$ -YFP were fixed and analyzed by confocal laser microscopy. *A*,  $D_3$  receptor immunoreactivity, in green. *B*,  $D_1$  receptor immunoreactivity in red. *C*, co-localization of both proteins is shown in yellow at the plasma membrane. *D*, overlapping of green and red pixels is shown in white. Images are representative of four coverslips from two independent transfections.

increased as a hyperbolic function of the concentration of the YFP-fusion construct added (assessed by the fluorescence emitted upon direct excitation at 480 nm) reaching an asymptote. From the saturation curve, a  $BRET_{max}$  of  $0.021 \pm 0.001$  and a  $BRET_{50}$  of  $2.2 \pm 0.6$  were calculated. The specificity of the interaction was demonstrated by including a negative control, in this case,  $D_3$ -*Rluc* and CXCR4-YFP. Because the pair  $D_3$ -*Rluc* and CXCR4-YFP led to a linear BRET signal (Fig. 4), the significant and hyperbolic BRET signal found for the  $D_3$ -*Rluc*- $D_1$ -YFP indicates that the interaction between  $D_3$  and  $D_1$  is specific.

**$D_1$ - $D_3$  Intramembrane Receptor Interaction**—Intramembrane receptor-receptor interactions are a common biochemical characteristic of receptor heteromers (25, 26). Therefore, we investigated the possible existence of striatal  $D_1$ - $D_3$  intramembrane receptor interactions in transfected HEK cells. Competition experiments with the  $D_1$  receptor antagonist [<sup>3</sup>H]SCH 23390 and increasing concentrations of SKF 81297 as a displacer produced biphasic competition curves (significantly better fitting than monophasic; *F* test:  $p < 0.05$ ) with  $K_{DH}$  and  $K_{DL}$  values for agonist binding to the  $D_1$  receptor of  $1.06 \pm 0.22$  nM and  $52.2 \pm 5.4$  nM, respectively (Fig. 5A). The addition of the  $D_3$ -selective agonist *R*(+)-7-OH-DPAT (10 nM) produced a significant decrease in  $K_{DH}$  and  $K_{DL}$  values to  $0.36 \pm 0.19$  nM and  $39.5 \pm 4.3$  nM, respectively (Student's paired *t* test;  $p < 0.01$  in both cases; five independent experiments); no significant differences were observed in the proportion of receptors in high and low affinity state (Fig. 5A). These results indicate that there is an increase in the affinity of both the high and low affinity states of the  $D_1$  receptor for agonists when the  $D_3$  receptor is activated. To investigate the possible existence of a  $D_1$  receptor modulation of agonist binding to the  $D_3$  receptor, competition experi-

## Dopamine $D_1$ - $D_3$ Receptor Heteromers



ROI	ROI_1	ROI_2	ROI_3	ROI_4	ROI_5	ROI_6	ROI_7
Donor pre-bleach	8.82	27.65	20.90	13.78	16.48	12.40	3.00
Donor post-bleach	10.33	33.87	24.75	17.89	20.55	12.08	2.97
Acceptor pre-bleach	21.95	69.28	57.98	55.22	56.74	32.69	1.97
Acceptor post-bleach	4.65	11.26	9.41	8.60	7.92	30.60	2.00
FRET efficiency (%)	14.62	18.36	15.54	23.02	19.82	0.00	0.00

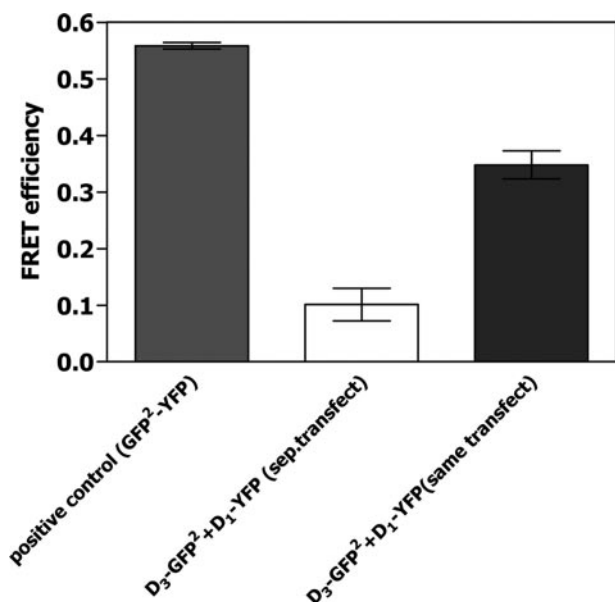
**FIGURE 2. Imaging FRET efficiency of the  $D_3R$ -GFP<sup>2</sup> and  $D_1R$ -YFP pair by acceptor photobleaching.** HEK-293T cells were transiently transfected with the plasmid DNA for the  $D_3R$ -GFP<sup>2</sup> and  $D_1R$ -YFP constructs using a ratio of donor to acceptor DNA of 1:2 and fixed 48 h after transfection. *Left panels* are images of the  $D_3R$ -GFP<sup>2</sup> donor before (*Donor pre-bleach*) and after (*Donor post-bleach*) photobleaching of the  $D_1R$ -YFP acceptor obtained by spectral imaging and subsequent linear un-mixing (see "Experimental Procedures") in several regions (ROI 1 to 5) of the lowest plane of the cell. The extent of the photobleaching is shown in the *central panels* as a lack of acceptor fluorescence in the selected region after photobleaching (*Acceptor post-bleach*) with respect to the image of the acceptor before photobleaching (*Acceptor pre-bleach*). The *right panel* represents donor un-quenching following acceptor photobleaching as donor post-bleach minus donor pre-bleach (subtraction) and a color representation of the FRET efficiency normalized to a scale from 0 to 1. The FRET efficiencies from different ROIs within the cell are given in the *table* below the *images*. As negative controls ROI 7 (out of the cell) and ROI 6 (cell section without photobleaching) were also analyzed. Images are representative of four coverslips from two independent transfections.

ments (five independent experiments) with the  $D_{2-3}$  receptor antagonist [<sup>3</sup>H]raclopride (which can only bind selectively to the  $D_3$  receptor in transfected cells) as radioligand and increasing concentrations of  $R(+)$ -7-OH-DPAT as displacer demonstrated monophasic competition curves, which were not modified by the addition of the  $D_1$  receptor agonist SKF 81297 (100 nM) (Fig. 5B).  $K_D$  values for  $R(+)$ -7-OH-DPAT binding to the  $D_3$  receptor in the presence and absence of SKF 38393 were  $4.3 \pm 0.6$  nM and  $3.9 \pm 0.6$  nM, respectively. These results demonstrate that  $D_3$  receptor stimulation enhances the binding of the  $D_1$  receptor agonist SKF 81297, but that  $D_1$  receptor stimulation does not modify the binding characteristics of the  $D_3$  receptor agonist  $R(+)$ -7-OH-DPAT, indicating that the intramembrane interaction is not reciprocal. Because, in addition, no  $R(+)$ -7-OH-DPAT-mediated modulation of  $D_1$  receptor agonist binding was observed with [<sup>3</sup>H]SCH 23390/SKF 81297 competition experiments in cells only transfected with the  $D_1$  receptor (data not shown), the synergistic  $D_1$ - $D_3$  intramembrane receptor-receptor interaction can be considered as a biochemical characteristic of the  $D_1$ - $D_3$  receptor heteromers.

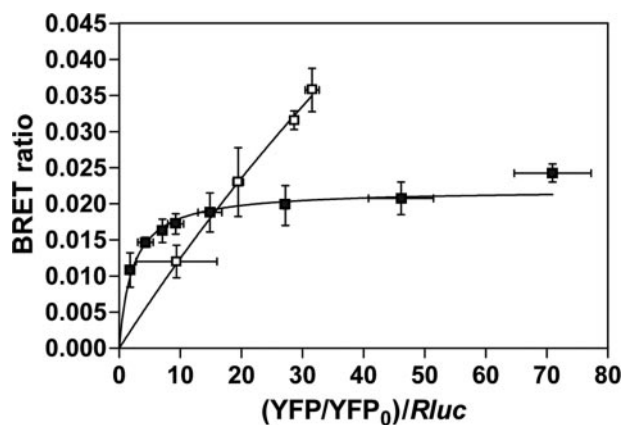
Using the synergistic  $D_1$ - $D_3$  intramembrane receptor-receptor interaction as a possible biochemical fingerprint to identify the  $D_1$ - $D_3$  receptor heteromer in the brain, we performed similar biochemical experiments in striatal membrane preparations. Competition experiments with the  $D_1$  receptor antagonist [<sup>3</sup>H]SCH 23390 as radioligand and increasing concentrations of the partial  $D_1$  receptor agonist SKF 38393 as displacer were carried out in the presence and in the absence of  $R(+)$ -7-OH-DPAT (10 nM). As shown in Fig. 6A, both competition curves were biphasic (significantly better fitting than monophasic;  $F$  test:  $p < 0.05$ ) and, the same as with transfected cells, there was a significant decrease in  $K_{DH}$  and  $K_{DL}$  values for agonist binding to the  $D_1$  receptor in the presence of  $R(+)$ -7-OH-DPAT, from  $21 \pm 3$  nM and  $1.1 \pm 0.1$   $\mu$ M to  $10 \pm 2$  nM and  $0.6 \pm 0.1$   $\mu$ M, respectively (Student's paired  $t$  test;  $p < 0.05$  in both cases; four independent experiments). There were no significant changes in the proportion of receptors in high and low affinity state. The  $D_3$  receptor antagonist ST 198 (1  $\mu$ M) did not produce any change in the binding properties of the  $D_1$  receptor (data not shown), indicating that modulation is due to  $D_3$  receptor activation. Competition experiments

with the  $D_3$  receptor agonist [<sup>3</sup>H] $R(+)$ -7-OH-DPAT as radioligand and increasing concentrations of  $R(+)$ -7-OH-DPAT as displacer were carried out in the presence or the absence of the selective  $D_1$  receptor agonist SKF 38393 (100 nM). Although there are some concerns about the *in vivo* selectivity of  $R(+)$ -7-OH-DPAT, it is important to point out that [<sup>3</sup>H] $R(+)$ -7-OH-DPAT has been previously shown to bind *in vitro* to the  $D_3$  receptors (27, 28). As shown in Fig. 6B, both competition curves were monophasic and the presence of the  $D_1$  receptor agonist did not significantly change the equilibrium binding parameters. From four independent experiments in the absence of  $D_1$  receptor agonist the  $K_D$  values for  $R(+)$ -7-OH-DPAT binding to  $D_3$  receptor was  $3.2 \pm 0.8$  nM, whereas in the presence of SKF 38393 the  $K_D$  value was  $2.9 \pm 0.7$  nM. These results demonstrate the existence of the same kind of  $D_1$ - $D_3$  intramembrane receptor-receptor interaction in the striatum and transfected cells, demonstrating the existence of  $D_1$ - $D_3$  receptor heteromers in the striatum.

*$D_1$ - $D_3$  Behavioral Receptor Interaction in Reserpinized Mice*—Administration of the selective  $D_1$  receptor agonist SKF 38393 (15 mg/kg, i.p.) or the putative non-selective  $D_{2-3}$  recep-

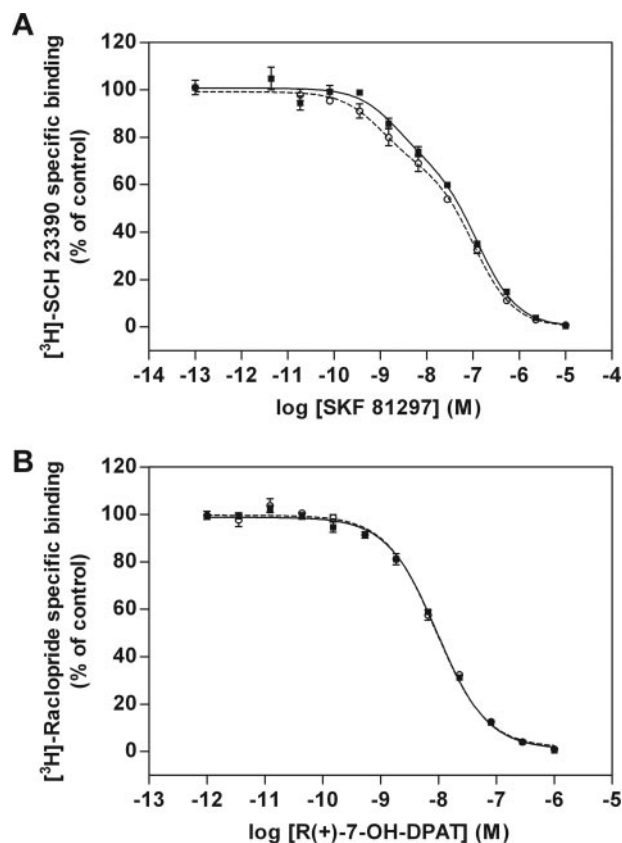


**FIGURE 3. FRET efficiency of the D<sub>3</sub>-GFP<sup>2</sup> and D<sub>1</sub>-YFP pair by sensitized emission in living cells.** HEK-293T cells were transiently transfected with the plasmid DNA corresponding to D<sub>3</sub>-GFP<sup>2</sup> (donor) and D<sub>1</sub>-YFP (acceptor) proteins using a ratio of donor to acceptor DNA of 1:2 (same transfect), or with the positive control plasmid GFP<sup>2</sup>-YFP. Fluorescence readings were performed 48 h after transfection, and linear un-mixing of the emission signals was applied to the data (see "Experimental Procedures"). Results are shown as the sensitized emission of the acceptor when the cells were excited at 400 nm. Cells separately transfected with either the D<sub>3</sub>-GFP<sup>2</sup> (donor) or D<sub>1</sub>-YFP (acceptor) proteins and subsequently mixed before FRET measurements (*sep. transfect*) were used as a negative control. Data are the mean ± S.D. of five independent experiments performed in duplicate. One-way ANOVA followed by Newman-Keuls test shows significant differences between GFP<sup>2</sup>-YFP and D<sub>3</sub>-GFP<sup>2</sup> + D<sub>1</sub>-YFP (*same transfect*) and between D<sub>3</sub>-GFP<sup>2</sup> + D<sub>1</sub>-YFP (*same transfect*) and D<sub>3</sub>-GFP<sup>2</sup> + D<sub>1</sub>-YFP (*sep. transfect*) ( $p < 0.001$  in all cases).



**FIGURE 4. BRET experiments in HEK-293 cells.** BRET was measured in HEK-293T cells co-expressing D<sub>3</sub>-Rluc and D<sub>1</sub>-YFP (*closed squares*) or D<sub>3</sub>-Rluc and CXCR4-YFP (*open squares*) constructs. Co-transfections were performed with increasing amounts of plasmid DNA for the YFP construct (1 μg of DNA to 10 μg of DNA), whereas the DNA for the Rluc construct was maintained constant (3 μg). Both fluorescence and luminescence of each sample were measured prior to every experiment to confirm equal expression of Rluc while monitoring the increase of YFP expression. The relative amounts of BRET acceptor are expressed as the ratio between the fluorescence of the acceptor and the luciferase activity of the donor. "YFP<sub>0</sub>" corresponds to the fluorescence value in cells expressing the BRET donor alone. BRET data are expressed as means ± S.D. of three to nine different experiments grouped as a function of the amount of BRET acceptor.

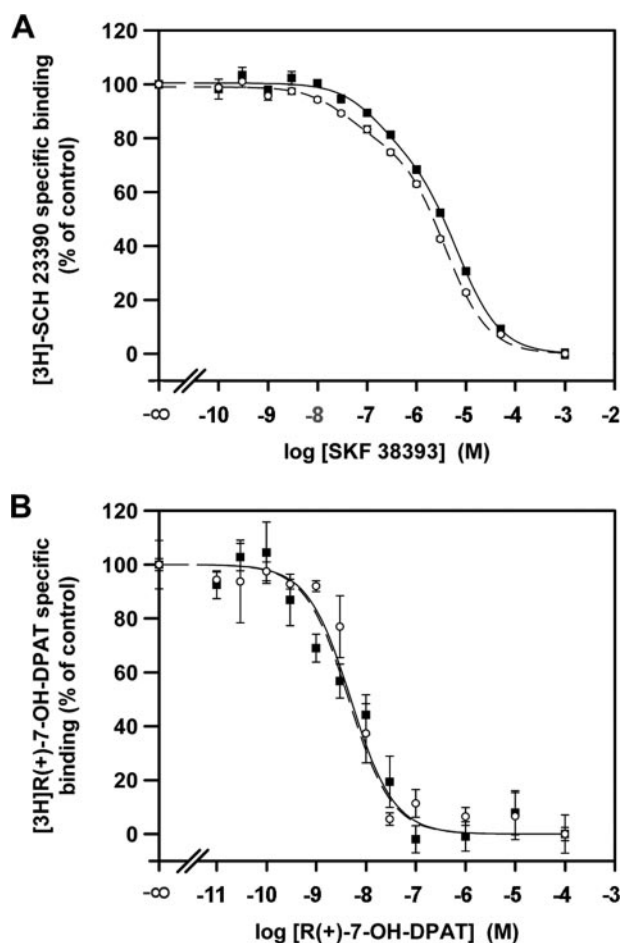
tor agonist quinpirole (0.5 mg/kg, i.p.) (minimal doses producing significant effects; see Table 1) produced a similar degree of motor activation in Swiss Webster reserpinized mice (Table 1),



**FIGURE 5. D<sub>1</sub>-D<sub>3</sub> intramembrane receptor cross-talk in transfected cells.** *A*, D<sub>3</sub> receptor agonist-mediated modulation of D<sub>1</sub> receptor agonist binding. Competition experiments of the D<sub>1</sub> receptor antagonist [<sup>3</sup>H]SCH 23390 (2.5 nM) versus increasing concentrations of the D<sub>1</sub> receptor agonist SKF 81297 in transfected cells were performed in the presence (○) or in the absence (■) of the D<sub>3</sub> receptor agonist R(+)-7-OH-DPAT (10 nM). *B*, D<sub>1</sub> receptor agonist-mediated modulation of D<sub>3</sub> receptor agonist binding. Competition experiments of the D<sub>2-3</sub> receptor antagonist [<sup>3</sup>H]raclopride (5 nM) versus increasing concentrations of R(+)-7-OH-DPAT in transfected cells were performed in the presence (○) or in the absence (■) of the D<sub>1</sub> receptor agonist SKF 81297 (100 nM). Values are expressed as percentage of specific binding of the sample without competing ligand (control). Data are means ± S.D. from a representative experiment performed in triplicate.

and their co-administration produced a synergistic effect (Fig. 7). On the contrary, the administration of the putative selective D<sub>3</sub> receptor agonist PD 128907 (29) did not produce any significant motor activation (up to 3 mg/kg, i.p.). Nevertheless, PD 128907 selectively and dose-dependently potentiated the locomotor activation induced by SKF 38393, but not when induced by quinpirole, a D<sub>2-3</sub> receptor agonist *in vitro* (Fig. 7). In pilot studies, higher doses of the D<sub>1</sub> receptor agonist were not potentiated by the co-administration of the D<sub>3</sub> receptor agonist. In addition, locomotor activation induced by co-administration of SKF 38393 (15 mg/kg, i.p.) and quinpirole (0.5 mg/kg, i.p.) was also significantly potentiated by PD 128907 (0.3–3 mg/kg, i.p.) (Fig. 7). Interestingly, when co-administered, PD 128907 and quinpirole exerted additive effects on D<sub>1</sub> receptor-mediated behavior, suggesting that simultaneous stimulation of D<sub>3</sub> and D<sub>2</sub> receptors induces a potentiation of D<sub>1</sub> receptor effects through different mechanisms of action. In fact, the selective D<sub>2</sub> receptor agonist L741626 (3 mg/kg, i.p. (30)) significantly counteracted the potentiating effect of quinpirole (0.5 mg/kg, i.p.), but not the potentiating effect of PD 128907 (3 mg/kg, i.p.) on

## Dopamine D<sub>1</sub>-D<sub>3</sub> Receptor Heteromers



**FIGURE 6. D<sub>1</sub>-D<sub>3</sub> intramembrane receptor cross-talk in the striatum.** A, D<sub>3</sub> receptor agonist-mediated modulation of D<sub>1</sub> receptor agonist binding. Competition experiments of the D<sub>1</sub> receptor antagonist [<sup>3</sup>H]SCH 23390 (2.5 nM) versus increasing concentrations of the D<sub>1</sub> receptor agonist SKF 38393 in calf striatal membranes were performed in the presence (○) or in the absence (■) of the D<sub>3</sub> receptor agonist R(+)-7-OH-DPAT (10 nM). B, D<sub>1</sub> receptor agonist-mediated modulation of D<sub>3</sub> receptor agonist binding. Competition experiments of the D<sub>3</sub> receptor agonist [<sup>3</sup>H]R(+)-7-OH-DPAT (1.7 nM) versus increasing concentrations of R(+)-7-OH-DPAT in calf striatal membranes were performed in the presence (○) or in the absence (■) of the D<sub>1</sub> receptor agonist SKF 38393 (100 nM). Values are expressed as percentage of specific binding of the sample without competing ligand (control). Data are means ± S.D. from a representative experiment performed in triplicate.

**TABLE 1**

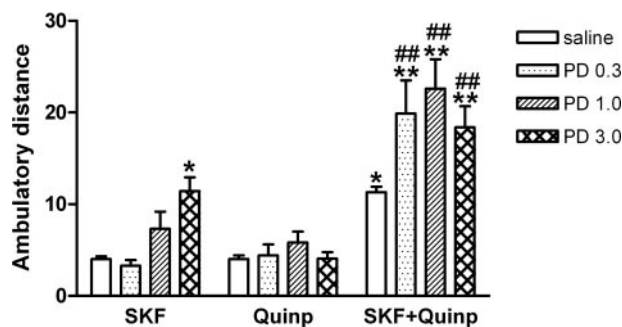
**Effects of the D<sub>1</sub> receptor agonist SKF 38393 (5 and 15 mg/kg, i.p.), the D<sub>2-3</sub> receptor agonist quinpirole (0.15 and 0.5 mg/kg, i.p.), and the D<sub>3</sub> receptor agonist PD 128907 (0.3–3 mg/kg, i.p.) on the locomotor activation in reserpinized Swiss Webster mice**

Results represent means ± S.E. of the average of the values obtained during the 10-min periods of the first hour of recording.

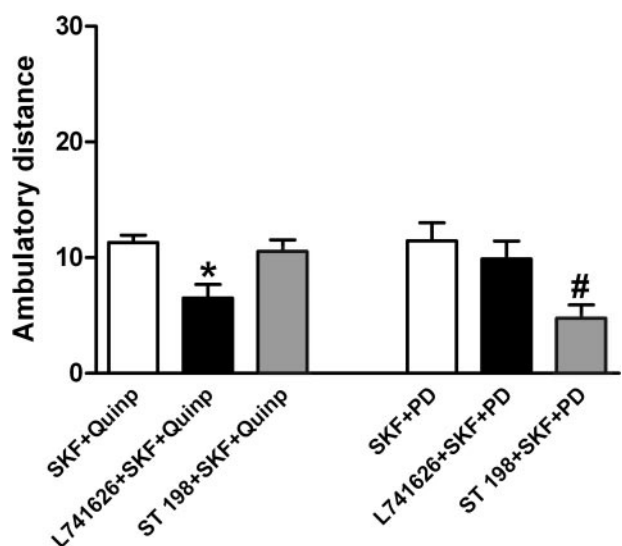
Treatment	Locomotor activity
mg/kg	mean ± S.E.
Saline	1.5 ± 0.2
SKF 38393 (5)	2.7 ± 0.7
SKF 38393 (15)	4.5 ± 0.8 <sup>a</sup>
Quinpirole (0.15)	2.5 ± 1.5
Quinpirole (0.5)	4.4 ± 1.2 <sup>a</sup>
PD 128907 (0.3)	1.1 ± 0.4
PD 128907 (1)	2.2 ± 0.6
PD 128907 (3)	2.3 ± 0.4

<sup>a</sup> Significantly different compared to the control, saline-treated group (ANOVA:  $p < 0.05$ ).

the locomotor activation induced by SKF 38393 (15 mg/kg, i.p.) (Fig. 8). On the other hand, the selective D<sub>3</sub> receptor antagonist ST 198 (10 mg/kg i.p. (28, 31)) significantly counteracted the



**FIGURE 7. D<sub>1</sub>-D<sub>3</sub> behavioral receptor interactions in reserpinized Swiss Webster mice.** Effects of the D<sub>3</sub> receptor agonist PD 128907 (PD, 0.3–3 mg/kg, i.p.) on the locomotor activation induced by the D<sub>1</sub> receptor agonist SKF 38393 (SKF, 15 mg/kg, i.p.) and/or the D<sub>2-3</sub> receptor agonist quinpirole (Quinp, 0.5 mg/kg, i.p.) in reserpinized mice. Results represent means ± S.E. of the average of the values obtained during 10-min periods of the first hour of recording. \* and \*\*, significantly different compared with SKF (ANOVA:  $p < 0.05$  and  $p < 0.01$ , respectively); ##, significantly different compared with SKF+Quinp without PD co-administration (ANOVA:  $p < 0.01$ ).



**FIGURE 8. D<sub>1</sub>-D<sub>3</sub> behavioral receptor interactions in reserpinized Swiss Webster mice.** Differential effects of the D<sub>2</sub> receptor antagonist L741626PD (3 mg/kg, i.p.) and the D<sub>3</sub> receptor antagonist ST198 (10 mg/kg, per os) on the locomotor activation induced by the co-administration of the D<sub>1</sub> receptor agonist SKF 38393 (SKF, 15 mg/kg, i.p.) plus the D<sub>2-3</sub> receptor agonist quinpirole (Quinp, 0.5 mg/kg, i.p.) and the co-administration of SKF plus the D<sub>3</sub> receptor agonist PD 128907 (PD, 3 mg/kg, i.p.) in reserpinized mice. Results represent means ± S.E. of the average of the values obtained during 10-min periods of the first hour of recording. \*, significantly different compared with SKF+Quinp (ANOVA:  $p < 0.05$ ); #, significantly different compared with SKF+PD (ANOVA:  $p < 0.05$ ).

potentiating effect of PD 128907 (3 mg/kg, i.p.) but not the potentiating effect of quinpirole (0.5 mg/kg, i.p.), on the locomotor activation induced by SKF 38393 (15 mg/kg, i.p.) (Fig. 8). Thus, these results strongly suggest that D<sub>3</sub> receptor stimulation potentiates D<sub>1</sub> receptor-mediated behavioral effects by a different mechanism than D<sub>2</sub> receptor stimulation.

In view of the debated D<sub>3</sub> receptor selectivity of PD 128907, its motor-activating effects were also analyzed in reserpinized D3KO mice and their WT littermates. Four different treated groups were studied in the two different genotypes: saline, PD 1218907 (3 mg/kg, i.p.), SKF 38393 (15 mg/kg, i.p.), and PD 1218907 plus SKF 38393. A two-way ANOVA demonstrated a significant genotype effect ( $F_{1,36} = 58.1, p < 0.0001$ ) and a sig-

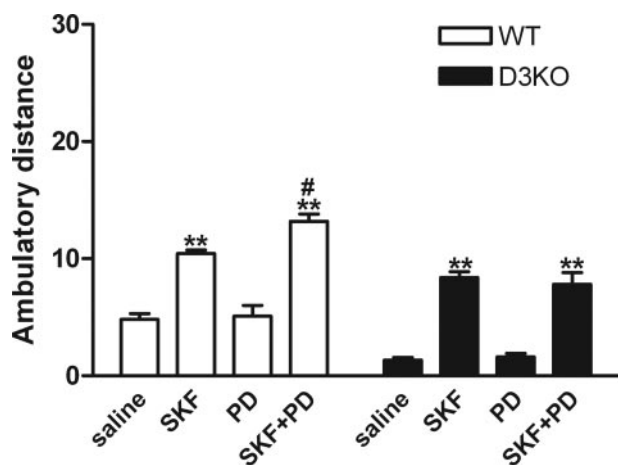


FIGURE 9.  $D_1$ - $D_3$  behavioral receptor interactions in D3KO reserpinized mice and their WT littermates. Effects of the  $D_3$  receptor agonist PD 128907 (PD, 3 mg/kg, i.p.) on the locomotor activation induced by the  $D_1$  receptor agonist SKF 38393 (SKF, 15 mg/kg, i.p.) in reserpinized D3KO and WT mice. Results represent means  $\pm$  S.E. of the average of the values obtained during 10-min periods of the first hour of recording. \*\*, significantly different compared with respective saline (ANOVA:  $p < 0.01$ , respectively); #, significantly different compared with SKF alone, without PD co-administration (ANOVA:  $p < 0.05$ ).

nificant treatment effect ( $F_{3,36} = 71.9$ ,  $p < 0.0001$ ) with no significant interaction. The significant genotype effect was due to higher locomotor activity in reserpinized wild-type mice compared to with reserpinized D3KO mice. In fact, motor activity in reserpinized wild-type mice (C57BL/6J strain) was higher than in reserpinized Swiss Webster mice (compare Fig. 9 with Fig. 7). The results indicate a higher sensitivity to the motor-depressant effects of reserpine in D3KO mice. The  $D_1$  receptor agonist SKF 38393 significantly increased locomotor activity in both wild-type and D3KO mice, and the putative selective  $D_3$  receptor agonist PD 12807 was ineffective on its own, but significantly potentiated the locomotor activation induced by SKF 38393 in wild-type, but not in D3KO mice (Fig. 9). Finally, the effect of quinpirole was also analyzed in D3KO mice and their WT littermates. Quinpirole (5 mg/kg, i.p.) produced a similar locomotor activation to that of SKF 38393 (15 mg/kg, i.p.) in both D3KO and WT mice (in means  $\pm$  S.E.,  $8.9 \pm 0.5$  and  $12.2 \pm 0.9$ , respectively; non-paired Student's  $t$  test:  $p < 0.01$  compared with the respective saline-treated groups in both cases). In view of the lower locomotor activity of D3KO mice compared with their WT littermates, these results confirm that  $D_3$  receptors do not play a significant role in the locomotor-activating effects of quinpirole.

## DISCUSSION

By using FRET and BRET techniques we demonstrate that  $D_1$  and  $D_3$  receptors can form heteromers if co-expressed in the same cell. This opens up the possibility of understanding the functional significance of the previously known co-localization of these receptors in the GABAergic SP-dynorphinergic striatal neuron (see the introduction). Receptor heteromerization does not only bring together two receptors to allow the interaction between their intracellular signaling cascades. Often these receptors show "intramembrane receptor-receptor interactions," promoting changes in their ability to bind ligands (25,

26, 32). Intramembrane receptor-receptor interactions are shown in crude membrane preparations of brain tissue or cells in culture, therefore not requiring the involvement of receptor signaling mechanisms. This implies the existence of an intermolecular interaction between two adjacent receptors and is a strong evidence of receptor heteromerization. Furthermore, intramembrane receptor-receptor interactions observed with cell membranes in which heteromerization is demonstrated can be used as a "biochemical fingerprint." Thus, the demonstration in the brain tissue of a specific intramembrane receptor-receptor interaction previously shown to depend on receptor heteromerization in an artificial cell system indicates the presence of the receptor heteromer in the brain (26).

In the present work we demonstrate the existence of the same kind of  $D_1$ - $D_3$  intramembrane receptor-receptor interaction in membrane preparations of both striatal tissue and co-transfected cells in which expression of  $D_1$ - $D_3$  receptor heteromers has been demonstrated, strongly suggesting the existence of  $D_1$ - $D_3$  receptor heteromers in the striatum. Because by far the main striatal localization of  $D_1$  receptors is postsynaptic to dopaminergic terminals, predominantly in the GABAergic dynorphinergic neurons (see the introduction), the  $D_1$ - $D_3$  intramembrane receptor-receptor interaction also indicates the existence of a postsynaptic striatal co-localization of both receptors. In this  $D_1$ - $D_3$  interaction,  $D_3$  receptor stimulation enhances the binding of  $D_1$  receptor agonists, indicating that  $D_3$  receptor stimulation could potentiate the effects of endogenous dopamine on  $D_1$  receptor. On the other hand,  $D_1$  receptor stimulation did not modify the binding of the  $D_3$  receptor agonist  $R(+)$ -7-OH-DPAT, indicating that the intramembrane modulatory interaction is not reciprocal within the  $D_1$ - $D_3$  receptor heteromer.

Previous studies about  $D_1$ - $D_3$  receptor interactions at the biochemical level have shown either facilitation or inhibition of the effects of  $D_1$  receptor stimulation upon  $D_3$  receptor activation, depending on the striatal compartment and on the signaling pathway (15, 33). On the one hand, stimulation of  $D_3$  receptor counteracted  $D_1$  receptor-mediated increase in *c-fos* expression in the islands of Calleja, but not in other striatal regions (15). This antagonistic interaction is in line with the differential coupling of  $D_1$  and  $D_3$  receptors to G proteins.  $D_1$  receptor is a  $G_{s-olr}$ -coupled receptor, the stimulation of which activates adenylyl cyclase (1, 2), whereas the  $D_3$  receptor, like any other  $D_2$ -like receptor, couples mostly to  $G_{i-o}$  proteins, and its stimulation inhibits adenylyl cyclase. On the other hand,  $\beta\gamma$ -subunit-mediated mechanisms of activation of other signaling pathways, such as mitogen-activated protein kinases, have been suggested to be involved in the ability of  $D_3$  receptor stimulation to potentiate an increase in SP expression in the nucleus accumbens induced by  $D_1$  receptor agonist (15). The present radioligand binding experiments provide an additional biochemical mechanism for a  $D_3$  receptor-mediated facilitation of  $D_1$  receptor function, which could be independent to, or be responsible for the  $D_1$ - $D_3$  synergistic interaction demonstrated at the signaling level.

Some studies have analyzed the existence of  $D_1$ - $D_3$  receptor interactions at the behavioral level, but without isolating pre- and post-synaptic mechanisms (29, 34). Thus, although it was



## Dopamine D<sub>1</sub>-D<sub>3</sub> Receptor Heteromers

initially a matter of debate, it is now quite well established that D<sub>3</sub> receptors are expressed on dopaminergic neurons, where they act as autoreceptors (reviewed in Ref. 28). It is also generally accepted that low doses of D<sub>3</sub> receptor agonists produce motor depression through the involvement of presynaptically located D<sub>3</sub> receptors and that the motor activation induced by higher doses of D<sub>3</sub> agonists are most probably related to loss of ligand selectivity and activation of postsynaptically located D<sub>2</sub> receptors (29, 35, 36). Thus, the *in vivo* selectivity of putative selective D<sub>3</sub> receptor ligands has been repeatedly questioned (28). Consequently, it has been suggested that the D<sub>3</sub> receptor is not involved in the potentiating effects of putative D<sub>3</sub> receptor agonists on D<sub>1</sub> receptor agonist-induced motor activation (29, 34). In contrast, in the present study we found clear evidence for a selective D<sub>3</sub> receptor-mediated potentiation of D<sub>1</sub> receptor agonist-induced locomotor activation.

The evaluation of locomotor activity induced by dopamine receptor agonists in reserpinized mice is a very useful *in vivo* model to study the function of striatal postsynaptic D<sub>1</sub>-like and D<sub>2</sub>-like receptors localized in GABAergic dynorphinergic and GABAergic enkephalinergic neurons without the influence of endogenous dopamine (24, 37, 38). In the present study we found that the D<sub>3</sub> receptor agonist PD 128907 potentiates the D<sub>1</sub>-mediated locomotor activation and that the effect of PD 128907 was counteracted by a specific D<sub>3</sub> but not by a specific D<sub>2</sub> receptor antagonist, indicating a selective involvement of the D<sub>3</sub> receptor. The specific involvement of D<sub>3</sub> receptors in the effects of PD 128907 was further demonstrated in experiments with D3KO mice. A rather surprising result was that quinpirole, a non-selective D<sub>2-3</sub> receptor agonist *in vitro*, demonstrated a selective D<sub>2</sub> receptor-mediated potentiation of D<sub>1</sub> receptor agonist-induced locomotor activation. Thus, the effect of quinpirole was counteracted by a D<sub>2</sub> but not by a D<sub>3</sub> receptor antagonist. Therefore, our results indicate that the dose of quinpirole used in the present experiments did not produce a significant *in vivo* stimulation of striatal postsynaptic D<sub>3</sub> receptors in mice. Accordingly, quinpirole produced the same degree of motor activation in D3KO mice and their WT littermates. Furthermore, co-administration of quinpirole or PD 128907 produced a very significant potentiation of the locomotor activation induced by the D<sub>1</sub> receptor agonist SKF 38393, strongly suggesting the existence of independent D<sub>2</sub>- and D<sub>3</sub>-mediated potentiating mechanisms of D<sub>1</sub> receptor action.

In addition to the intramembrane receptor-receptor interaction, D<sub>1</sub>-D<sub>3</sub> receptor heteromerization can potentially modify D<sub>1</sub> receptor signaling and internalization as it has been shown for other receptor heteromers (25, 26). The demonstration of tight intermolecular physical and functional interactions between both receptors gives a new framework for the understanding of dopamine receptor physiology and pharmacology. GABAergic enkephalinergic and GABAergic dynorphinergic neurons give rise to two striatal efferent systems, which connect the striatum with the output structures of the basal ganglia: the substantia nigra pars reticulata and the internal segment of the globus pallidus (entopeduncular nucleus in rodents) (3). These are called "direct" and "indirect" pathways. The direct pathway is made of GABAergic dynorphinergic neurons, which directly connect the striatum with the output structures. The indirect

pathway consists mostly of GABAergic enkephalinergic neurons, which connect the striatum with the external segment of the globus pallidus (globus pallidus in rodents), GABAergic neurons, which connect the globus pallidus with the subthalamic nucleus, and glutamatergic neurons, which connect the subthalamic nucleus with the output structures. Stimulation of the direct pathway results in motor activation and stimulation of the indirect pathway produces motor inhibition (39). It is generally accepted that dopamine, or dopamine agonists, induces motor activation by activating the direct pathway (acting on stimulatory D<sub>1</sub> receptors localized in GABAergic dynorphinergic neurons) and by depressing the indirect pathway (acting on inhibitory D<sub>2</sub> receptors localized in GABAergic enkephalinergic neurons) (3, 6–8). An important locus of D<sub>1</sub>-D<sub>2</sub> receptor agonist behavioral synergism is at the circuit level, due to the simultaneous inhibition (direct pathway) and release of stimulation (indirect pathway) of the tonic neuronal activity of the output structures (40, 41). However, the D<sub>1</sub>-D<sub>3</sub> receptor agonist behavioral synergism most probably takes place at the cellular level, within the GABAergic SP-dynorphinergic neurons (direct pathway). The present results add strong support for this hypothesis, although we cannot discard a D<sub>1</sub>-D<sub>3</sub> receptor interaction at the circuit level. However, because the binding properties of D<sub>1</sub> receptors that are not co-localized with D<sub>3</sub> receptors cannot be modulated by D<sub>3</sub> receptor ligands (as we demonstrate in cells only transfected with D<sub>1</sub> receptors), the results obtained with radioligand binding experiments in membranes preparations demonstrate that a significant amount of D<sub>1</sub> receptors are co-localized with D<sub>3</sub> receptors in the striatum. The two mechanisms, intercellular D<sub>1</sub>-D<sub>2</sub> and intracellular D<sub>1</sub>-D<sub>3</sub> receptor interactions, could explain the remarkable locomotor activation obtained with the simultaneous administration of D<sub>1</sub>, D<sub>2</sub>, and D<sub>3</sub> receptor agonists reported herein and fit very well with the role of D<sub>3</sub> receptors in behavioral sensitization to L-dopa in different animal models of Parkinson disease (31, 42). Chronic striatal dopamine depletion followed by L-dopa treatment leads to an increased sensitivity to L-dopa or dopamine D<sub>1</sub> receptor agonists, which seems to be causally related to an up-regulation of D<sub>3</sub> receptors in the GABAergic SP-dynorphinergic neurons (42). These experimental findings suggest that targeting D<sub>1</sub>-D<sub>3</sub> receptor heteromers can have important implications for the treatment of basal ganglia disorders.

During the resubmission of this manuscript another research group also demonstrated D<sub>1</sub>-D<sub>3</sub> receptor heteromerization in co-transfected cells with BRET techniques (43). Evidence was also obtained for a D<sub>3</sub> receptor-mediated potentiation of D<sub>1</sub> receptor signaling (cAMP accumulation) and modifications of agonist-mediated internalization with D<sub>1</sub>-D<sub>3</sub> receptor heteromerization (43).

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