Identification of Dopamine $D_1$–$D_3$ Receptor Heteromers

**INDICATIONS FOR A ROLE OF SYNERGISTIC $D_1$–$D_3$ RECEPTOR INTERACTIONS IN THE STRIATUM**

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The function of dopamine $D_3$ receptors present in the striatum has remained elusive. In the present study evidence is provided for the existence of dopamine $D_1$–$D_3$ receptor heteromers and for an intramembrane $D_1$–$D_3$ receptor cross-talk in living cells and in the striatum. The formation of $D_1$–$D_3$ 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 $D_1$–$D_3$ intramembrane receptor-receptor interaction was observed, by which $D_3$ receptor stimulation enhances $D_1$ receptor agonist affinity, indicating that the $D_1$–$D_3$ intramembrane receptor-receptor interaction is a biochemical characteristic of the $D_1$–$D_3$ receptor heteromer. The same biochemical characteristic was also observed in membrane preparations from brain striatum, demonstrating the striatal co-localization and heteromerization of $D_1$ and $D_3$ receptors. According to the synergistic $D_1$–$D_3$ intramembrane receptor-receptor interaction, experiments in reserpinized mice showed that $D_3$ receptor stimulation potentiates $D_1$ receptor-mediated physiological effects by a different mechanism than $D_2$ receptor stimulation. The present study shows that a functional significance of the $D_1$ 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, $D_1$-like receptors, which include $D_1$, and $D_3$ receptors, and $D_2$-like receptors, which include $D_2$ (with two isofoms, $D_{2S}$ and $D_{2L}$), and $D_3$ and $D_4$ 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 $D_1$ and $D_2$ receptors. It is widely accepted that $D_1$ and $D_2$ receptor subtypes are largely segregated in the two most populated types of striatal neurons, the $\gamma$-aminobutyric-acidergic (GABAergic) 2 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 $D_1$ receptors localized in the GABAergic SP-dynorphinergic neurons and inhibitory $D_2$ receptors localized in the GABAergic enkephalinergic neurons (6–8). However, functional $D_1$-like and $D_2$-like receptors, as well as significant levels of $D_1$ and $D_2$ 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 $D_1$ and $D_2$ receptors (10). This co-expression of $D_1$ and $D_2$ receptors has been confirmed in neostriatal neurons at the confocal microscopy level (11, 12). George and coworkers (12, 13) have also found evidence for $D_1$–$D_2$ receptor heteromerization (by co-immunoprecipitation) and for the generation of a unique pharmacology of the $D_1$–$D_2$ receptor heteromer, with binding to selective ligands...
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and with selective coupling to G_{q/11} and phospholipase C-mediated calcium signaling.

In contrast to the few GABAergic neurons expressing D₁ and D₂ 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 D₃ receptors, which have a very similar pharmacology to the D₂ receptors. Therefore, the D₃ receptors could in fact mediate most of the reported D₁–D₂-like receptor interactions demonstrated at the striatal neuronal level (reviewed in ref. 14). In the striatum, D₃ 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 D₁–D₃ receptor interactions could occur at the single cell level (15).

The evidence for striatal co-localization of D₁ and D₃ receptors gives a frame for the possible existence of direct D₁–D₃ receptor intermolecular interactions. In the present study we demonstrate that D₁ and D₃ receptors can, in fact, interact at the molecular level, with the ability to form D₁–D₃ receptor heteromers in transfected cells and in the striatum. These receptor heteromers show a synergistic D₁–D₃ intramembrane receptor-receptor interaction that is most probably involved in the ability of D₃ receptor to potentiate the effects of D₁ receptor stimulation at the neuronal and behavioral level.

EXPERIMENTAL PROCEDURES

Animals—Male Swiss Webster mice (Charles River Laboratories, Inc., Wilmington, MA) and homozygous D₃ 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/6 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 D₃ receptor or the PGK (phosphoglycerate kinase 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% CO₂. 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 experiments in which the transfection reagent, FuGENE-6™ (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 D₃ 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 protein² (GFP²) into the EcoRI and KpnI restriction site of a Rluc-expressing vector (pRluc-N1, PerkinElmer Life Sciences) or a variant of GFP (pGFYP²-N3, Clontech, Heidelberg, Germany), respectively, to give the two plasmids, pD₃-Rluc and pD₃-GFP², that express D₃ receptors fused to Rluc or GFP² on the C-terminal ends of the receptor (D₃-Rluc and D₃-GFP²). The human D₁ 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) (D₁-YFP). The positive control vector used for the FRET experiments, pGFYP²-EYFP (encoding for a fusion protein of GFP² 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 × 10⁶ cells/sample) stimulated with different concentrations of the D₁ receptor agonist SKF 38393 (Tocris, Ellisville, MO) or a variant of SKF (Sigma) (D₂,₃ receptor agonist quinpirole (Sigma) and/or forskolin (Sigma) for 15 min prior to the determination of cAMP levels by a [³H]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 200-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 flowmetry, 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, GFP² 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$^2$ 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 $D_2$-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 fluorimeter (BMG Labtechnologies, Offenburg, Germany) using a 10 nm bandwidth excitation filter at 485 nm for $D_1$R-GFP$^2$ reading or 400 nm for $D_2$R-GFP$^2$ reading or 485 nm for YFP reading. Rotor–fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing $D_2$-Rluc alone. For BRET measurements, the protocol previously described was used (18). To quantify $D_3$-Rluc expression, luminescence readings were performed after 10 min of adding 5 μM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] − C; where C 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 30-s periods in 10 volumes of 20 mM Tris-HCl buffer containing 100 mM NaCl, 7 mM MgCl$_2$, 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 homogenate was centrifuged at 1,500 × g for 10 min at 4 °C. Membrane suspensions from striatal tissue were obtained by tissue homogenate was centrifuged at 1,500 × g for 10 min at 4 °C. Membrane suspensions from striatal tissue were obtained by tissue homogenate was centrifuged at 1,500 × g for 10 min at 4 °C. Membrane suspensions from striatal tissue were obtained by tissue homogenate was centrifuged at 1,500 × g for 10 min at 4 °C. Membrane suspensions from striatal tissue were obtained by tissue suspension was determined from IC$_{50}$ values using the Cheng-Prusoff relation (23). Differences in $K_{DH}$ and $K_{DL}$ in the presence and absence of $R(+)\text{-}7$-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$_2$-3 receptor agonist quinpirole, the D$_3$ receptor agonist (4aR,10bR)-3,4a,4,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride (PD 128907) and the D2 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.
RESULTS

Heteromerization of D1 and D3 Receptors in Living Cells—The formation of D1–D3 receptor heteromers was demonstrated by FRET and BRET techniques in cells transfected with fusion proteins consisting of each receptor and either a fluorescent protein (GFP2 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, -GFP2, 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 D1 receptor to the adenyl cyclase, the D1 receptor agonist SKF 38393 properly induced cAMP accumulation in cells transfected with D1-YFP similar to the wild-type untagged receptor. On the other hand, in agreement with the inhibitory role of D3 receptor on adenyl cyclase activity, the D2–3 receptor agonist quinpirole was able to reduce forskolin-induced cAMP levels in cells transfected with either D3-Rluc or D3-GFP2 similar to the wild-type untagged receptor. 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 D3-GFP2 and D1-YFP both receptors were expressed, and highly co-localized, at the membrane level (Fig. 1).

By using the FRET approach with the D3-GFP2 and D1-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 D1 and D3 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 fluorescence emitted upon direct excitation at 480 nm) reaching an asymptote. From the saturation curve, a BRETmax of 0.021 ± 0.001 and a BRET50 of 2.2 ± 0.6 were calculated. The specificity of the interaction was demonstrated by including a negative control, in this case, D3-Rluc and CXCR4-YFP. Because the pair D3-Rluc and CXCR4-YFP led to a linear BRET signal (Fig. 4), the significant and hyperbolic BRET signal found for the D3-Rluc-D1-YFP indicates that the interaction between D3 and D1 is specific.

D1–D3 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 D1–D3 intramembrane receptor interactions in transfected HEK cells. Competition experiments with the D1 receptor antagonist [3H]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 KD values to 0.36 nM and 0.19 nM for agonist binding to the D1 receptor of 1.06 ± 0.22 nM and 52.2 ± 5.4 nM, respectively (Fig. 5A). The addition of the D3-selective agonist R(+)7-OH-DPAT (10 nM) produced a significant decrease in KD and KD values to 0.36 ± 0.19 nM and 39.5 ± 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 D1 receptor for agonists when the D3 receptor is activated.

To investigate the possible existence of a D1 receptor modulation of agonist binding to the D3 receptor, competition experi-
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Using the synergistic D₁–D₃ intramembrane receptor-receptor interaction as a possible biochemical fingerprint to identify the D₁–D₃ receptor heteromer in the brain, we performed similar biochemical experiments in striatal membrane preparations. Competition experiments with the D₁ receptor antagonist [³H]SCH 23390 as radioligand and increasing concentrations of the partial D₁ 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 Kᵢ values for agonist binding to the D₁ receptor in the presence of R(+)-7-OH-DPAT, from 21 ± 3 nM and 1.1 ± 0.1 μM to 10 ± 2 nM and 0.6 ± 0.1 μM, 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 D₃ receptor antagonist ST 198 (1 μM) did not produce any change in the binding properties of the D₃ receptor (data not shown), indicating that modulation is due to D₃ receptor activation. Competition experiments with the D₃ receptor agonist [³H]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 D₁ 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 [³H]R(+)-7-OH-DPAT has been previously shown to bind in vitro to the D₃ receptors (27, 28). As shown in Fig. 6B, both competition curves were monophasic and the presence of the D₁ receptor agonist did not significantly change the equilibrium binding parameters. From four independent experiments in the absence of D₁ receptor agonist the Kᵢ values for R(+)-7-OH-DPAT binding to D₁ receptor was 3.2 ± 0.8 nM, whereas in the presence of SKF 38393 the Kᵢ value was 2.9 ± 0.7 nM. These results demonstrate the existence of the same kind of D₁–D₃ intramembrane receptor-receptor interaction in the striatum and transfected cells, demonstrating the existence of D₁–D₃ receptor heteromers in the striatum.

D₁–D₃ Behavioral Receptor Interaction in Reserpinized Mice—Administration of the selective D₁ receptor agonist SKF 38393 (15 mg/kg, i.p.) or the putative non-selective D₂–D₃ recep-

![FIGURE 2. Imaging FRET efficiency of the D₁R-GFP² and D₃R-YFP pair by acceptor photobleaching. HEK-293T cells were transiently transfected with the plasmid DNA for the D₁R-GFP² and D₃R-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 D₁R-GFP² donor before (Donor pre-bleach) and after (Donor post-bleach) photobleaching of the D₁R-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.]

| ROI    | ROI_1  | ROI_2  | ROI_3  | ROI_4  | ROI_5  | ROI_6  | ROI_7  |
|--------|--------|--------|--------|--------|--------|--------|--------|
| Donor pre-bleach | 8.82   | 7.65   | 26.90  | 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   |

FRETeff = Dpost − Dpre / Dpost for all Dpost > Dpre.
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), and their co-administration produced a synergistic effect (Fig. 7). On the contrary, the administration of the putative selective D₃ 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 D₂–3 receptor agonist in vitro (Fig. 7). In pilot studies, higher doses of the D₁ receptor agonist were not potentiated by the co-administration of the D₃ 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 D₁ receptor-mediated behavior, suggesting that simultaneous stimulation of D₁ and D₂ receptors induces a potentiation of D₁ receptor effects through different mechanisms of action. In fact, the selective D₂ 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...
the locomotor activation induced by SKF 38393 (15 mg/kg, i.p.) (Fig. 8). On the other hand, the selective D₃ receptor antagonist ST 198 (10 mg/kg i.p. (28, 31)) significantly counteracted the 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 D₃ receptor stimulation potentiates D₁ receptor-mediated behavioral effects by a different mechanism than D₂ receptor stimulation.

In view of the debated D₃ 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-
In view of the lower locomotor activity of D3KO compared with the respective saline-treated groups in 0.5 and 12.2 mg/kg, i.p.) 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 D1 receptor agonist SKF 38393 (SKF, 15 mg/kg, i.p.) in reserpinized D3KO and WT 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 respective saline (ANOVA: p < 0.01, respectively); †, significantly different compared with SKF alone, without PD co-administration (ANOVA: p < 0.05).

**FIGURE 9.** D₁–D₃ behavioral receptor interactions in D3KO reserpinized mice and their WT littermates. Effects of the D₁ receptor agonist PD 128907 (PD, 3 mg/kg, i.p.) on the locomotor activation induced by the D₃ receptor agonist SKF 38393 (SKF, 15 mg/kg, i.p.) in reserpinized D3KO and WT mice. The D₁ receptor agonist SKF 38393 significantly increased locomotor activity in both wild-type and D3KO mice, and the putative selective D₃ 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 ± S.E., 8.9 ± 0.5 and 12.2 ± 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 D₃ receptors do not play a significant role in the locomotor-activating effects of quinpirole.

**DISCUSSION**

By using FRET and BRET techniques we demonstrate that D₁ and D₃ 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 those 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 D₁–D₃ intramembrane receptor-receptor interaction in membrane preparations of both striatal tissue and co-transfected cells in which expression of D₁–D₃ receptor heteromers has been demonstrated, strongly suggesting the existence of D₁–D₃ receptor heteromers in the striatum. Because by far the main striatal localization of D₁ receptors is postsynaptic to dopaminergic terminals, predominantly in the GABAergic dynorphinergic neurons (see the introduction), the D₁–D₃ intramembrane receptor-receptor interaction also indicates the existence of a postsynaptic striatal co-localization of both receptors. In this D₁–D₃ interaction, D₁ receptor stimulation enhances the binding of D₃ receptor agonists, indicating that D₃ receptor stimulation could potentiate the effects of endogenous dopamine on D₃ receptor. On the other hand, D₁ receptor stimulation did not modify the binding of the D₃ receptor agonist R(+)-7-OH-DPAT, indicating that the intramembrane modulatory interaction is not reciprocal within the D₁–D₃ receptor heteromer.

Previous studies about D₁–D₃ receptor interactions at the biochemical level have shown either facilitation or inhibition of the effects of D₁ receptor stimulation upon D₃ receptor activation, depending on the striatal compartment and on the signaling pathway (15, 33). On the one hand, stimulation of D₃ receptor counteracted D₁ 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 D₁ and D₃ receptors to G proteins. D₁ receptor is a Gαolf-coupled receptor, the stimulation of which activates adenylyl cyclase (1, 2), whereas the D₃ receptor, like any other D₂-like receptor, couples mostly to Gₛα₃ proteins, and its stimulation inhibits adenylyl cyclase. On the other hand, βγ-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 D₃ receptor stimulation to potentiate an increase in SP expression in the nucleus accumbens induced by D₁ receptor agonist (15). The present radioligand binding experiments provide an additional biochemical mechanism for a D₃ receptor-mediated facilitation of D₁ receptor function, which could be independent to, or be responsible for the D₁–D₃ synergistic interaction demonstrated at the signaling level.

Some studies have analyzed the existence of D₁–D₃ receptor interactions at the behavioral level, but without isolating pre- and post-synaptic mechanisms (29, 34). Thus, although it was
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initially a matter of debate, it is now quite well established that D$_3$ 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$_3$ receptor agonists produce motor depression through the involvement of presynaptically located D$_3$ receptors and that the motor activation induced by higher doses of D$_3$ agonists are most probably related to loss of ligand selectivity and activation of postsynaptically located D$_2$ receptors (29, 35, 36). Thus, the in vivo selectivity of putative selective D$_3$ receptor ligands has been repeatedly questioned (28). Consequently, it has been suggested that the D$_3$ receptor is not involved in the potentiating effects of putative D$_3$ receptor agonists on D$_1$ receptor agonist-induced motor activation (29, 34). In contrast, in the present study we found clear evidence for a selective D$_3$ receptor-mediated potentiation of D$_1$ 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$_1$-like and D$_2$-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$_3$ receptor agonist PD 128907 potentiates the D$_1$-mediated locomotor activation and that the effect of PD 128907 was counteracted by a specific D$_3$ but not by a specific D$_2$ receptor antagonist, indicating a selective involvement of the D$_3$ receptor. The specific involvement of D$_3$ 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$_2$–3 receptor agonist in vitro, demonstrated a selective D$_2$ receptor-mediated potentiation of D$_1$ receptor agonist-induced locomotor activation. Thus, the effect of quinpirole was counteracted by a D$_2$ but not by a D$_3$ 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$_1$ 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$_1$ receptor agonist SKF 38393, strongly suggesting the existence of independent D$_2$- and D$_3$-mediated potentiating mechanisms of D$_1$ receptor action.

In addition to the intramembrane receptor-receptor interaction, D$_1$–D$_3$ receptor heteromerization can potentially modify D$_1$ 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$_1$ receptors localized in GABAergic dynorphinergic neurons) and by depressing the indirect pathway (acting on inhibitory D$_2$ receptors localized in GABAergic enkephalinergic neurons) (3, 6–8). An important locus of D$_1$–D$_3$ receptor agonist behavioral synergism is at the circuit level, due to the simultaneous depression (indirect pathway) and release of stimulation (indirect pathway) of the tonic neuronal activity of the output structures (40, 41). However, the D$_1$–D$_3$ 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$_1$–D$_3$ receptor interaction at the circuit level. However, because the binding properties of D$_1$ receptors that are not co-localized with D$_3$ receptors cannot be modulated by D$_3$ receptor ligands (as we demonstrate in cells only transfected with D$_1$ receptors), the results obtained with radioligand binding experiments in membranes preparations demonstrate that a significant amount of D$_1$ receptors are co-localized with D$_3$ receptors in the striatum. The two mechanisms, intercellular D$_1$–D$_3$ and intracellular D$_1$–D$_3$ receptor interactions, could explain the remarkable locomotor activation obtained with the simultaneous administration of D$_1$, D$_2$, and D$_3$ receptor agonists reported herein and fit very well with the role of D$_1$ 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$_1$ receptor agonists, which seems to be causally related to an up-regulation of D$_3$ receptors in the GABAergic SP-dynorphinergic neurons (42). These experimental findings suggest that targeting D$_1$–D$_3$ 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$_1$–D$_2$ receptor heteromerization in co-transfected cells with BRET techniques (43). Evidence was also obtained for a D$_3$ receptor-mediated potentiation of D$_1$ receptor signaling (cAMP accumulation) and modifications of agonist-mediated internalization with D$_1$–D$_3$ receptor heteromerization (43).

REFERENCES
1. Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., and Caron, M. G. (1998) Physiol. Rev. 78, 189–225
2. Neve, K. A., Seamans, J. K., and Trantham-Davidson, H. (2004) J. Recept. Signal Transduct. Res. 24, 165–205
3. Gerfen, C. R. (2004) The Rat Nervous System (Paxinos, G., ed) pp. 445–508, Elsevier Academic Press, Amsterdam
4. Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N.,
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Monsma, F. J., Jr., and Sibley, D. R. (1990) Science 250, 1429–1432
5. Le Moine, C., Normand, E., and Bloch, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4205–4209
6. Gerfen, C. R. (1992) Trends Neurosci. 15, 133–139
7. Ferré, S., O’Connor, W. T., Fuxe, K., and Ungerstedt, U. (1993) J. Neurosci. 13, 5402–5406
8. Ferré, S., O’Connor, W. T., Svenningsson, P., Bjorklund, L., Lindberg, J., Tinner, B., Stromberg, I., Goldstein, M., Ogren, S. O., Ungerstedt, U., Fredholm, B. B., and Fuxe, K. (1996) Eur. J. Neurosci. 8, 1545–1553
9. Surmeier, D. J., Eberwine, J., Wilson, C. J., Cao, Y., Stefani, A., and Kitai, S. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10178–10182
10. Surmeier, D. J., Song, W. J., and Yan, Z. (1996) J. Neurosci. 16, 6579–6591
11. Aizman, O., Brismar, H., Uhlen, P., Zettergren, E., Levey, A. I., Forsberg, H., Greengard, P., and Aperia, A. (2000) Nat. Neurosci. 3, 226–230
12. Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca, A. J., O’Dowd, B. F., and George, S. R. (2007) J. Pharmacol. Exp. Ther. 293, 1063–1073
13. Bezard, E., Ferry, S., Mach, U., Stark, H., Leriche, L., Boraud, T., Gross, C., and Sokoloff, P. (2003) Trends Biochem. Sci. 28, 238–243
14. Surmeier, D. J., Reiner, A., Levine, M. S., and Ariano, M. A. (1993) Trends Neurosci. 16, 299–305
15. Ridray, S., Griffin, N., Mignon, V., Soulé, E., Carboni, S., Diaz, J., Schwartz, J. C., and Sokoloff, P. (1998) Eur. J. Neurosci. 10, 1676–1686
16. Accili, D., Fishburn, C. S., Drago, I., Steiner, H., Lachowicz, J. E., Park, B. H., Gauda, E. B., Lee, E. J., Cool, M. H., Sibley, D. R., Gerfen, C. R., Westphal, H., and Fuchs, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 103, 1945–1949
17. Jordan, M., Schallhorn, A., and Wurm, F. M. (1996) Nucleic Acids Res. 24, 596–601
18. Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S. R., Neve, K., Fuxe, K., Agnati, L. F., Woods, A. S., Ferré, S., Lluis, C., Bouvier, M., and Franco, R. (2000) J. Biol. Chem. 275, 46741–46749
19. Zimmermann, T., Rietdorf, J., Girod, A., Georget, V., and Pepperkok, R. (2002) FEBS Lett. 531, 245–249
20. Sarriò, S., Casadó, V., Escriché, M., Ciruela, F., Mallol, J., Canela, E. I., Lluis, C., and Franco, R. (2000) Mol. Cell. Biol. 20, 5164–5174
21. Casadó, V., Canti, C., Mallol, J., Canela, E. I., Lluis, C., and Franco, R. (1990) J. Neurosci. Res. 26, 461–473
22. Ciruela, F., Casado, V., Rodrigues, R. J., Luján, R., Burgués, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S. R., Mallol, J., Cortes, A., Canela, E. I., Lopez-Gimenez, J. F., Milligan, G., Lluis, C., Cunha, R. A., Ferré, S., and Franco, R. (2006) J. Neurosci. 26, 2080–2087
23. Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108
24. Ferré, S., Gimenez-Lloret, L., Artigas, F., and Martinez, E. (1994) Eur. J. Pharmacol. 255, 203–213
25. Agnati, L. F., Ferré, S., Lluis, C., Franco, R., and Fuxe, K. (2003) Pharmacol. Rev. 55, 509–550
26. Ferré, S., Ciruela, F., Woods, A. S., Lluis, C., and Franco, R. (2007) Trends Neurosci. 30, 440–446
27. Schoemaker, H. (1993) Eur. J. Pharmacol. 242, R1–R2
28. Sokoloff, P., Diaz, J., Le Foll, B., Guillou, O., Leriche, L., Bezard, E., and Gross, C. (2006) CNS Neurol. Disord. Drug Targets 5, 25–43
29. Pugsley, T. A., Davis, M. D., Akunne, H. C., MacKenzie, R. G., Shih, Y. H., Damsma, G., Wikstrom, H., Whetzel, S. Z., Georgic, L. M., Cooke, L. W., Demattos, S. B., Corbin, A. E., Glase, S. A., Wise, L. D., Dijkstra, D., and Heftner, T. G. (1995) J. Pharmacol. Exp. Ther. 275, 1355–1366
30. Millan, M. J., Dekeyne, A., Rivet, J. M., Dubuffet, T., Lavielle, G., and Brocco, M. (2000) J. Pharmacol. Exp. Ther. 293, 1063–1073
31. Bezard, E., Ferry, S., Mach, U., Stark, H., Leriche, L., Boraud, T., Gross, C., and Sokoloff, P. (2003) Nat. Med. 9, 762–767
32. Franco, R., Canals, M., Marcellino, D., Ferré, S., Agnati, L., Mallol, J., Casado, V., Ciruela, F., Fuxe, K., Lluis, C., and Canela, E. I. (2003) Trends Biochem. Sci. 28, 238–243
33. Schwartz, J. C., Diaz, J., Bordet, R., Griffin, N., Perachon, S., Pilon, C., Ridray, S., and Sokoloff, P. (1998) Brain Res. 26, 236–242
34. Mori, T., Murase, K., Tanaka, J., and Ichimaru, Y. (1997) Jpn. J. Pharmacol. 73, 251–254
35. Pittard, L. M., Logue, A. D., Hayes, S., Welge, J. A., Xu, M., Zhang, J., Berger, S. P., and Richtand, N. M. (2003) Neuropsychopharmacology 28, 100–107
36. Millan, M. J., Seguin, L., Gobert, A., Cussac, D., and Brocco, M. (2004) Psychopharmacology 174, 341–357
37. Rubinstein, M., Gershon, O., and Stefano, F. J. (1988) Eur. J. Pharmacol. 148, 419–426
38. Starr, M. S., and Starr, B. S. (1989) Pharmacol. Biochem. Behav. 33, 41–44
39. Alexander, G. E., and Crutcher, M. D. (1990) Trends Neurosci. 13, 266–271
40. Albin, R. L., Young, A. B., and Penney, J. B. (1989) Trends Neurosci. 12, 366–375
41. Surmeier, D. J., Ding, J., Day, M., Wang, Z., and Shen, W. (2007) Trends Neurosci. 30, 228–235
42. Bordet, R., Ridray, S., Carboni, S., Diaz, J., Sokoloff, P., and Schwartz, J. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3363–3367
43. Fiorentini, C., Busi, C., Gorumuso, E., Gotti, C., Spano P., and Missale, C. (2008) Mol. Pharmacol. 74, 59–69