Regulation of Dopamine D₁ Receptor Trafficking and Desensitization by Oligomerization with Glutamate N-Methyl-d-aspartate Receptors*

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Activation of dopamine D₁ receptors is critical for the generation of glutamate-induced long-term potentiation at corticostriatal synapses. In this study, we report that, in striatal neurons, D₁ receptors are co-localized with N-methyl-d-aspartate (NMDA) receptors in the postsynaptic density and that they co-immunoprecipitate with NMDA receptor subunits from postsynaptic density preparations. Using modified bioluminescence resonance energy transfer, we demonstrate that D₁ and NMDA receptor clustering reflects the existence of direct interactions. The tagged D₁ receptor and NR1 subunit cotransfected in COS-7 cells generated a significant bioluminescence resonance energy transfer signal that was insensitive to agonist stimulation and that did not change in the presence of the NR2B subunit, suggesting that the D₁ receptor constitutively and selectively interacts with the NR1 subunit of the NMDA channel. Oligomerization with the NR1 subunit substantially modified D₁ receptor trafficking. In intrinsically transfected HEK293 cells, NR1 was localized in the endoplasmic reticulum, whereas the D₁ receptor was targeted to the plasma membrane. In cotransfected cells, both the D₁ receptor and NR1 subunit were retained in cytoplasmic compartments. In the presence of the NR2B subunit, the NR1-D₁ receptor complex was translocated to the plasma membrane. These data suggest that D₁ and NMDA receptors are assembled within intracellular compartments as constitutive heteromeric complexes that are delivered to functional sites. Coexpression with NR1 and NR2B subunits also abolished agonist-induced D₁ receptor cytoplasmic sequestration, indicating that oligomerization with the NMDA receptor could represent a novel regulatory mechanism modulating D₁ receptor desensitization and cellular trafficking.

Dopaminergic fibers originating in the substantia nigra and cortical glutamatergic neurons extensively interact in the striatum to drive the physiological functions of this structure from motor planning to reward seeking and procedural learning (1, 2). The critical importance of dopamine in this system is such that the degeneration of nigral dopaminergic neurons leads to the motor and cognitive deficits of Parkinson’s disease (3).

At the cellular level, nigral and cortical fibers converge on the medium spiny projection neurons (4), where dopamine D₁- and D₂-like receptors are coexpressed to high degree with glutamate NMDA¹ and non-NMDA receptor channels (5–8). From a functional point of view, it is well established that dopamine modulates the firing pattern of these neurons. In particular, there is evidence that dopamine, while attenuating the responses mediated by non-NMDA receptors, potentiates those associated with activation of NMDA receptors (2). The D₁ receptor appears to be involved in these interactions. In fact, activation of D₁ receptors in medium spiny neurons enhances NMDA-induced whole cell currents (2, 9) and is a critical requirement for the formation of NMDA-mediated long-term potentiation at corticostriatal synapses (2, 10–12). Moreover, activation of NMDA receptors in striatal neurons triggers the translocation of cytoplasmic D₁ receptors to the plasma membrane and spines (13). Within neuronal spines, D₁ receptors are mainly localized in the spine shaft and, to a lesser extent, also in the spine head and in the postsynaptic density (PSD) (14–16). This cell structure is typical of the glutamatergic synapse and consists of a complex network of critical proteins involved in synaptic plasticity, many of which bind directly or indirectly to the NMDA receptor, which is an abundant component of the fraction (17, 18). The mechanisms that specifically drive D₁ receptor delivery to different spine domains are still unknown. The partial overlap in the subcellular distribution of NMDA and D₁ receptors and the observation that both D₁ and NMDA receptor delivery to synapses is dependent on glutamate translocation (13, 19) suggest that direct protein-protein interactions might direct the trafficking of these receptors to the same subcellular domain.

In this study, we report that the dopamine D₁ receptor forms a heteromeric complex with the NR1 subunit of the NMDA receptor in both purified striatal PSDs and cotransfected cells. This interaction is constitutive, occurs in the endoplasmic reticulum (ER), influences D₁ receptor targeting to the...
EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney cells (HEK293) were provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Tissue culture media and fetal bovine serum were obtained from Euroclone Celnbio (Milano, Italy). Dexamethasone, glutamate, 6-butaenal, SKF-81297, and the rat monoclonal anti-D1 receptor antibody (clone 1-1F11) were purchased from Sigma. Glycerine was obtained from Tocris (Avonmouth, UK). The rabbit anti-FDI antibody was raised against Streptomyces Biotech Corp. (Victoria, British Columbia, Canada). Cy3-labeled anti-rat and anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The anti-NR1 and anti-NR2A/B mouse monoclonal anti-D1 receptor antibodies were from Chemicon International (Temecula, CA). The horseradish peroxidase-conjugated anti-mouse antibody was purchased from DAKO (Milano), and the horseradish peroxidase-conjugated anti-rabbit antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Dr. Marc Caron (Duke University, Durham, NC) kindly provided D1 and D3 receptor cDNAs. Dr. Hannah Monyer (Heidelberg University) kindly provided the NR2B cDNA, and the NR1 cDNA was a gift of Dr. Shigetada Nakanishi (Kyoto University, Kyoto, Japan).

PSD and Triton-insoluble Fraction Preparation—Striatal PSD was isolated according to Carlin et al. (20) with minor modifications as described previously (21). Briefly, the tissue was homogenized in ice-cold 0.32 M sucrose containing 1 mM Hepes, 1 mM MgCl2, 1 mM NaHCO3, and 0.1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Complete, Roche Diagnostic, Milano) at pH 7.4 (buffer A) and centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 3000 × g for 15 min. The resulting pellet (containing mitochondria and synaptosomes) was resuspended in ice-cold 0.32 M sucrose containing 1 mM Hepes, 1 mM NaHCO3, and 0.1 mM phenylmethylsulfonyl fluoride (buffer B); overlaid on a sucrose gradient (0.85 to 1.0 to 1.2 M); and centrifuged at 82,500 × g for 2 h. The fraction between 1.0 and 1.2 M was diluted with buffer B containing 1% Triton X-100, stirred at 4 °C for 15 min, and centrifuged at 82,500 × g for 30 min. The resulting pellet was resuspended, layered on a sucrose gradient (1.0 to 1.5 to 2.1 M), and centrifuged at 100,000 × g for 2 h at 4 °C. The fraction between 1.5 and 2.1 M was removed and diluted with 150 mM KCl containing 1% Triton X-100. PSD were collected by centrifugation at 100,000 × g for 30 min at 4 °C.

To isolate the Triton-insoluble fraction (TIF), tissue was homogenized in ice-cold buffer A and centrifuged at 1000 × g for 10 min. The resulting supernatant was centrifuged at 3000 × g for 15 min, and the pellet was resuspended in 1 mM Hepes and centrifuged at 100,000 × g for 1 h. The pellet was resuspended in 70 mM KCl containing 1% Triton X-100, and TIF was collected by centrifugation at 100,000 × g for 1 h. TIF was characterized by enrichment in PSD proteins as previously described (22).

Immunoprecipitation and Western Blotting—Ten micrograms of PSD were incubated overnight at 4 °C with antibodies against either the NR1 subunit (1 μg/ml) or the D1 receptor (1.25 μg/ml; mouse monoclonal) in 200 mM NaCl, 10 mM EDTA, 10 mM NaHPO4, 0.5% Nonidet P-40, and 0.1% SDS (buffer C). Protein A-agarose beads (Santa Cruz Biotechnology) were added, and incubation was continued for 2 h at room temperature. The beads were washed and extensively washed with buffer C. The resulting proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted on for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% low fat dry milk. Membranes were incubated for 2 h at room temperature with the anti-NR1 (1 μg/ml) or anti-D1 receptor (1.25 μg/ml) antibodies. Detection was performed by chemiluminescence (ECL, Amersham Biosciences, Milano) with horseradish peroxidase-conjugated secondary antibodies (1:1500 dilution).

Cloning, Expression, and Purification of GST Fusion Proteins—The C-terminal regions of the D1 receptor (D1-CT, 321-446) and of the D3 receptor (D3-CT, 373-477) and two fragments of the NR1 subunit C terminus (NR1-CT, 834-930 and NR1-CT, 834-892) were generated by PCR amplification, cloned into the pGEX-KG plasmid, and expressed in E. coli competent cells. Synthesis of recombinant proteins was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) for 2–4 h. The bacteria were lysed, and the proteins were purified by incubation with glutathione-agarose beads (50% v/v) in PBS for 12 h at 4 °C as previously described (23).

Affinity Purification (“Pull-out”)—TIF proteins (35 μg) were diluted with PBS containing 0.1% SDS and incubated for 1 h at room temperature with glutathione-agarose beads saturated with GST fusion proteins. Beads were washed with PBS containing 0.1% Triton X-100, and bound proteins were released by SDS-PAGE and immunoblotted with anti-NR1 and anti-NR2A/B antibodies.

Generation of Biotinylated Resonance Energy Transfer (BRET) Fusion Constructs—The D1 receptor and NR1a subunit coding sequence were amplified out of their original vectors using sense and antisense primers containing unique XhoI and BamHI sites and HindIII and BamHI sites, respectively, and the native PstI DNA polymerase (Strategene, Milano) to generate stop codon-free fragments. The D1 receptor fragment was cloned in-frame into the Renilla luciferase-containing vector pBlue-N2/h (PerkinElmer Life Sciences, Milano) to generate the plasmid D1-Blue. The NR1a fragment was cloned into the pGFP-N2/h vector containing the green fluorescent protein (GFP) (PerkinElmer Life Sciences) to generate the plasmid NR1-GFP. The D1-Blue receptor was tested for its efficiency in activating adenyl cyclase in transfected COS-7 cells as previously described (24). The influence of GFP on glutamate-mediated Ca2+ influx in COS-7 cells cotransfected with NR1-GFP and NR2B was assessed by standard methods.

Cell Culture, Transfection, and BRET Assay—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Semiconfluent cells were cotransfected for 3 h with D1, NR1-GFP, and NR1a RNA, which was shown to give the best BRET signal, in the absence or presence of NR2B using the LipofectAMINE technique (Invitrogen, Milano). The total amount of DNA was kept at 10 μg. Forty-eight hours post-transfection, cells were harvested, centrifuged, and resuspended in PBS containing 0.1% CaCl2, 0.1% mg/ml MgCl2, and 1 mg/ml n-glucose. Approximately 50,000 cells/well were distributed in a 96-well microplate (white Optiplate, PerkinElmer Life Sciences) and incubated in the absence or presence of 50 μM dopamine, 100 μM glutamate, and 10 μM glycine for 30 min at 37 °C. DeepBlueTM coelenterazine (PerkinElmer Life Sciences) was added at a final concentration of 5 μM, and BRET signals were determined using a FusionTM universal microplate analyzer (PerkinElmer Life Sciences), which allows for the potent integrative signal discrimination detected at 390/400 and 505/510 nm. Untransfected cells and cells transfected with D1-Blue alone were used to define the nonspecific signals, and cells transfected with the pBlue-GFP control vector (PerkinElmer Life Sciences) were used as positive controls. The BRET signal was calculated as the difference in the ratio between emission at 510 and 395 nm of cotransfected RLuc and GFP fusion proteins and the ratio between emission at 510 and 395 nm of the RLuc fusion protein alone.

Immunofluorescence and Confocal Microscopy—HEK293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Semiconfluent cells were transfected with different combinations of D1 receptor, NR1-GFP, and NR2B cDNAs using LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours after transfection, cells were plated onto poly-L-lysine-coated cover slips, fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS containing 5% and normal goat serum for 10 min at room temperature. Cells were incubated overnight at 4 °C with either the rat monoclonal anti-D1 receptor antibody (1.600 dilution in PBS containing 1% normal goat serum) or the anti-FDI antibody (1.400 dilution in PBS containing 1% normal goat serum) and then for 45 min at room temperature with the Cy3-conjugated anti-goat secondary antibody (1.1000 dilution). The immunofluorescent cells were recorded with a Biophotoneconfocal microscope. Untransfected cells and omission of the primary antibodies were used as negative controls.

Sequestration Assay—HEK293 cells, which spontaneously express different G protein-coupled receptor kinases and arrestin (25), were transfected with the D1 receptor in the absence or presence of NR1 and each subunit using the LipofectAMINE 2000 reagent onto poly-L-lysine-coated glass coverslips, and allowed to recover for 1 day. Cells were incubated for 1 h at 37 °C in the absence of 10 μM SKF-81297 and processed as described above for confocal microscopy detection of the D1 receptor.

Membrane Preparation and [3H]SCH23390 Binding—Cells were rinsed, harvested, and centrifuged at 100 × g for 10 min. Cells were homogenized with a Polytron homogenizer in 5 mM Tris-HCl containing 2 mM EDTA and a mixture of protease inhibitors (pH 7.8) and centrifuged at 80 × g for 10 min to pellet unbroken cells and nuclei. The supernatant was centrifuged at 30,000 × g for 20 min at 4 °C. The
RESULTS

Dopamine D1 and Glutamate NMDA Receptors Are Co-clustered in Striatal PSD—Striatal PSD were isolated and analyzed for the presence of D1 receptors and other PSD-associated proteins. Fig. 1A shows the results from Western blot analysis performed with different tissue fractions with antibodies recognizing the D1 receptor, the NR1 subunit, α-Ca\(^{2+}\)/calmodulin-dependent protein kinase II (αCaMKII), and protein kinase Ce (PKCe). Five micrograms of proteins were loaded in each lane. A, co-immunoprecipitation of striatal PSD D1 receptors by the anti-NR1 antibody (lane 2) and of the NR1 subunit by the anti-D1 receptor antibody (lane 3), but not by an irrelevant (PKCe) antibody (lane 4) or by omission of a precipitating antibody (lane 5). Ten micrograms of PSD were used in each immunoprecipitation (IP). Data are representative of three independent experiments.

Pull-out experiments were then performed with GST fusion proteins containing the C-terminal domains of both the D1 receptor and NR1 subunit. Striatal TIF proteins were incubated with GST fusion proteins containing the D1 receptor C-terminal tail or, as a control, the D5 receptor C terminus. D1 and D5 receptors display, in fact, particular sequence divergence within the C-terminal domain, a region that might confer subtype-selective properties (29). As shown in Fig. 2A, a 116-kDa species, detected by the anti-NR1 antibody, was pulled out from striatal TIF by GST-D1-C(373–477) (lane 3) or GST alone (lane 2). By contrast, the NR2A/B subunits that were present in our TIF prepared from HEK293 cells expressing the D1 receptor fused to luciferase (D1-Luc), which was detected by the anti-D1 receptor antibody, was unable to bind the NR1 subunit, but not the D5 receptor C-terminal tail (lane 4), was able to bind the NR1 subunit, but not the NR2A/B subunits. B, fusion proteins of GST with two different fragments of the NR1 subunit C terminus (GST-NR1-CT-(834–930) and GST-NR1-CT-(834–930) were incubated with membranes obtained from HEK293 cells expressing the D1 receptor fused to luciferase (D1-Luc). After extensive washing, the glutathione-agarose beads containing the pulled out proteins were assayed for luciferase activity using DeepBlueC coelenterazine as a substrate. Both NR1 C-terminal fragments were able to bind the D1 receptor. Bars represent the means ± S.E. of three experiments. *p < 0.001 versus GST (Student’s t test). WB, Western blot; RLU, relative light units.

FIG. 1. Co-localization and association of D1 and NMDA receptors in striatal PSD. A, Western blot (WB) analysis carried out with the striatal homogenate (H), P1 fraction, purified membranes (P2), and PSD using antibodies recognizing the D1 receptor, the NR1 subunit, α-Ca\(^{2+}\)/calmodulin-dependent protein kinase II (αCaMKII), and protein kinase Ce (PKCe). Five micrograms of proteins were loaded in each lane. B, co-immunoprecipitation of striatal PSD D1 receptors by the anti-NR1 antibody (lane 2) and of the NR1 subunit by the anti-D1 receptor antibody (lane 3), but not by an irrelevant (PKCe) antibody (lane 4) or by omission of a precipitating antibody (lane 5). Ten micrograms of PSD were used in each immunoprecipitation (IP). Data are representative of three independent experiments.

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From the provided text:

**Fig. 3. Detection of D1 receptor and NR1 subunit interaction by BRET.** The D1 receptor fused to luciferase (D1-Rluc) and the NR1 subunit fused to GFP2 (NR1-GFP2) were transfected either individually or simultaneously in COS-7 cells. The DeepBlueC coelonerase substrate was added at a final concentration of 5 μM, and BRET2 signals were determined using the Fusion™ universal microplate analyzer. A, quantification of BRET2 data (means ± S.E., n = 5) from a series of control experiments with a single receptor construct (D1-Rluc, NR1-GFP2, and pRluc-GFP2) and of BRET2 data obtained when both D1-Rluc (donor) and NR1-GFP2 (acceptor) were coexpressed in the same cells (D1-Rluc/NR1-GFP2). B, D1-Rluc and NR1-GFP2 coexpressed in COS-7 cells generated a significant BRET2 signal in both the absence and presence of NR2B. Bars represent the means ± S.E. of five experiments. *p < 0.001 versus D1-Rluc. B, agonist stimulation does not affect the BRET2 signal generated by D1-Rluc and NR1-GFP2. Bars are the means ± S.E. of five experiments. DA, dopamine.

**Fig. 4. Confocal image of D1 receptor and NR1 subunit localization in HEK293 cells.** Cells were transfected with the D1 receptor cDNA and NR1-GFP2 individually or simultaneously either with or without the NR2B subunit cDNA. a, membrane localization of the D1 receptor revealed by immunostaining with the rat monoclonal anti-D1 receptor antibody and the Cy3-conjugated secondary antibody; b, nonspecific signal obtained with the anti-D1 receptor antibody in untransfected cells; c, nonspecific spontaneous green fluorescence of HEK293 cells; d, ER localization of NR1-GFP2 individually transfected in HEK293 cells; e, cells transfected with NR1-GFP2 immunostained with the anti-PDI antibody and revealed with the Cy3-conjugated secondary antibody; f, merge of NR1-GFP2 and PDI staining showing that the two proteins co-localized; g and h, intracellular localization of NR1-GFP2 and the D1 receptor, respectively, in HEK293 cells expressing both proteins; i, merge of D1 receptor and NR1-GFP2 staining showing that the majority of D1 receptor staining co-localized with NR1-GFP2 in intracellular compartments and only a fraction of the D1 receptor translocated to the membrane; j–n, the NR1 subunit and the D1 receptor co-localized at the plasma membrane of cells expressing also NR2B: j, NR1-GFP2 staining; m, D1 receptor immunostaining; n, merge of D1 receptor and NR1-GFP2 fluorescence, respectively.

BRET partners for signal detection. Coexpression of the tagged D1 receptor and NR1 subunit yielded a BRET2 ratio that was significantly higher than that observed with cells expressing D1-Rluc alone or with cells individually expressing D1-Rluc and NR1-GFP2 and mixed before analysis. The specificity of this interaction is illustrated by the absence of significant energy transfer between the D1-Rluc construct and the pGFP2-N2(h) vector (Fig. 3A). This BRET2 ratio was unchanged when the NR2B subunit of the NRMDA receptor was also expressed, suggesting that there is no competition between NR1 and NR2B for interaction with the D1 receptor. Moreover as shown in Fig. 3B, the BRET2 signal recorded in cells cotransfected with D1-Rluc, NR1-GFP2, and NR2B was insensitive to stimulation by 50 μM dopamine with or without 100 μM glutamate and 10 μM glycine. These data demonstrate a physical proximity between D1-Rluc and NR1-GFP2 that can be explained best by the formation of constitutive protein dimers.

**Oligomerization with the NRMDA Receptor Regulates D1 Receptor Targeting to the Plasma Membrane.** To identify the cellular compartment in which the D1 receptor and NR1 subunit are assembled, HEK293 cells transfected with the D1 receptor and NR1-GFP2 construct, either individually or simultaneously, were analyzed by confocal microscopy. As shown in Fig. 4a, the D1 receptor expressed in HEK293 cells was completely targeted to the plasma membrane. By contrast, as previously reported (19, 30), when expressed alone, the NR1 subunit accumulated in the perinuclear region and in cytoplasmic compartments with a reticular staining pattern (Fig. 4d) that was identified as the ER using an
antibody to PDI, a specific marker for this structure (Fig. 4c).

Virtually all the intracellular NR1 staining was in fact colocalized with PDI (Fig. 4f). When the D1 receptor and NR1 subunit were coexpressed in the same cells, the D1 receptor was only partially targeted to the cell membrane (Fig. 4, h and i), with the majority of D1 receptor staining retained in cytoplasmic structures (Fig. 4h), where it was co-localized with NR1 (Fig. 4, g and i). Coexpression of the D1 receptor with both the NR1 and NR2B subunits relieved the cytoplasmic retention of the complex, allowing insertion of both the NR1 subunit (Fig. 4l) and D1 receptor (Fig. 4m) at the plasma membrane, where they were completely co-localized (Fig. 4n). These data suggest that D1 and NMDA receptors are assembled as oligomeric units in the ER and transported to the cell surface as a preformed complex.

Oligomerization with the NMDA Receptor Abolishes Agonist-mediated D1 Receptor Sequestration—A common adaptive response of G protein-coupled receptors to agonist stimulation is redistribution from the plasma membrane to cytosolic compartments. Using confocal microscopy and receptor binding in transfected HEK293 cells, which spontaneously express different G protein-coupled receptor kinases and β-arrestin (25), we investigated whether interaction with NMDA receptors alters D1 receptor sequestration induced by agonist administration (26, 31). As shown in Fig. 5A, in unstimulated cells, the fluorescence distribution of the D1 receptor was exclusively localized at the plasma membrane (panel a). Exposure to 10 μM SKF-81297 for 1 h resulted in D1 receptor sequestration into cytosolic compartments, as shown by the D1 receptor fluorescence that was detectable also in the cytoplasm with a punctate appearance (panel b). In contrast, when the D1 receptor was coexpressed with NR1 and NR2B subunits, SKF-81297 failed to induce D1 receptor internalization. Under these conditions, D1 receptor immunofluorescence was in fact retained at the plasma membrane (panel c). Similar results were obtained by [3H]SCH23390 binding in the purified heavy membrane fraction. As shown in Fig. 5 (B and C), pretreatment with 10 μM SKF-81297 resulted in a 20-28% reduction of cell-surface [3H]SCH23390 binding in HEK293 cells expressing only the D1 receptor. On the other hand, exposure to 10 μM SKF-81297 did not modify cell-surface [3H]SCH23390 binding in cells expressing both the D1 receptor and NR1 and NR2B subunits (Fig. 5, B and C). The dose-response curve and the time course of SKF-81297-induced D1 receptor sequestration in HEK293 cells expressing the D1 receptor either alone or in combination with NR1 and NR2B subunits are shown in Fig. 6. The SKF-81297-induced decrease in membrane [3H]SCH23390 binding was dose-dependent, with an EC50 of 80 ± 2 nM in cells expressing the D1 receptor, but not in those coexpressing also the NR1 and NR2B subunits (Fig. 6A). Moreover, in cells expressing only the D1 receptor, SKF-81297-induced receptor internalization was detectable after 10 min of incubation and reached a maximum within 30 min (Fig. 6B). By contrast, in cells coexpressing the D1 receptor and the NR1 and NR2B subunits, no decrease in membrane [3H]SCH23390 binding was detectable at any time tested. Increasing SKF-81297 incubation to 2 h did not modify [3H]SCH23390 binding as well (data not shown). Taken together, these data suggest that interaction with the NMDA receptor immobilizes the D1 receptor at the plasma membrane, impairing the mechanisms of the receptor plasticity that normally occurs as an adaptive response to agonist stimulation.

**DISCUSSION**

In this study, we have shown that, in striatal neurons and in transfected cells, the dopamine D1 receptor directly and selectively interacts with the NR1 subunit of the NMDA receptor to form a constitutive oligomeric complex that is recruited to the plasma membrane by the NR2B subunit. In medium spiny neurons, a direct protein-protein interaction with the NMDA receptor is thus one of the mechanisms directing the trafficking of D1 receptors to specific subcellular compartments. Furthermore, we have shown that this interaction abolishes D1 receptor internalization, a crucial adaptive response that normally
occurs upon agonist stimulation (26, 31, 32).

Using classical biochemical approaches, we have clearly shown that the D1 receptor is concentrated in purified striatal PSD, displaying a subcellular distribution that is consistent with the reported localization of NMDA receptors (17, 18). In addition, the D1 receptor was co-immunoprecipitated from striatal PSD with NMDA receptor subunits, suggesting that these proteins are co-clustered in this structure. Recently, energy transfer approaches such as fluorescence resonance energy transfer and BRET have been developed as the systems of choice to study protein-protein interactions (33). These techniques have the advantage of monitoring protein oligomerization in living cells without disrupting the natural environment where they are clustered, thus eliminating the possibility of artifactual aggregation that could happen during the solubilization and concentration of membrane proteins. Using BRET2, we have demonstrated that D1 and NMDA receptor clustering reflects the existence of direct protein-protein binding. In fact, the tagged D1 receptor and NR1 subunit generated a significant and specific BRET2 signal for energy transfer when co- transfected in COS-7 cells. This signal did not change when the NR2B subunit was also expressed in the same cells, suggesting that this subunit does not compete with the NR1 subunit for binding to the D1 receptor. In addition, the association of the NR1 subunit with the D1 receptor was insensitive to agonist stimulation. Taken together, these observations point to a constitutive, direct, and selective interaction of the D1 receptor with the NR1 subunit of the NMDA channel. Using specific GST fusion proteins, we have also shown that the interaction between D1 and NMDA receptors involves the binding of the D1 receptor C-terminal tail to the C-terminal sequence of the NR1α/b and NR1e/f isoforms, with no contribution from NR2 subunits. The NR1 subunit, the essential component of the NMDA receptor, gives rise to eight splice variants, with four possible C termini (34, 35). These isoforms differ in their physiological and pharmacological properties and show different regional and cellular distribution (34, 36). Our present data point to the capability of interacting with the D1 receptor as a further difference among these isoforms and suggest that the interaction between D1 and NMDA receptors might be a specific feature of certain neuronal populations. In line with our findings, it was reported, while this manuscript was in preparation, that D1 and NMDA receptors directly interact in the hippocampus (37). In particular, in this brain area, the D1 receptor apparently associates with both the NR1 and NR2A subunits, but not with the NR2B subunit. Our observation that the D1 receptor does not interact with NR2 subunits in striatal PSD may reflect the fact that NR2B is the prevalent species in this structure (36).

Oligomerization may play important roles in receptor trafficking and/or signaling. In several cases, receptors appear to fold as constitutive dimers early after biosynthesis, whereas ligand-promoted dimerization at the cell surface has been proposed for others (33). Our data obtained by BRET showing that the D1 receptor and NR1 subunit interact in the absence of the NR2B subunit and in an agonist-independent way suggest that this interaction is constitutive. The results obtained by confocal microscopy give support to this concept and indicate that the trafficking properties of the D1 receptor are substantially modified by heteromerization with the NMDA receptor. When the NR1 subunit and D1 receptor were individually transfected in HEK293 cells, NR1 was retained in the ER, whereas the D1 receptor was targeted to the plasma membrane. In cotransfected cells, both the D1 receptor and NR1 subunit were colocalized in cytoplasmic compartments, suggesting that interaction with NR1 blocks D1 receptor delivery to the plasma membrane. In the presence of the NR2B subunit, however, the NR1-D1 receptor complex was completely translocated to the plasma membrane. These observations are consistent with previous data showing that, when expressed alone in both heterologous cells and cultured hippocampal neurons, the NR1 subunit accumulates in the ER (19, 30) due to the presence of an ER retention motif in the alternatively spliced C1 domain in its C terminus (38) and that coexpression of NR2 subunits is necessary to drive the complex to the cell membrane (19, 30).

Taken together, these data suggest that, in striatal medium spiny neurons, D1 and NMDA receptors are assembled within intracellular compartments as constitutive heteromeric complexes that are delivered to functional sites. Interaction with the NMDA receptor thus represents a critical mechanism to recruit the D1 receptor to the PSD. The postsynaptic specialization of corticostriatal glutamatergic synapses finely regulates the strength of synaptic transmission, thus determining the activity of medium spiny neurons. Several lines of evidence suggest that the efficacy of corticostriatal transmission is highly dependent on the concurrent activation of D1 and NMDA receptors. In particular, it has been shown that NMDA currents are potentiated by activation of D1 receptors, which is also an essential requirement for long-term potentiation generation (2, 9–12). In this context, the direct interaction between D1 and NMDA receptors may be crucial to recruit the D1 receptor in the place of synaptic plasticity and to keep it in close proximity with the NMDA receptor to allow rapid AMPA-protein kinase A/DARPP32-mediated potentiation of NMDA transmission (39–42).

The interaction of the D1 receptor with the NR1 subunit does not reflect simply a chaperon-like strategy to deliver the D1 receptor to the PSD, but also implies regulation of D1 receptor function by interfering with the mechanisms of receptor plasticity. A common adaptive response of G protein-coupled receptors to agonist stimulation is desensitization involving both G protein-coupled receptor kinase-mediated phosphorylation and arrestin binding and internalization (43). In line with this paradigm and with in vitro studies (26, 31), there is morphological evidence that, in striatal medium spiny neurons, extrasynaptic D1 receptors, localized in cell bodies and dendrites, respond to agonist administration by massive internalization (32). We have shown here that association with the NMDA receptor abolishes agonist-induced D1 receptor cytoplasmic sequestration, indicating that oligomerization with NMDA receptors could represent a novel regulatory mechanism modulating D1 receptor function. Taken together, these observations suggest that, within a single neuron, D1 receptor plasticity may be subjected to different regulatory mechanisms in different neuronal microdomains. In particular, agonist stimulation would induce D1 receptor sequestration in all neuronal compartments except the PSD, where this receptor is immobilized at the plasma membrane by association with the NMDA receptor. Along this line, Dumartin et al. (32) have reported that, in striatal medium spiny neurons, the localization of the perisynaptic D1 receptor in dendritic spines is apparently unmodified by agonist treatment. It is well known that agonist-induced internalization dynamically calibrates receptor availability for extracellular ligands. Disruption of D1 receptor cytoplasmic sequestration in response to agonist stimulation due to heteromerization with the NMDA receptor might represent a neuronal mechanism to preserve the optimal synaptic strength at corticostriatal synapses in the presence of alterations in the dopamine environment as occurs, for instance, during drug administration. In conclusion, our present data suggesting that, in striatal medium spiny neurons, D1 and NMDA receptors are assembled within intracellular compartments and re-
cruciated to the PSD as a constitutive heteromeric complex may provide a new rationale for a better understanding of the mechanisms that control corticostratal synaptic transmission under both physiological and pathological conditions.

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Regulation of Dopamine D₁ Receptor Trafficking and Desensitization by 
Oligomerization with Glutamate N-Methyl-D-aspartate Receptors
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