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Loss of Synaptic D1 Dopamine/*N*-Methyl-D-aspartate Glutamate Receptor Complexes in L-DOPA-Induced Dyskinesia in the Rat

Chiara Fiorentini, Maria Cristina Rizzetti, Chiara Busi, Sandra Bontempi, Ginetta Collo, PierFranco Spano, and Cristina Missale

Division of Pharmacology, Department of Biomedical Sciences and Biotechnology and Centre of Excellence on Diagnostic and Therapeutic Innovation, University of Brescia, Brescia, Italy (C.F., M.C.R., C.B., S.B., G.C., P.F.S., C.M.); and Istituto di Ricovero e Cura a Carattere Scientifico, Venezia, Italy (P.F.S., C.M.)

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ABSTRACT

Glutamate-mediated mechanisms are related to the motor complications of L-DOPA therapy in Parkinson's disease (PD). In striatal postsynaptic densities (PSD), the dopamine D1 receptor (D1R) is part of an oligomeric complex with the glutamate *N*-methyl-D-aspartate receptor (NMDAR), determining the strength of corticostriatal transmission. We studied D1R/NMDAR complex alterations induced by L-DOPA in the 6-hydroxydopamine-lesioned rat model of PD. L-DOPA-treated hemiparkinsonian rats were determined to be dyskinetic or nondyskinetic based on behavioral testing. D1R/NMDAR assemblies containing NR1-C2 and NR2B subunits were decreased in the PSD of lesioned striatum. Short-term L-DOPA administration improved akinesia and restored the synaptic abundance of D1R, NR1-C2 and NR2B. Prolonged L-DOPA treatment also normalized synaptic D1R/NMDAR complexes in nondyskinetic rats, but remarkably reduced them in the dyskinetic group without changing their interaction. This decrease involved NR1-C2, NR1-C2′, NR2A, and NR2B subunits. The composition of residual synaptic D1R/NMDAR complexes in dyskinetic rats may thus be different from that observed in lesioned rats, suggesting that expression of different motor dysfunctions might be related to the receptor profile at corticostriatal synapses. The levels of D1R/NMDAR complexes were unchanged in total striatal membrane proteins, suggesting that the decrease of these species in the PSD is likely to reflect an altered receptor trafficking. In human embryonic kidney 293 cells expressing the D1R/NMDAR, complex costimulation of both D1R and NMDAR, but not individual receptor activation, promoted internalization, suggesting that development of dyskinesias might be related to agonist-mediated down-regulation of the D1R/NMDAR complex at corticostriatal synapses.

The striatum is a key area of the basal ganglia controlling motor function (Nicola et al., 2000; Olanow et al., 2000). The activity of this nucleus is intimately linked to its massive dopaminergic innervation originating in the substantia nigra. In addition to dopamine (DA), the striatum receives a major glutamatergic innervation from the cortex conveying sensorimotor information. This corticostriatal pathway converges with DA fibers on dendritic spines of striatal medium spiny neurons, and there is general agreement that an integrated interplay between DA and glutamate inputs is essential to drive correct motor behavior processing.

Parkinson's disease (PD) is a neurological disorder that is caused by the degeneration of nigral dopaminergic neurons and the consequent massive drop of DA content in the striatum (Obeso et al., 2000). The most effective therapy to alleviate the main motor symptoms of this disease is treatment with the DA precursor L-DOPA, which increases DA availability (Obeso et al., 2000). However, long-term L-DOPA administration is associated with the development of involuntary movements, known as dyskinesias, that represent the most debilitating complication in the vast majority of pa-

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ABBREVIATIONS: DA, dopamine; PD, Parkinson's disease; LID, L-DOPA-induced dyskinesias; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor; PSD, postsynaptic density; D1R, dopamine D1 receptor; 6-OHDA, 6-hydroxydopamine; AIM, abnormal involuntary movement; ANOVA, analysis of variance; TIF, triton-insoluble fraction; HEK, human embryonic kidney; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; GFP, green fluorescent protein; SKF 81297, (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide.

tients (Obeso et al., 2000; Bezard et al., 2001). Despite the clinical importance of these side effects, little is known about their causes (Bezard et al., 2001). Although alterations of DA receptor function have been suggested as the most plausible mechanism of L-DOPA-induced dyskinesias (LID) (Bezard et al., 2001), increasing evidence supports the idea that glutamate-mediated mechanisms may also contribute to the development of these complications (Chase and Oh, 2000; Robelet et al., 2004; Brotchie, 2005). Furthermore, increased glutamate release has been described in hemiparkinsonian rats undergoing long-term treatment with L-DOPA (Robelet et al., 2004). Moreover, the observations that the glutamate NMDA receptor (NMDAR) is altered in experimental parkinsonism (Chase and Oh, 2000; Dunah et al., 2000; Bezard et al., 2001) and that LID may be ameliorated by NMDAR antagonists (Papa and Chase, 1996; Chase and Oh, 2000; Lundblad et al., 2002; Brotchie, 2005) point to this receptor subtype as a major player in the development of motor dysfunctions.

NMDARs are heteromeric complexes formed by two major families of subunits, NR1 and NR2A-NR2D (Dingledine et al., 1999). At glutamatergic synapses, NMDAR are concentrated in the postsynaptic density (PSD), where they interact with specific scaffolding and signaling proteins to form a dynamic complex that is critical to determine the strength of synaptic transmission (Kennedy, 2000). In striatal PSD, this complex also includes the DA D1 receptor (D1R). In particular, the D1R directly binds to the NR1 subunit of the NMDAR, and this interaction strongly influences the trafficking, signaling and desensitization of both interacting receptors (Lee et al., 2002; Fiorentini et al., 2003). Because the concurrent activation of both D1R and NMDAR is crucial to determine the efficacy of corticostriatal transmission (Nicola et al., 2000), it is possible that alterations involving the D1R/NMDAR complex in the PSD could contribute to the generation of motor dysfunctions.

The most common rodent model of PD is the rat with unilateral lesion of the nigrostriatal DA pathway obtained by 6-hydroxydopamine (6-OHDA). It is noteworthy that the gradual development of abnormal involuntary movements has been observed in approximately 50% of hemiparkinsonian rats undergoing long-term treatment with low doses of L-DOPA (Cenci et al., 1998; Lundblad et al., 2002). By using this paradigm, an aberrant form of striatal synaptic plasticity has been detected in dyskinetic rats, but not in nondyskinetic animals (Picconi et al., 2003), suggesting that this experimental model may be useful to distinguish molecular changes involved in the therapeutic effects of L-DOPA from those related to the development of dyskinesias. The aim of this study was thus to define the expression and regulation of synaptic D1R/NMDAR complexes in hemiparkinsonian rats treated with L-DOPA.

Materials and Methods

6-Hydroxydopamine Lesion. Experiments were performed according to the European Community Council Directive, November 1986 (86/609/EEC). Eighty male Wistar rats (290–300 g) were deeply anesthetized with chloral hydrate (400 mg/kg; Sigma-Aldrich, Milan, Italy), mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA) and injected with 12 μg of 6-OHDA (Sigma-Aldrich), dissolved in 4 μl of saline containing 0.2% ascorbic acid, at the rate of 0.38 μl/min into the left medial forebrain bundle (from bregma: anteroposterior, −3.6; lateral, 1.9; dorsoventral, −8.8; tooth bar, −3.3) according to the atlas of Paxinos and Watson (1986). Sham-operated rats (n = 15) were injected with vehicle. Two weeks after surgery, rats were tested for contralateral turning behavior induced by a low dose of apomorphine (0.05 mg/kg i.p.; Sigma-Aldrich). Only rats showing more than 200 turns contralateral to the lesion in 40 min were included in the study (n = 73). In previous experiments, we found that, in agreement with Papa et al. (1994), this rotational score corresponds in fact to a greater than 95% depletion of striatal dopamine.

L-DOPA Treatment and Behavioral Testing. Three weeks after lesion, rats were treated with either saline (n = 20) or L-DOPA (10 mg/kg i.p.; Sigma-Aldrich) plus benserazide (7.5 mg/kg; Sigma-Aldrich) for 5 days (n = 10) or 21 days (n = 43). This dose was chosen on the basis of preliminary results obtained with different doses of L-DOPA (6.5, 10, and 20 mg/kg) and according to previous studies showing a gradual development of abnormal involuntary movements (AIMs) in one group of rats during a 21-day treatment with low doses of this drug (Cenci et al., 1998; Lundblad et al., 2002; Picconi et al., 2003). Twice per week, limb-use asymmetry was evaluated, as an index of akinesia, with the cylinder test (Schallert et al., 2000). In brief, 45 min after L-DOPA administration, each rat was introduced into a Plexiglas cylinder and recorded for 5 min. The number of supporting wall contacts executed independently with the right or the left forelimb was counted. The difference between the percentage of wall contacts executed by the impaired and the normal forelimbs was calculated to obtain a limb-use asymmetry score. L-DOPA-induced AIMs were recorded in individual animals twice a week and, according to Cenci et al. (1998), were classified as axial (i.e., twisted posturing of the neck and the upper body contralateral to the lesion), limb (i.e., repetitive purposeless movements of the contralateral forelimb), orolingual (i.e., empty jaw movements and contralateral tongue protrusion), or locomotor (i.e., increased locomotion with contralateral turning). Each of these symptoms was scored on a validated severity scale (Cenci et al., 1998; Lundblad et al., 2002) from 0 to 4 as follows: 0 = absent; 1 = mild (present during less than half of the observation time); 2 = moderate (present during more than half of the observation time and not interfering with the normal exploratory activity); 3 = marked (present all the time and interfering with the normal exploratory activity but suppressible by threatening stimuli); 4 = severe (replacing the normal activity and not suppressible). In particular, each animal was recorded for 1 min every 20 min at 20 to 120 min after L-DOPA administration, for a total of six observations, and the AIM score represents the sum of these observations. The theoretical maximum score that can be accumulated by one animal in one testing session was 96 (maximum score per observation point = 16; number of observation points per session = 6). However, because the interpretation of locomotor dyskinesia is still controversial (Papa et al., 1994; Cenci et al., 1998), this motor response was removed from the analysis. Thus, in our experimental protocol, the maximum score that can be accumulated by one animal in each testing session was 72 (maximum score per observation point = 12; number of observation points per session = 6). Statistical significance of the data was determined by repeated-measure analysis of variance (ANOVA). Animals were killed after either 5 days (n = 10) or 21 days (n = 43) of L-DOPA treatment, 24 h after the last L-DOPA injection. The striata were rapidly dissected and stored at −80°C.

Preparation of Total Striatal Membrane Proteins and Triton-Insoluble Fraction. To prepare the total membrane protein fraction, striata from control (n = 5), lesioned (n = 5), nondyskinetic (n = 5), and dyskinetic (n = 5) rats were homogenized in ice-cold Tris-EDTA buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4) and centrifuged at 700g at 4°C. The supernatant was centrifuged at 10,000g at 4°C for 30 min, and the pellet was suspended in 10 mM Tris-HCl, 1 mM EDTA, and 1 mM EGTA, pH 7.4, and solubilized with 0.5% Nonidet P-40 and 0.1% SDS.

To isolate the Triton-insoluble fraction (TIF), which is enriched in PSD, the striatum from control (n = 10), lesioned (n = 15), nondys-

kinetic (n = 12), and dyskinetic (n = 21) rats was homogenized in ice-cold 0.32 M sucrose containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.1 mM phenylmethylsulfonyl fluoride, and a complete set of protease inhibitors (Roche, Milan, Italy), pH 7.4, and centrifuged at 1000*g* for 10 min. The supernatant was centrifuged at 3000*g* for 15 min, and the resulting pellet containing mitochondria and synaptosomes was resuspended in 1 mM HEPES and centrifuged at 100,000*g* for 1 h. The pellet was resuspended in 75 mM KCl containing 1% Triton X-100, and the TIF was collected by centrifugation at 100,000*g* for 1 h. The TIF was characterized by the enrichment in PSD proteins as described previously (Fiorentini et al., 2003). This fraction was used instead of purified PSD to limit the number of animals in each group. For protein quantification, the TIF was solubilized in 1% SDS. Protein concentration in both the TIF and total membrane proteins was measured according to a modified, detergent-compatible Lowry method (Bio-Rad, Hercules, CA).

Coimmunoprecipitation and Western Blot. Coimmunoprecipitation of both TIF and total membrane proteins was performed under nondenaturing conditions. TIF was solubilized in buffer A containing 200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP-40, 0.1% SDS for 1h at 4°C. These detergent concentrations are sufficient to solubilize the TIF proteins without dissociating the NMDA channel and its associated proteins (Apperson et al., 1996; Yu et al., 1997; Gardoni et al., 1998). Protein concentration in each sample was checked as described above. To further ensure the use of equal protein amounts in the immunoprecipitation experiments, aliquots of these proteins were also checked by Western blot for β-tubulin content. In the immunoprecipitation experiments, aliquots of either total membrane proteins (100 μg) or solubilized TIF (25 μg) were incubated overnight at 4°C in buffer A containing the anti-D1R antibody (Chemicon International, Temecula, CA). Protein-A agarose beads were added, and incubation was continued for 2 h at room temperature. Beads were collected and extensively washed with buffer A, and the resulting proteins were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and blotted for 1 h at room temperature in TBS containing 0.1% Tween 20 and 5% nonfat powdered milk. Membranes were incubated for 2 h at room temperature with the anti-NR1-C2 or the anti-NR1-C2′ antibody (1:500 dilution; Chemicon). Detection was performed by chemiluminescence with horseradish peroxidase-conjugated secondary antibodies (Chemicon). In Western blot experiments, 40 μg of total proteins or 20 μg of TIF were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and blotted as described previously. Membranes were incubated for 2 h at room temperature with anti-NR1-C2, anti-NR1-C2′ (1:500), anti-NR2A (1:500; Chemicon), anti-NR2B (1:500; Chemicon) and anti-D1R (1:250; Chemicon) antibodies. To ensure equal protein loading, membranes were stripped with a Western blot recycling kit (Chemicon) and reprobed with anti-β-tubulin (1:2500; Sigma) antibody. Detection was performed by chemiluminescence. Blots were analyzed by densitometry and statistical significance was evaluated by the Student's t test against sham-operated control rats.

Cell Culture and Transfection. HEK293 cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with the D1R cDNA using the LipofectAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen, Milan, Italy). Cell clones stably expressing D1R (HEK-D1R) were isolated by Zeocin (100 μg/ml) selection and characterized for receptor levels in binding studies with [^{3}H]SCH23390 according to Fiorentini et al. (2003). HEK-D1R or wild-type HEK293 cells were transiently transfected with green fluorescent protein (GFP)-conjugated NR1 cDNA (NR1-GFP), NR2B cDNA, and PSD95 cDNA, to stabilize the NMDA complex at the membrane (Roche et al., 2001), and tested in the sequestration assay 48 h after transfection.

Sequestration Assay and Immunofluorescence. Cells expressing the D1R and the NMDAR, either alone or in combination, were plated onto poly-L-lysine-coated coverslips and allowed to recover for 1 day. Cells were incubated for 1 h at 37°C in the absence or in the presence of 10 μM SKF 81297, 100 μM glutamate/10 μM glycine, or both. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 5% bovine serum albumin and 5% normal goat serum for 10 min at room temperature, and incubated overnight at 4°C with the monoclonal anti-D1R antibody (1:700; Sigma) and then for 45 min at room temperature with Cy3-conjugated anti-goat secondary antibody (1:800; Jackson ImmunoResearch Laboratories, West Grove, PA). Immunolabeled cells were recorded with a fluorescence microscope (IX51; Olympus, Tokyo, Japan) at a 100× magnification. Nontransfected cells and omission of the primary antibody were used as negative controls.

Results

Long-Term L-DOPA Administration Induces Dyskinesias in 6-OHDA-Lesioned Rats. In preliminary experiments, 6-OHDA-lesioned rats were treated with different doses of L-DOPA for 21 days (6.5 mg/kg; n = 10; 10 mg/kg, n = 15; 20 mg/kg, n = 15) and tested for the development of AIMs. L-DOPA, given at a dose of 6.5 mg/kg, did not induce the appearance of severe AIMs but, at a dose of 20 mg/kg, produced dyskinesias in approximately 70 to 80% of rats. At a dose of 10 mg/kg, L-DOPA induced the development of AIMs in approximately 50 to 60% of animals. This dose was thus used in subsequent experiments. 6-OHDA-lesioned rats were treated with either saline (n = 20) or L-DOPA (10 mg/kg) plus benserazide (7.5 mg/kg) (n = 53) and individually tested for spontaneous motor behavior in the cylinder test and for the development of dyskinesias according to Cenci et al. (1998). As reported in Fig. 1A, all 6-OHDA-lesioned rats (n = 73) showed a high degree of forelimb use asymmetry in the cylinder test, suggesting that they developed severe akinesia of the parkinsonian forelimb. After 5 days of L-DOPA admin-

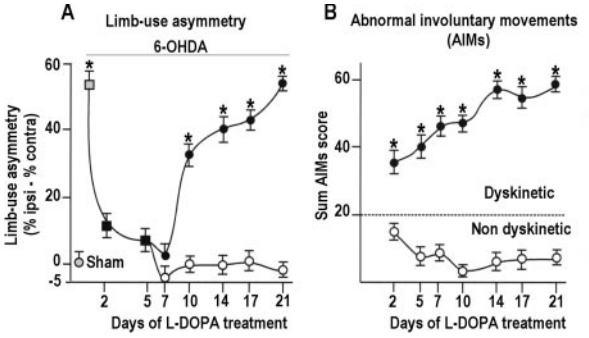

Fig. 1. Behavioral characterization of 6-OHDA-lesioned rats treated with L-DOPA. A, evaluation of limb-use asymmetry in the cylinder test. The number of supporting wall contacts executed independently with the right or the left forelimb was counted. The limb-use asymmetry score was calculated as the difference between the percentage of wall contacts executed by the impaired and the normal forelimbs. All 6-OHDA-lesioned rats (n = 73) showed a high degree of forelimb-use asymmetry (▨). Short-term L-DOPA administration improved akinesia in all treated animals (n = 53; ■). During long-term administration, one group of rats was still improved by L-DOPA (n = 17; ○), whereas a disabling effect of this drug, occurring as increased limb-use asymmetry, was detectable in the other group (n = 26; ●). B, L-DOPA-induced AIMs were recorded as described under *Materials and Methods*. Rats that during long-term treatment lost the antiakinetic effect of L-DOPA showed severe AIMs (n = 26; filled symbols, dyskinetic); rats that maintained the therapeutic effect of L-DOPA did not develop AIMs (n = 17; open symbols, nondyskinetic). Data are the means ± S.E.M. A, $*p < 0.001$ versus sham, ANOVA followed by Westfall test; n >10 in each group; B, $p < 0.001$ versus nondyskinetic, ANOVA followed by Westfall test; n >10 in each group.

istration, akinesia was significantly improved in all animals in the absence of dyskinesias (only 5 rats of 53 developed a mild dyskinesia), suggesting that this treatment restored the normal, spontaneous motor behavior. One group of rats (n = 10) was killed after a 5-day L-DOPA therapy. During prolonged L-DOPA treatment, the improvement of akinesia was maintained in one group of rats (n = 17), but was gradually lost in another group of animals (n = 26). Moreover, rats that were improved in the cylinder test did not develop AIMs during prolonged L-DOPA administration, although rats that lost the antiakinetic effect of L-DOPA during long-term treatment also developed AIMs (Fig. 1B). Administration of L-DOPA to unlesioned control rats did not induce the development of AIMs (data not shown). Two groups of rats were thus distinguished with this protocol: those showing a locomotor improvement in response to L-DOPA without dyskinesias (here referred to as nondyskinetic rats) and those that, after a short period of remission, lost the benefit of L-DOPA because of the development of severe dyskinesias (here referred to as dyskinetic rats).

Loss of Synaptic D1/NMDA Receptor Complex in Dyskinetic Rats. Within striatal neurons, the D1R is widely distributed in cell bodies, dendritic shafts, and dendritic spines (Missale et al., 1998), where it is localized also in the PSD of corticostriatal synapses as part of an oligomeric complex with the NMDAR (Fiorentini et al., 2003). To measure the fraction of synaptic D1R associated with the NMDA channel in denervated, nondyskinetic and dyskinetic rats, we thus used a PSD-enriched fraction (TIF) instead of a traditional membrane preparation. This fraction, which is rather insoluble in nonionic detergents (Kennedy, 2000), was dissolved by using a combination of 0.5% Nonidet P-40 and 0.1% SDS. These nondenaturing detergent concentrations efficiently solubilize TIF proteins without dissociating the NMDA channel complexes (Apperson et al., 1986; Yu et al., 1997; Gardoni et al., 1998). According to Fiorentini et al. (2003), the purity of our TIF preparation was confirmed by the enrichment in PSD proteins and by the absence of presynaptic markers (data not shown).

The NR1 subunit of the NMDAR consists of eight splice variants of a single gene (Zukin and Bennett, 1995). Because NMDAR interacts with D1R through its NR1 C-terminal region, the abundance of NR1-C2 and NR1-C2′ splice variants as well as of NR2A/B subunits in striatal TIF was determined by Western blot. Representative blots are reported in Fig. 2, A and C, and the densitometric analysis of three independent experiments with the specific signals normalized to the corresponding β-tubulin staining is reported in Fig. 2, B and D. As reported in Fig. 2, A and B, levels of NR1-C2, but not NR1-C2′, were remarkably decreased in the TIF from denervated rats. Analysis of NR2A and NR2B subunit expression in striatal TIF showed that NR2B content was significantly decreased in lesioned rats, whereas NR2A levels were unchanged (Fig. 2, C and D). A 5-day L-DOPA treatment that improved akinesia, restored NR1-C2 and NR2B to the physiological levels (Fig. 2, A–D). Prolonged L-DOPA therapy (21 days), on the other hand, induced a dramatic decrease of both NR1-C2 and NR1-C2′ and NR2A and NR2B subunits in dyskinetic rats (AIMs rating score = 3–4), leaving them unchanged in the nondyskinetic group (Fig. 2, A–D). No differences in NMDAR subunit levels were found in striatal TIF contralateral to the lesion. Because NMDAR and D1R are present in striatal PSD as an oligomeric complex (Fiorentini et al., 2003), we investigated whether 6-OHDA lesion and L-DOPA treatment modify D1R content and interaction with NMDAR. As shown in the representative Western blot reported in Fig. 3A and in the densitometric analysis of four independent blots normalized to the corresponding β-tubulin levels reported in Fig. 3B, 6-OHDA lesion reduced D1R levels in striatal TIF, a defect that was reversed by short-term L-DOPA administration. During prolonged L-DOPA treatment D1R content in striatal TIF was maintained at the control levels in nondyskinetic rats, but was significantly decreased in dyskinetic animals. Moreover, as shown in Fig. 4, A and B, L-DOPA administration to unlesioned control rats, which did not produce AIMs, did not change the abundance of D1R and NMDAR subunits in striatal TIF. The density of D1R and NMDAR subunits was also determined in total membrane proteins. The results are shown in the representative Western blot reported in Fig. 5A and in the densitometric analysis of three independent experiments reported in Fig. 5B. The abundance of all measured subunits of the NMDAR was similar in total membrane protein preparations from control, lesioned, nondyskinetic, and dyskinetic rats. Likewise, neither the lesion nor L-DOPA treatment modified D1R density in this tissue fraction. Taken together, these data thus suggest that the decrease of these species in the TIF fraction probably reflects an altered receptor trafficking.

We also investigated whether changes of DA transmission induced by denervation or L-DOPA treatment may influence the interaction between D1R and NMDAR. Because the formation of the D1R/NMDAR complex involves the NR1 subunit, the interaction between D1R and NR1 was measured, as an index of complex formation, in coimmunoprecipitation

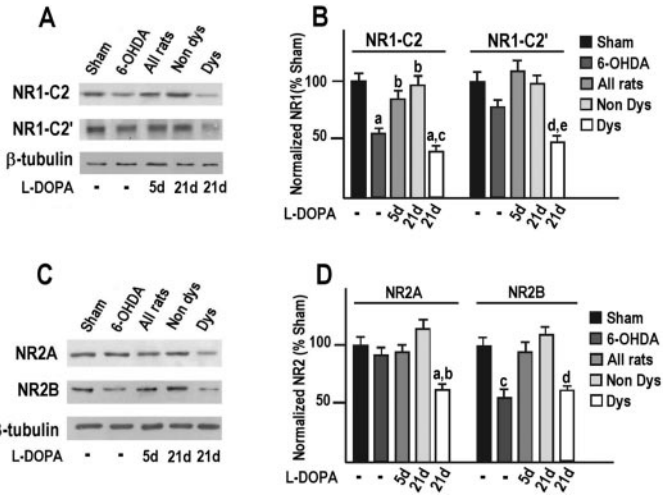

Fig. 2. Effects of L-DOPA on NMDAR density and composition in striatal TIF. Representative Western blot analysis of NR1-C2 and NR1-C2′ isoform levels (A) and of NR2A and NR2B levels (C) in striatal TIF are shown. B and D, densitometric analysis of three blots, with specific signals normalized to the corresponding β-tubulin levels. Bars represent the mean ± S.E. of three experiments. B, a, $p < 0.001$ to sham; b, $p < 0.01$ to 6-OHDA; c, $p < 0.001$ to nondyskinetic and 5-day L-DOPA treatment; d, $p < 0.05$ to sham and 6-OHDA; e, $p < 0.01$ to nondyskinetic and 5-day L-DOPA treatment. D, a, $p < 0.001$ to sham; b, $p < 0.05$ to 6-OHDA, 5-day L-DOPA treatment and nondyskinetic; c, $p < 0.001$ to sham, nondyskinetic, and 5-day L-DOPA treatment; d, $p < 0.001$ to sham, nondyskinetic, and 5-day L-DOPA treatment. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

experiments. Figure 6, A and B, shows representative coimmunoprecipitations of D1R and either NR1-C2 or NR1-C2′ in the TIF fraction; the densitometric analysis of three independent experiments is reported in Fig. 6, E and F. D1R and NR1-C2 coimmunoprecipitated in all experimental groups (Fig. 6A), although the abundance of immunoprecipitated D1R/NMDAR complexes was reduced in lesioned and dyskinetic rats compared with control and nondyskinetic rats (Fig. 6E). As reported in Fig. 6B, the anti-D1R antibody immunoprecipitated the NR1-C2′ isoform from the TIF fraction of control, lesioned, nondyskinetic, and dyskinetic rats. Moreover, according to the data obtained by Western blot, the abundance of immunoprecipitated D1R/NR1-C2′ complexes was decreased only in the TIF from dyskinetic rats (Fig. 6F). As reported in Fig. 6, C and D, the anti-D1R antibody immunoprecipitated both the NR1-C2 and NR1-C2′ subunits of NMDAR also from total membrane proteins of all experimental groups, and the abundance of immunoprecipitated D1R/NMDAR complexes was similar in control, lesioned, nondyskinetic, and dyskinetic rats (Fig. 6, E and F). To further control whether L-DOPA treatment might interfere with the interaction between D1R and NMDAR, coimmunoprecipitation experiments were performed in striatal TIF from unlesioned rats treated with L-DOPA for 21 days. The results showed that this treatment did not modify D1R/NMDAR interactions (Fig. 4C). Taken together, these data suggest that in lesioned and dyskinetic rats residual D1R and NMDAR are still associated at synaptic sites and that they are associated also at nonsynaptic sites and traffic together.

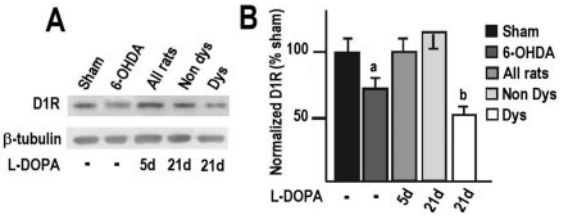

Fig. 3. Effects of L-DOPA on D1R content in striatal TIF. A, representative Western blot analysis of D1R in striatal TIF. B, densitometric analysis of four blots with D1R signals normalized to the corresponding β-tubulin levels. Bars represent the mean ± S.E. of four experiments. a, $p < 0.001$ to sham, nondyskinetic, and 5-day L-DOPA treatment; b, $p < 0.001$ to sham, nondyskinetic and 5-day L-DOPA treatment. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

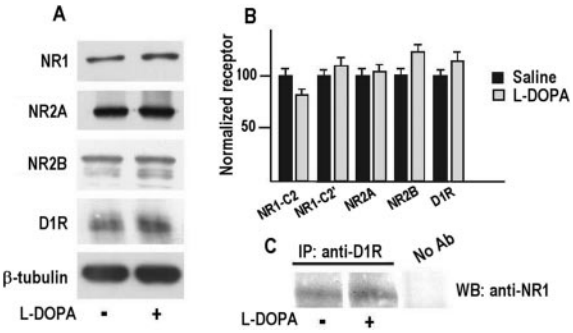

Fig. 4. Detection of NMDAR and D1R in the TIF fraction of unlesioned rats treated with L-DOPA. Rats were treated with either saline ($n = 6$) or L-DOPA (10 mg/kg i.p.) plus benserazide (7.5 mg/kg) ($n = 6$) for 21 days and killed 24 h after the last L-DOPA administration. Striatal TIF were isolated and analyzed for NMDAR subunit and D1R content as described under *Materials and Methods*. A, representative Western blot analysis of NMDAR subunits and D1R in striatal TIF. B, densitometric analysis of three blots with specific protein signals normalized to the corresponding β-tubulin levels. Bars represent the mean ± S.E. of three experiments. C, coimmunoprecipitation of NR1 subunit by the anti-D1R antibody in striatal TIF of unlesioned rats treated with either saline or L-DOPA.

Costimulation of D1R and NMDAR Induces D1R/NMDAR Complex Sequestration in Transfected Cells. We have reported that interaction with the NMDAR blocks D1R internalization induced by agonist stimulation (Fiorentini et al., 2003), a mechanism that could preserve the optimal synaptic strength at corticostriatal synapses during alterations of DA transmission. However, the data reported so far could be suggestive of a redistribution of the D1R/NMDAR complex from the postsynaptic membrane to intracellular sites in dyskinetic rats. Because in experimental parkinsonism the development of LID seems to be related to enhanced glutamate transmission (Chase and Oh, 2000; Robelet et al., 2004), it is possible that the nonphysiological coincident stimulation of both D1R and NMDAR induces desensitization of the D1R/NMDAR complex. To support this possibility, we took advantage of a HEK293 cell model stably expressing D1R, and transiently transfected with NR1-GFP and NR2B and PSD-95. The functional characteristics of expressed receptors were checked by measuring cAMP formation induced by the D1R agonist SKF 81297 (1 nM–10 μM) and by measuring $^{45}Ca^{2+}$ influx in response to glutamate/glycine (100 μM/10 μM) stimulation (data not shown). That D1R and NMDAR expressed in a host cell system do interact was previously demonstrated by bioluminescence resonance energy transfer (Fiorentini et al., 2003). The cellular localization of the D1R/NMDAR complex in transfected cells was evaluated by fluorescence microscopy. As shown in the representative photomicrograph reported in Fig. 7, in unstimulated cells the fluorescence of both D1R and NR1-GFP was localized at the plasma membrane (a and e). Exposure of cells to either the D1R agonist SKF 81297 (10 μM; b, and f) or glutamate/glycine (100 μM/10 μM; c and g) for 1 h did not modify D1R and NMDAR membrane localization.

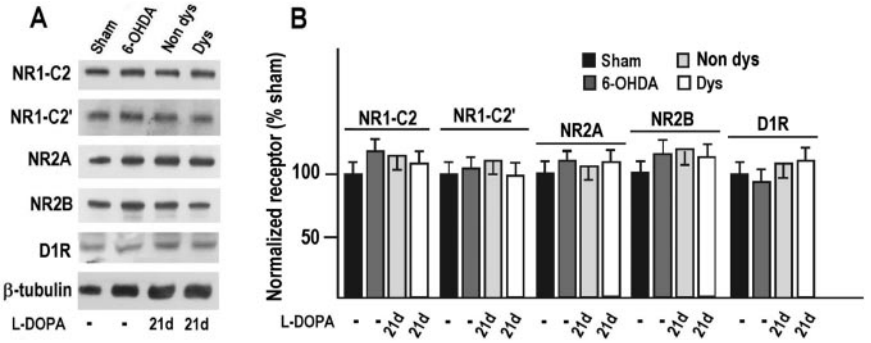

Fig. 5. Detection of NMDAR subunits and D1R in striatal membrane proteins. 6-OHDA-lesioned rats were treated with L-DOPA (10 mg/kg) plus benserazide (7.5 mg/kg) for 21 days and total striatal membrane proteins were isolated from controls, lesioned, nondyskinetic, and dyskinetic rats as described under *Materials and Methods*. A, representative Western blot analysis of NR1-C2, NR1-C2′ NR2A, NR2B, and D1R. B, densitometric analysis of three independent blots with each specific signal normalized to the corresponding β-tubulin level. Bars represent the mean ± S.E. of three experiments.

However, the simultaneous stimulation with both SKF 81297 and glutamate/glycine for 1 h resulted in the internalization of the D1R/NMDAR complex in the majority of transfected cells, as shown by the D1R and NR1-GFP fluorescence that was detectable also in the cytoplasm with a punctate appearance (d and h). The coincident stimulation of both interacting receptors thus promotes the cytoplasmic sequestration of the D1R/NMDAR complex. These data support the hypothesis that hyperfunctioning of glutamatergic transmission combined with the nonphysiological stimulation of DA receptors, both induced by L-DOPA treatment, might result in a remarkable down-regulation of synaptic D1R/NMDAR complex in dyskinetic rats.

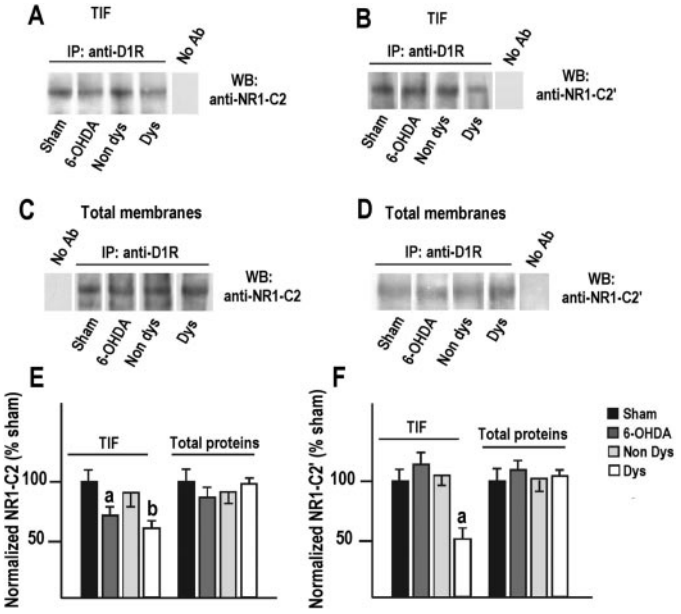

Fig. 6. Effects of L-DOPA on the interaction between D1R and NMDAR in both striatal TIF and total membrane proteins. Coimmunoprecipitation of D1R and NR1 splice variants was measured as an index of D1R/NMDAR interaction. Representative coimmunoprecipitation of NR1-C2 subunit by the anti-D1R antibody in striatal TIF and in total striatal membrane protein preparations from control, lesioned, nondyskinetic, and dyskinetic rats are shown in A and B. C, representative coimmunoprecipitation of NR1-C2′ splice variant by the anti-D1R antibody in striatal TIF and D, representative coimmunoprecipitation of NR1-C2′ by the anti-D1R antibody in total striatal membrane protein preparations from control, lesioned, nondyskinetic, and dyskinetic rats. E and F, densitometric analysis of three independent coimmunoprecipitations with each specific signal expressed as percentage of control. Bars represent the mean ± S.E. of three experiments. E, a, $p < 0.05$ to sham; b, $p < 0.05$ to sham and nondyskinetic. F, a, $p < 0.05$ to sham, 6-OHDA and nondyskinetic, one-way ANOVA followed by Tukey's multiple comparison test.

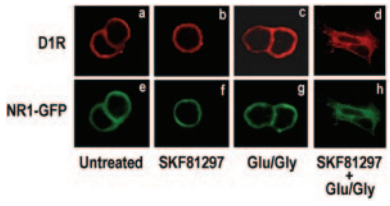

Fig. 7. Sequestration of D1R/NMDAR complex in response to agonist stimulation in transfected HEK293 cells. HEK293 cells expressing D1R, NR1-GFP, and NR2B subunits were exposed to agonists (10 μM SKF 81297 and 100 μM glutamate/10 μM glycine), either given alone or in combination, for 1 h and analyzed by immunofluorescence as described under *Materials and Methods*. a–c, representative cells detected at 100× magnification showing the membrane localization of D1R in untreated cells (a) and in cells individually treated with D1R (b) or NMDAR (c) agonists; e–g, representative cells showing the membrane localization of NMDAR in untreated cells (e) and in cells individually treated with D1R (f) or NMDAR (g) agonists; d and h, representative cells showing the cytoplasmic localization of both D1R (d) and NMDAR (h) in cells exposed to both D1R and NMDAR agonists.

Discussion

DA and glutamate interact in the striatum to control motor activity (Nicola et al., 2000), and loss of DA transmission results in dysregulations of glutamate function, suggesting that concurrent modifications of these systems contribute to the symptoms of both PD and LID. In particular, LID has been viewed as an aberrant form of motor learning resulting from DA and glutamate-dependent molecular alterations at corticostriatal synapses (Chase and Oh, 2000; Picconi et al., 2003). In this study, by using the 6-OHDA rat model of PD, we report that the development of LID is associated with a remarkable down-regulation of D1R/NMDAR oligomeric complexes at corticostriatal synapses.

DA denervation induced akinesia that was associated with a selective decrease of D1R, NR1-C2 splice variant and NR2B subunit in striatal TIF ipsilateral to the lesion, implying relative enrichment of NR1-C2′ and NR2A subunit-containing assemblies. Moreover, the results showing that D1R and NMDAR coimmunoprecipitate in lesioned rats suggest that DA depletion does not induce the complex to dissociate. The properties of NMDAR depend on its subunit composition and phosphorylation (Dingledine et al., 1999). Both binary NMDAR containing either NR1/NR2A or NR1/NR2B and ternary complexes containing all three subunits are present in striatal synaptosomal membranes (Dunah and Standaert, 2003), and it is known that NMDAR containing NR2A are rapidly inactivating compared with those containing NR2B (reviewd in Dingledine et al., 1999). Moreover, although the role of NR2A and NR2B in synaptic plasticity is still controversial, it has been recently reported that replacement of synaptic NR2B with NR2A reduces LTP in hippocampal neurons (Barria and Malinow, 2005). Interaction with the D1R adds further complexity to the NMDAR system. D1R interacts with both NR1 and NR2A (Lee et al., 2002; Fiorentini et al., 2003). Because NR1 is the fundamental subunit of the NMDAR, the D1R/NMDAR complex may indifferently contain NR2A, NR2B, or both. From a functional point of view, however, the interaction with NR2A is specifically responsible for inhibition of NMDA-mediated currents (Lee et al., 2002). Thus, the alterations in the D1R/NMDAR complex detected in lesioned rats could be responsible for the production of faster and smaller NMDA currents and for the loss of LTP formation that has been previously reported in this model (Centonze et al., 1999).

Previous studies showed a decreased density of NR1 and NR2B subunits and alterations of their phosphorylation in DA-depleted rat striatum (Oh et al., 1998, 1999; Chase and Oh, 2000; Dunah et al., 2000). Our results extend these data demonstrating that the decrease of NR1 subunit is due to one of its splice variants, occurs in the PSD, and also involves the D1R. That the receptor profile of striatal PSD is related to motor activity is further supported by the observation that short-term L-DOPA administration to lesioned rats improved akinesia and restored the synaptic abundance of D1R, NR1-C2, and NR2B. During prolonged treatment, the antiakinetic effect of L-DOPA was maintained in one group of rats but was

lost in another group because of the development of severe AIMs. It is noteworthy that although the levels of D1R and NMDAR subunits in the PSD of nondyskinetic rats were identical to those detected in controls and in rats treated with L-DOPA for 5 days, the PSD of dyskinetic rats showed a remarkable reduction in the density of NMDAR and D1R with no alterations in their interaction. This decrease involved NR1-C2, NR1-C2′, NR2A, and NR2B subunits and suggests that D1R/NMDAR assemblies in dyskinetic rats is profoundly different from that observed in both control and lesioned rats. Thus, the expression of different motor dysfunctions might be related to definite receptor changes at corticostriatal synapses. The observation that L-DOPA administration to unlesioned rats produced neither behavioral nor biochemical alterations supports this view and is in line with the assumption that DA depletion is required for the development of LID (Bezard et al., 2001). It is noteworthy that LID-associated increase of NR2A has been reported in synaptosomal membranes of MPTP-lesioned primates (Hallett et al., 2005). This preparation, however, mainly contains the presynaptic compartment and is therefore different from the postsynaptic TIF fraction used in our study. The difference in the tissue fractions used could thus explain the apparent discrepancy and could perhaps reflect the existence of different mechanisms regulating NMDAR in the PSD or at extrasynaptic sites. Moreover, our analysis has been performed 24 h after the end of L-DOPA treatment, whereas biochemical changes in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–lesioned primates were studied 1 h after treatment (Hallett et al., 2005). Thus further studies are necessary to define whether the changes we observed also occur at the peak of L-DOPA action, are transitory, or are persistent.

Changes of D1R function have been correlated with the development of LID. In particular, increased density and functional efficiency of D1R have been described in animal models of LID (Gerfen et al., 2002; Picconi et al., 2003; Aubert et al., 2005). Our results, showing a reduction of synaptic D1R associated with the NMDAR in dyskinetic rats, are apparently in contrast with these observations. Taken together, however, these data point to a more complex alteration of D1R function in LID than previously thought. In particular, they suggest that, within a single neuron, the D1R may be differentially affected by perturbations of DA transmission in different neuronal microdomains depending on its interaction with other membrane components. These differential modifications, by changing the relative proportion of D1R in discrete subcellular regions, might result in severe neuronal dysfunctions.

The finding that the levels of NMDAR subunits and D1R are unchanged in total membrane proteins suggests that alterations in the trafficking of these receptors, rather than changes of their expression, might be involved. Moreover, analysis of NMDAR subunit abundance, showing specific alterations in lesioned and dyskinetic rats, suggests that different mechanisms could be likely involved in these changes. In lesioned rats, the decrease of D1R/NMDAR containing NR1-C2 and NR2B subunits could be related to abnormal trafficking of the complex to synaptic sites. The observation that 1) DA, by phosphorylating NR2B, triggers the delivery of NMDAR to synaptic sites (Dunah and Standaert 2001), 2) the phosphorylation of striatal NR2B is decreased in lesioned rats (Dunah et al., 2000), 3) DA deprivation alters the interaction of NR2A/B subunits with PSD-95 (Picconi et al., 2004), a scaffolding protein anchoring the NMDA complex to the PSD, and 4) the expression and membrane insertion of different NR1 splice variants is dependent on neuronal activity (Mu et al., 2003) supports this idea. On the other hand, agonist-induced desensitization could perhaps underlie the decrease of all measured NMDAR subunits and D1R in the PSD of dyskinetic rats. Prolonged L-DOPA treatment, in fact, increases not only DA but also extracellular glutamate in the striatum of dyskinetic rats (Robelet et al., 2004). These changes, resulting in the coincident stimulation of both interacting receptors, could lead to D1R/NMDAR complex internalization, independently of the subunit composition of the NMDAR. The observation that the intracellular localization of D1R is increased in PD patients treated with L-DOPA (Muriel et al., 1999) is in line with this view. Moreover, our observation that in HEK293 cells expressing D1R/NMDAR complex, the coincident stimulation of both D1R and NMDAR, but not individual receptor activation, promotes internalization of the complex gives support to this hypothesis, although this should be confirmed in a neuronal cell model. The observation that the synaptic D1R/NMDAR complex is down-regulated in dyskinetic rats is apparently in contrast with the reported NMDAR sensitization in LID (Bezard et al., 2001; Brotchie, 2005). Our preliminary observation that in transfected cells internalized D1R/NMDAR complex undergoes rapid recycling to the plasma membrane (data not shown) suggests the possibility that in dyskinetic rats, this complex could be redistributed to extrasynaptic sites, an event that may affect synaptic plasticity. Moreover, according to the paradigm developed by Luttrell and Lefkowitz (2002), the possibility should also be considered that internalized D1R/NMDAR could be responsible for the activation of the extracellular signal-regulated kinase 1/2 (Erk1/2), which plays an important role in synaptic plasticity (Thomas and Huganir, 2004). In line with this hypothesis, aberrant activation of this intracellular pathway, apparently due to stimulation of both D1R and NMDAR, has been reported in dyskinetic rats (Gerfen et al., 2002; Paul et al., 2003). On the other hand, because the coincident stimulation of both D1R and NMDAR is required to induce D1R/NMDAR complex internalization, the antidyskinetic effect of NMDAR antagonists (Papa and Chase, 1996; Chase and Oh, 2000; Lundblad et al., 2002; Brotchie, 2005) could be related to inhibition of this agonist-mediated adaptive response. All these mechanisms require further investigation to be fully clarified.

Previous studies demonstrated that D1R and NMDAR physically and functionally interact in striatal PSD (Fiorentini et al., 2003). Our present data suggesting remarkable alterations of synaptic D1R/NMDAR complexes in dyskinetic rats provide a pathophysiological implication of this receptor complex in the most debilitating side effect of L-DOPA therapy. The finding that PSD-95, which may play an important role in the organization of the D1R/NMDAR complex within the PSD, is involved in DA-mediated synaptic plasticity (Yao et al., 2004) supports this view.

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