Molecular Pharmacology Fast Forward. Published on December 19, 2005 as doi: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2005/016667

LOSS OF SYNAPTIC D1 DOPAMINE/NMDA 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, Viale Europa 11, 25124 Brescia, Italy (C.F., M.C.R., C.B., S.B., G.C., P.F.S., C.M.); and Scientific Institute IRCCS S. Camillo, Venezia, Italy (P.F.S., C.M.).

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2005/016667

Running title: Synaptic D1/NMDA receptor complexes and L-DOPA-induced dyskinesia

Corresponding author:

Cristina Missale, Division of Pharmacology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Viale Europa 11, 25124 Brescia, Italy

Tel. 00390303717518; Fax 00390303717529; E-mail: cmissale@med.unibs.it

Number of text pages: 34

Number of tables : 0

Number of figures: 7

Number of references: 40

Number of words in the Abstract: 241

Number of words in the Introduction: 620

Number of words in the Discussion: 1498

Abbreviations: NMDA, N-methyl-D-aspartate; PSD, postsynaptic densities; TIF, triton-insoluble fraction; GFP, green fluorescent protein; DA, dopamine; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; L-DOPA, L-3,4-dihyroxyphenylalanine; LID, L-DOPA-induced dyskinesias; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; AIMs, abnormal involuntary movements; TH, tyrosine hydroxylase; HEK 293, human embryonic kidney 293; DMEM, Dulbecco's modified Eagle's Medium.

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2005/016667

ABSTRACT

Glutamate-mediated mechanisms are related to the motor complications of L-DOPA therapy in Parkinson's disease (PD). In striatal post synaptic densities (PSD) the dopamine D1 receptor (D1R) is part of an oligomeric complex with the glutamate NMDA 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 divided into dyskinetic and non dyskinetic on the basis of behavioural 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 non dyskinetic 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 likely reflects an altered receptor trafficking. In HEK293 cells expressing the D1R/NMDAR complex co-stimulation of both D1R and NMDAR, but not individual receptor activation, promotes internalization, suggesting that development of dyskinesias may be related to agonist-mediated downregulation of the D1R/NMDAR complex at cortico-striatal synapses.

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

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 fibres on dendritic spines of striatal medium spiny neurons and there is a general agreement that an integrated interplay between DA and glutamate inputs is essential to drive correct motor behaviour 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 (Olanow et al., 2000). The most effective therapy to alleviate the main motor symptoms of this disease is treatment with the DA precursor L-DOPA, that increases DA availability (Olanow 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 patients (Olanow 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), there is now increasing evidence that glutamate-mediated mechanisms may also contribute to the development of these complications (Brotchie JM, 2005; Robelet et al., 2004; Chase and Oh, 2000). On this line, increased glutamate release has been described in hemiparkinsonian rats chronically treated with L-DOPA (Robelet et al., 2004). Moreover, the observations that the glutamate NMDA receptor (NMDAR) is altered in experimental

parkinsonism (Olanow et al., 2000; Bezard et al., 2001; Dunah et al., 2000) and that LID may be ameliorated by NMDAR antagonists (Chase and Oh, 2000; Papa and Chase 1996; Lundblad et al., 2002; Brotchie JM, 2005) point to this receptor subtype as a major player in the development of motor dysfunctions.

NMDAR 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 post synaptic density (PSD), where they interact with specific scaffolding and signalling 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, signalling and desensitization of both interacting receptors (Fiorentini et al., 2003; Lee et al., 2002). Since the concurrent activation of both D1R and NMDAR is crucial to determine the efficacy of cortico-striatal 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). Interestingly, the gradual development of abnormal involuntary movements has been observed in about 50% of hemiparkinsonian rats chronically treated 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 non dyskinetic 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

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

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.

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

6-Hydroxydopamine (6-OHDA) 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, Milano, Italy), mounted on a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and injected with 12 μg of 6-OHDA (Sigma-Aldrich), dissolved in 4μl of saline containing 0.2% ascorbic acid, at the rate of 0.38 μl/min into the left medial forebrain bundle (from bregma: AP = -3.6, L = 1.9, DV = -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 controlateral turning behaviour 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 behavioural 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 mg/kg, 10 mg/kg 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). Limb-use asymmetry was evaluated, as an index of akinesia, with the cylinder test (Schallert et al.,

2000) twice a week. Briefly, 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 into axial, i.e. twisted posturing of the neck and the upper body controlateral to the lesion; limb, i.e. repetitive purposeless movements of the controlateral forelimb; orolingual, i.e. empty jaw movements and controlateral tongue protrusion; locomotor, i.e. increased locomotion with controlateral 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 20-120 min after L-DOPA administration, for a total of six observations and the AIMs 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, since 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, 24h 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 (TIF). To prepare the total membrane protein fraction the striatum from control (n = 5), lesioned (n =5), non dyskinetic (n = 5) and dyskinetic (n = 5) rats was homogenized in ice-cold Tris-EDTA buffer (10mM Tris-HCl, 5mM EDTA, pH 7.4) and centrifuged at 700 x g at 4°C. The supernatant was centrifuged at 10,000 x g at 4°C for 30 min and the pellet was suspended in 10 mM Tris-HCl, 1mM EDTA, 1mM EGTA, pH 7.4 and solubilized with 0.5% NP-40, 0.1% SDS.

To isolate the triton-insoluble fraction (TIF), which is enriched in PSD, the striatum from controls (n = 10), lesioned (n = 15), non dyskinetic (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 phenyl-methyl-sulfonyl fluoride (PMSF) and a complete set of protease inhibitors (Roche, Milano, Italy), pH 7.4, and centrifuged at 1,000 x g for 10 min. The supernatant was centrifuged at 3,000 x g for 15 min and the resulting pellet containing mitochondria and synaptosomes was resuspended in 1 mM Hepes and centrifuged at 100,000 x g for 1h. The pellet was resuspended in 75 mM KCl containing 1% Triton X-100 and TIF was collected by centrifugation at 100,000 x g for 1h. TIF was characterized by the enrichment in PSD proteins as previously described (Fiorentini et al., 2003). This fraction was used instead of purified PSD to limit the number of animals in each group. For protein

quantification, TIF was solubilized in 1% SDS. Protein concentration in both TIF and total membrane proteins was measured according to a modified, detergent-compatible Lowry method (BioRad).

Co-immunoprecipitation and Western Blot. Co-immunoprecipitation of both TIF and total membrane proteins was performed under non denaturing 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 (Gardoni et al., 1998; Apperson et al., 1986; Yu et al., 1997). 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 beta-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). Protein-A agarose beads were added and incubation was continued for 2h at room temperature. Beads were collected, extensively washed with buffer A and the resulting proteins were resolved by SDS-PAGE, transferred onto PVDF membranes and blotted for 1h at room temperature in TBS containing 0.1% Tween 20 and 5% non fat powdered milk. Membranes were incubated for 2h at room temperature with the anti-NR1-C2 or the anti-NR1-C2' antibody (1:500 dilution, Chemicon). Detection was performed by chemiluminescence with HRP-conjugated secondary antibodies (Chemicon). In Western blot experiments 40 μg of total proteins or 20 μg of TIF were resolved by SDS-PAGE, transferred onto PVDF membranes and blotted as previously

described. Membranes were incubated for 2h at room temperature with anti-NR1-C2, anti-NR1-C2' (1:500 dilution), anti-NR2A (1:500 dilution, Chemicon), anti-NR2B (1:500 dilution, Chemicon) and anti-D1R (1:250 dilution, Chemicon) antibodies. To ensure equal protein loading, membranes were stripped with a Western blot recycling kit (Chemicon) and reprobed with anti-β-tubulin (1:2,500 dilution; 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 controls.

Cell culture and transfection. HEK293 cells were cultured in high glucose DMEM containing 10% FBS, 2 mM glutamine, 0.1 mM non-essential aminoacids, 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-Life Technology, Milano 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 hours post-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 1h at 37°C in the absence or in the presence of

10 μM SKF 81297 or 100 μM glutamate/10 μM glycine or a combination of SKF 81297 (10 μM) and glutamate (100μM)/glycine (10μM). Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% triton X-100 in PBS containing 5% bovine serum albumin (BSA) 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 dilution, Sigma) and then for 45 min at room temperature with Cy3-conjugated anti-goat secondary antibody (1:800 dilution, Jackson ImmunoResearch, West Grove, PA). Immunolabeled cells were recorded with a Olympus IX51 fluorescence microscope at a 100x magnification. Non transfected cells and omission of the primary antibody were used as negative controls.

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Chronic 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 the dose of 6.5 mg/kg, did not induce the appearance of severe AIMs, while at the dose of 20 mg/kg produced dyskinesias in about 70-80% of rats. At the dose of 10 mg/kg L-DOPA induced the development of AIMs in about 50-60% 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 behaviour 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 administration akinesia was significantly improved in all animals in the absence of dyskinesias (only 5 rats over 53 developed a mild dyskinesia), suggesting that this treatment restored the normal, spontaneous motor behaviour. 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, while rats that during long-term treatment lost the anti-akinetic effect of L-DOPA 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

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

showing a locomotor improvement in response to L-DOPA without dyskinesias (here referred to as non dyskinetic rats) and those that, after a short period of remission, lost the benefit of L-DOPA due to 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 cortico-striatal 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, non dyskinetic and dyskinetic rats, we thus used a PSD-enriched fraction (Triton-insoluble fraction, TIF) instead of a classical membrane preparation. This fraction, which is rather insoluble in non-ionic detergents (Kennedy, 2000), was dissolved by using a combination of 0.5% NP-40, and 0.1% SDS. These non denaturing detergent concentrations efficiently solubilize TIF proteins without dissociating the NMDA channel complexes (Gardoni et al., 1998; Apperson et al., 1986; Yu et al., 1997). 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 no shown).

The NR1 subunit of the NMDAR consists of eight splice variants of a single gene (Zukin and Bennett, 1995). Since 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. 2A and 2C and the densitometric analysis of three independent experiments with the specific signals normalized to the corresponding β-tubulin staining is reported in fig. 2B and

3D. As reported in fig. 2A and 2B, NR1-C2, but not NR1-C2' levels 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, while NR2A levels were unchanged (Fig. 2C and 2D). A 5-day L-DOPA treatment, that improved akinesia, restored NR1-C2 and NR2B to the physiological levels (Fig. 2A-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 non dyskinetic group (Fig. 2A-D). No differences in NMDAR subunit levels were found in striatal TIF contralateral to the lesion. Since 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 non dyskinetic rats, but was significantly decreased in dyskinetic animals. Moreover, as shown in fig. 4A and B, L-DOPA administration to unlesioned control rats, that 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, non

dyskinetic and dyskinetic rats. Similarly, 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 likely 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. Since 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 co-immunoprecipitation experiments. Fig 6A and 6B show representative co-immunoprecipitations of D1R and either NR1-C2 or NR1-C2' in the TIF fraction; the densitometric analysis of three independent experiments is reported in fig 6E and 6F. D1R and NR1-C2 co-immunoprecipitated in all experimental groups (fig 6A), although the abundance of immunoprecipitated D1R/NMDAR complexes was reduced in lesioned and dyskinetic rats when compared to control and non dyskinetic rats (fig. 6E). As reported in fig. 6B, the anti-D1R antibody immunoprecipitated the NR1-C2' isoform from the TIF fraction of control, lesioned, non dyskinetic 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. 6C and 6D, 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, non dyskinetic and dyskinetic rats (Fig. 6E and 6F). To further control whether L-DOPA treatment may interfere with the interaction between D1R and NMDAR, co-immunoprecipitation experiments were performed in striatal TIF from unlesioned rats treated with L-DOPA for 21 days. The results showed that this treatment did

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

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 non-synaptic sites and traffic together.

Co-stimulation 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 cortico-striatal 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. Since in experimental parkinsonism the development of LID appears to be related to enhanced glutamate transmission (Robelet et al., 2004; Chase and Oh, 2000), it is possible that the non-physiological 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 BRET (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

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

fluorescence of both D1R and NR1-GFP was localized at the plasma membrane (panels a and e). Exposure of cells to either the D1R agonist SKF 81297 (10 μM; panels b, and f) or glutamate/glycine (100 μM/10 μM; panels c and g) for 1h did not modify D1R and NMDAR membrane localization. However, the simultaneous stimulation with both SKF 81297 and glutamate/glycine for 1h 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 (panels 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 non-physiological 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.

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 motor learning resulting from DA and glutamate-dependent molecular alterations at cortico-striatal 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 cortico-striatal 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 co-immunoprecipitate 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). Since NR1 is the fundamental subunit of the NMDAR, the D1R/NMDAR complex may indifferently contain either NR2A or 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 (Chase and Oh, 2000; Dunah et al., 2000; Oh et al., 1998; Oh et al., 1999). 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 anti-akinetic effect of L-DOPA was maintained in one group of rats but was lost in another group due to the development of severe AIMs. Interestingly, while the levels of D1R and NMDAR subunits in the PSD of non-dyskinetic 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 cortico-striatal synapses. The

observation that L-DOPA administration to unlesioned rats did not produce neither behavioural nor biochemical alterations support 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 worth noting 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 pre-synaptic compartment and is therefore different from the postsynaptic TIF fraction used in our study. The difference in the tissue fractions used could thus explain this apparent discrepancy and could perhaps reflect the existence of different mechanisms regulating NMDAR in the PSD or at extra-synaptic sites. Moreover, our analysis has been performed 24h after the end of L-DOPA treatment, while biochemical changes in MPTP-lesioned primates were studied 1h 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 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 (Picconi et al., 2003; Aubert et al., 2005; Gerfen et al., 2002). 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 increases, in fact, 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, although should be confirmed in a neuronal cell model, give support to this hypothesis. The observation that

the synaptic D1R/NMDAR complex is downregulated in dyskinetic rats is apparently in contrast with the reported NMDAR sensitization in LID (Bezard et al., 2001; Brotchie JM, 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), that plays an important role in synaptic plasticity (Thomas and Huganir, 2004). On this line 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, since the coincident stimulation of both D1R and NMDAR is required to induce D1R/NMDAR complex internalization, the antidyskinetic effect of NMDAR antagonists (Chase and Oh, 2000; Papa and Chase, 1996; Lundblad et al., 2002; Brotchie JM, 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, that may play a 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), support this view.

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

ACNOWLEGEMENTS

We thank Dr. M.G. Caron (Duke University) for providing the human D1R cDNA; Dr. H. Monyer (Heidelberg University) for providing the NR2B cDNA; Dr. S. Nakanishi (Kyoto University) for providing the NR1 cDNA; Dr. M. Passafaro (University of Milano) for providing the PSD95 cDNA; Dr. S. Calza (University of Brescia) for his help with the statistical analysis of the data.

REFERENCES

Apperson ML, Moon IS and Kennedy MB (1996) Characterization of densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family. *J Neurosci* 16:6839-6852.

Aubert I, Guigoni C, Hakansson K, Li Q, Barche N, Bioulac BH, Gross CE, Fisone G, Bloch B and Bezard E (2005) Increased D1 dopamine receptor signaling in levodopa-induced dyskinesia. *Ann Neurol* 57:17-26.

Barria A and Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48:289-301

Bezard E, Brotchie JM and Gross CE (2001) Pathophysiology of levodopa-induced dyskinesia: potential for new therapies. *Nat Rev Neurosci* 2:577-588.

Brotchie JM (2005) Nondopaminergic mechanisms in levodopa-induced dyskinesia. *Mov Dis* 20: 919-931.

Cenci MA, Lee CS, and Bjorklund A (1998) L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. *Eur J Neurosci* 10:2694-2706.

Centonze D, Gubellini P, Picconi B, Calabresi P, Giacomini P and Bernardi G (1999) Unilateral dopamine denervation blocks corticostriatal LTP. *J Neurophysiol* 82:3575-3579.

Chase TN and Oh JD (2000) Striatal dopamine- and glutamate-mediated dysregulation in experimental parkinsonism. *Trends Neurosci* 23 (Suppl. Basal Ganglia, Parkinson's Disease and Levodopa Therapy):86-91.

Dingledine RT, Borges K, Bowie D and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61.

Dunah AW and Standaert DG (2001) Dopamine D1 receptor-dependent trafficking of striatal

NMDA glutamate receptors to the postsynaptic membrane. *J Neurosci* 21:5546-5558.

Dunah AW and Standaert DG (2003) Subcellular segregation of distinct heteromeric NMDA glutamate receptors in the striatum. *J Neurochem* 85:935-943.

Dunah AW, Wang Y, Yasuda RP, Kameyama K, Huganir RL, Wolfe BB and Standaert DG (2000) Alterations in subunit expression, composition and phosphorylation of striatal N-methyl-D-aspartate glutamate receptors in a rat 6-hydroxydopamine model of Parkinson's disease. *Mol Pharmacol* 57:342-352.

Fiorentini C, Gardoni F, Spano PF, Di Luca M and Missale C (2003) Regulation of dopamine D1 receptor trafficking and desensitization by oligomerization with glutamate N-methyl-D-aspartate receptors. *J Biol Chem* 278:20196-20202.

Gardoni F, Caputi A, Cimino M, Pastorino L, Cattabeni F and Di Luca M (1998) Calcium/Calmodulin-dependent protein kinase II is associated with NR2A/B subunits of NMDA receptor in postsynaptic densities. *J Neurochem* 71:1733-1741.

Gerfen CR, Miyachi S, Paletzi R and Brown P (2002) D1 dopamine receptor supersensitivity in the dopamine-depleted striatum results from a switch in the regulation of Erk1/2MAPkinase. *J Neurosci* 22:5042-5054.

Hallett PJ, Dunah AW, Ravenscroft P, Zhou S, Bezard E, Crossman AR, Brotchie JM, Standaert DG (2005) Alterations of striatal NMDA receptor subunits associated with the development of dyskinesia in the MPTP-lesioned primate model of Pakinson's disease. *Neuropharmacol* 48:503-516.

Kennedy MB (2000) Signal-processing machines at the postsynaptic density. *Science* 290:750-754.

Lee FJ, Xue S, Pei L, Vukusic B, Chéry N, Wang Y, Wang YT, Niznik HB, Yu X and Liu F (2002) Dual regulation of NMDA receptor functions by direct protein-protein interaction with the dopamine D1 receptor. *Cell* 111:219-230.

Lundblad M, Andersson M, Winkler C, Kirk D, Wierup N and Cenci MA (2002) Pharmacological validation of behavioural measures of akinesia and dyskinesia in a rat model of Parkinson's disease. *Eur J Neurosci* 15:120-132.

Luttrell LM and Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115:455-465.

Missale C, Nash R, Robinson SW, Jaber M and Caron MG (1998) Dopamine receptors: from structure to function. *Physiological Review 78:189-225.*

Mu Y, Otsuka T, Horton AC, Scott DB and Ehlers MD (2003) Activity-dependent mRNA splicing controls ER export and synaptic delivery of NMDA receptors. *Neuron* 40:581-594.

Muriel MP, Bernard V, Levey AI, Laribi O, Abrous DN, Agid Y, Bloch B and Hirsch HC (1999) Levodopa induces a cytoplasmic localization of D1 dopamine receptors in striatal neurons in Parkinson's disease. *Ann Neurol* 46:103-111.

Nicola SM, Surmeier DJ and Malenka RC (2000) Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. *Annu Rev Neurosci* 23:185-215.

Oh JD, Russel D, Vaughan CL and Chase TN (1998) Enhanced tyrosine phosphorylation of striatal NMDA receptor subunits: effect of dopaminergic denervation and L-DOPA administration. *Brain Res* 813:150-159.

Oh JD, Vaughan CL and Chase TN (1999) Effect of dopamine denervation and dopamine agonist administration on serine phosphorylation of striatal NMDA receptor subunits. *Brain Res* 821:433-442.

Olanow CW, Obeso JA and Nutt JG (2000) *Trends Neurosci* 23 (suppl. Basal Ganglia, Parkinson's Disease and Levodopa Therapy):1-126.

Papa SM and Chase TN (1996) Levodopa-induced dyskinesias improved by a glutamate antagonist in Parkinsonian monkeys. *Ann Neurol* 39:574-578.

Papa SM, Engber TM, Kask AM and Chase TN (1994) Motor fluctuations in levodopa treated parkinsonian rats: relation to lesion extent and treatment duration. *Brain Res* 662:69-74.

Paul S, Nairn AC, Wang P and Lombroso PJ (2003) NMDA-mediated activation of tyrosine phosphatase STEP regulates the duration of ERK signalling. *Nat Neurosci* 6:34-42.

Paxinos G and Watson C (1986) *The rat brain in stereotaxic coordinates.* Academic Press

Picconi B, Centonze D, Hakansson K, Bernardi G, Greengard P, Fisone G, Cenci MA and Calabresi P (2003) Loss of bidirectional striatal plasticity in L-DOPA-induced dyskinesia. *Nat Neurosci* 6:501-506.

Picconi B, Gardoni F, Centonze D, Mauceri D, Cenci MA, Bernardi G, Calabresi P and Di Luca M (2004) Abnormal Ca^{2+}-calmodulin-dependent protein kinase II function mediates synaptic and motor deficits in experimental parkinsonism. *J Neurosci* 24:5283-5291.

Robelet S, Melon C, Guillet B, Salin P and Kerkerian-Le Goff L (2004) Chronic L-DOPA treatment increases extracellular glutamate levels and GLT1 expression in the basal ganglia in a rat model of Parkinson's disease. *Eur J Neurosci* 20:1255-1266.

Roche KW, Standley S, McCallum J, Ly CD, Ehlers MD and Wenthold RJ (2001) Molecular determinants of NMDA receptor internalization. *Nat Neurosci* 4:794-802.

Schallert T, Fleming SM, Leasure JL, Tillerson JL and Bland ST (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacol* 39:777-787.

Thomas GM and Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci* 5:173-183.

Zukin RS and Bennett MLV (1995) Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci* 18:306-313.

Yao WD, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu JM, Torres GE, Grant SGN and Caron M (2004) Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioural plasticity. *Neuron* 41:625-638**.**

Yu XM, Askalan R, Keil GJ and Salter MW (1997) NMDA channel regulation by channel-associated protein tyrosine kinase src. *Science* 275:674-678.

FOOTNOTES

This study was supported by Ministero della Sanità; CNR and MIUR – Fondo FISR and, in part, by MIUR and University of Brescia (PRIN MM06272753) to C.M.

Please send reprint request 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

Molecular Pharmacology Fast Forward. Published on December 19, 2005 as DOI: 10.1124/mol.105.016667
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Fig 1. Behavioural 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 (gray square). Short term L-DOPA administration improved akinesia in all treated animals (n = 53; filled squares). During chronic administration one group of rats was still improved by L-DOPA (n = 17; open circles) while a disabling effect of this drug, occurring as increased limb-use asymmetry, was detectable in the other group (n = 26; filled circles). B, L-DOPA-induced AIMs were recorded as described in Materials and Methods. Rats that during chronic treatment lost the anti-akinetic 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, non dyskinetic). Data are the means ± SEM. A, * $p < 0.001$ vs. sham, ANOVA followed by Westfall test; $n > 10$ in each group; B, $p < 0.001$ vs. non dyskinetic, ANOVA followed by Westfall test; $n > 10$ in each group.

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 ± SE of three experiments. (B) a, $p < 0.001$ to sham; b, $p < 0.01$ to 6-OHDA; c, $p < 0.001$ to non dyskinetic and 5-day L-DOPA treatment; d, $p < 0.05$ to sham and 6-OHDA, e, $p < 0,01$ to non dyskinetic 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 non dyskinetic; c, $p < 0.001$ to sham, non dyskinetic and 5-day L-DOPA treatment; d, $p < 0.001$ to sham, non dyskinetic and 5-day L-DOPA treatment. Data were statistically analyzed by one-way ANOVA followed by Tuckey's multiple comparison test.

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 ± SE of four experiments. a, $p < 0.001$ to sham, non dyskinetic and 5-day L-DOPA treatment; b, $p < 0.001$ to sham, non dyskinetic and 5-day L-DOPA treatment. Data were statistically analyzed by one-way ANOVA followed by Tuckey'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 24h after the last L-DOPA administration. Striatal TIF were isolated and analyzed for NMDAR subunit and D1R content as described in 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 ± SE of three experiments. C,

co-immunoprecipitation of NR1 subunit by the anti-D1R antibody in striatal TIF of unlesioned rats treated with either saline or L-DOPA.

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, non-dyskinetic and dyskinetic rats as described in 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 ± SE of three experiments.

Fig. 6. Effects of L-DOPA on the interaction between D1R and NMDAR in both striatal TIF and total membrane proteins. Co-immunoprecipitation of D1R and NR1 splice variants was measured as an index of D1R/NMDAR interaction. Representative co-immunoprecipitation of NR1-C2 subunit by the anti-D1R antibody in striatal TIF and in total striatal membrane protein preparations from control, lesioned, non dyskinetic and dyskinetic rats are shown in panels A and B. C, representative co-immunoprecipitation of NR1-C2' splice variant by the anti-D1R antibody in striatal TIF and D, representative co-immunoprecipitation of NR1-C2' by the anti-D1R antibody in total striatal membrane protein preparations from control, lesioned, non dyskinetic and dyskinetic rats. E and F, densitometric analysis of three independent co-immunoprecipitations with each specific signal expressed as percentage of control. Bars represent the mean ± SE of three experiments. (E) a, $p < 0.05$ to sham; b, $p < 0.05$ to sham and non dyskinetic. (F) a, $p < 0.05$ to sham, 6-OHDA and non dyskinetic,

one-way ANOVA followed by Tuckey'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 1h and analyzed by immunofluorescence as described in Materials and Methods. Panels a-c: representative cells detected at 100x magnification showing the membrane localization of D1R in untreated cells (a) and in cells individually treated with D1R (b) or NMDAR (c) agonists; panels 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; panels 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.

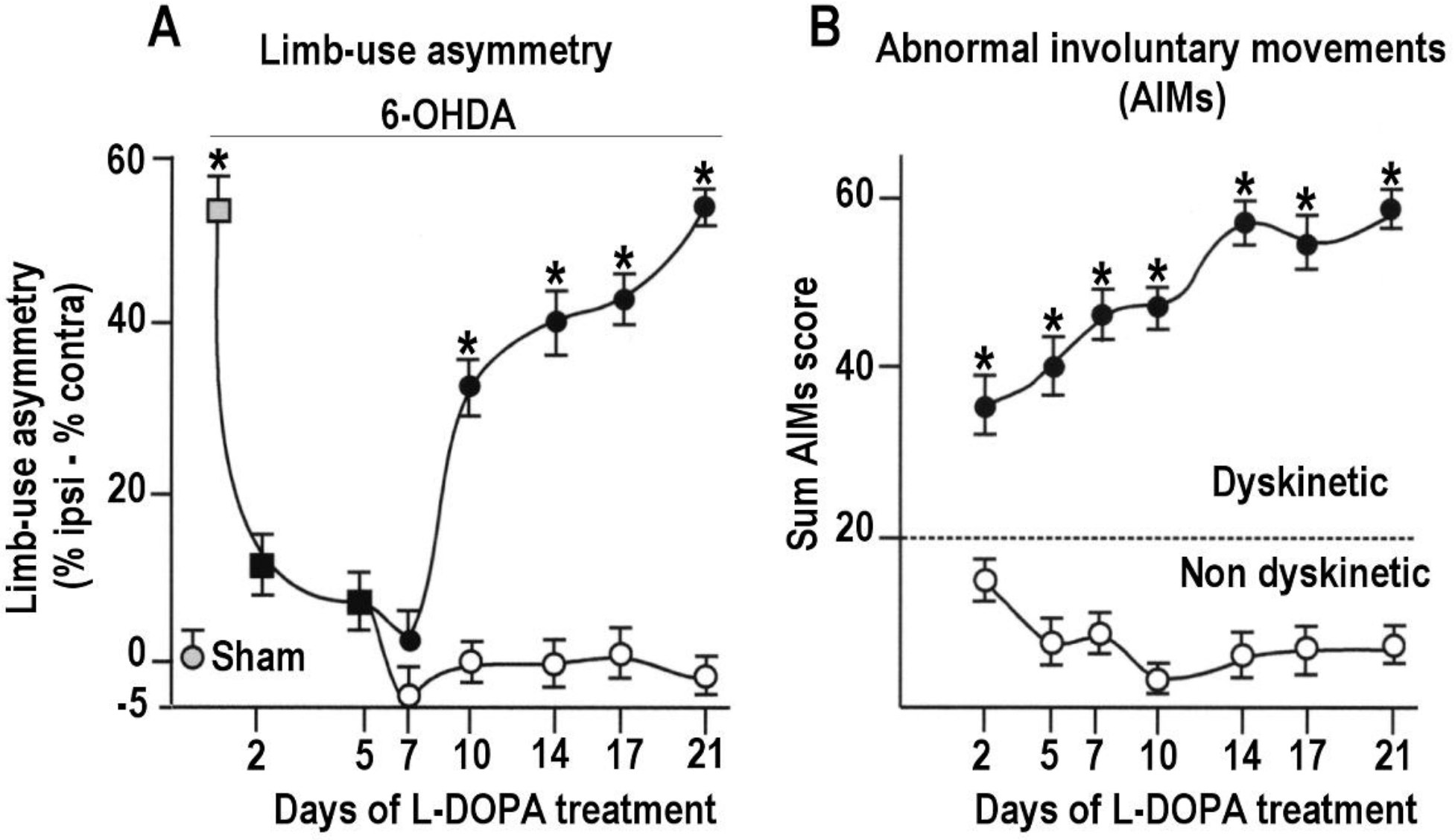
A
Limb-use asymmetry
6-OHDA
Limb-use asymmetry
(% ipsi - % contra)
60
40
20
0
-5
Sham
2
5
7
10
14
17
21
Days of L-DOPA treatment
B
Abnormal involuntary movements
(AIMs)
Sum AIMs score
60
40
20
Dyskinetic
Non dyskinetic
2
5
7
10
14
17
21
Days of L-DOPA treatment

Figure 1

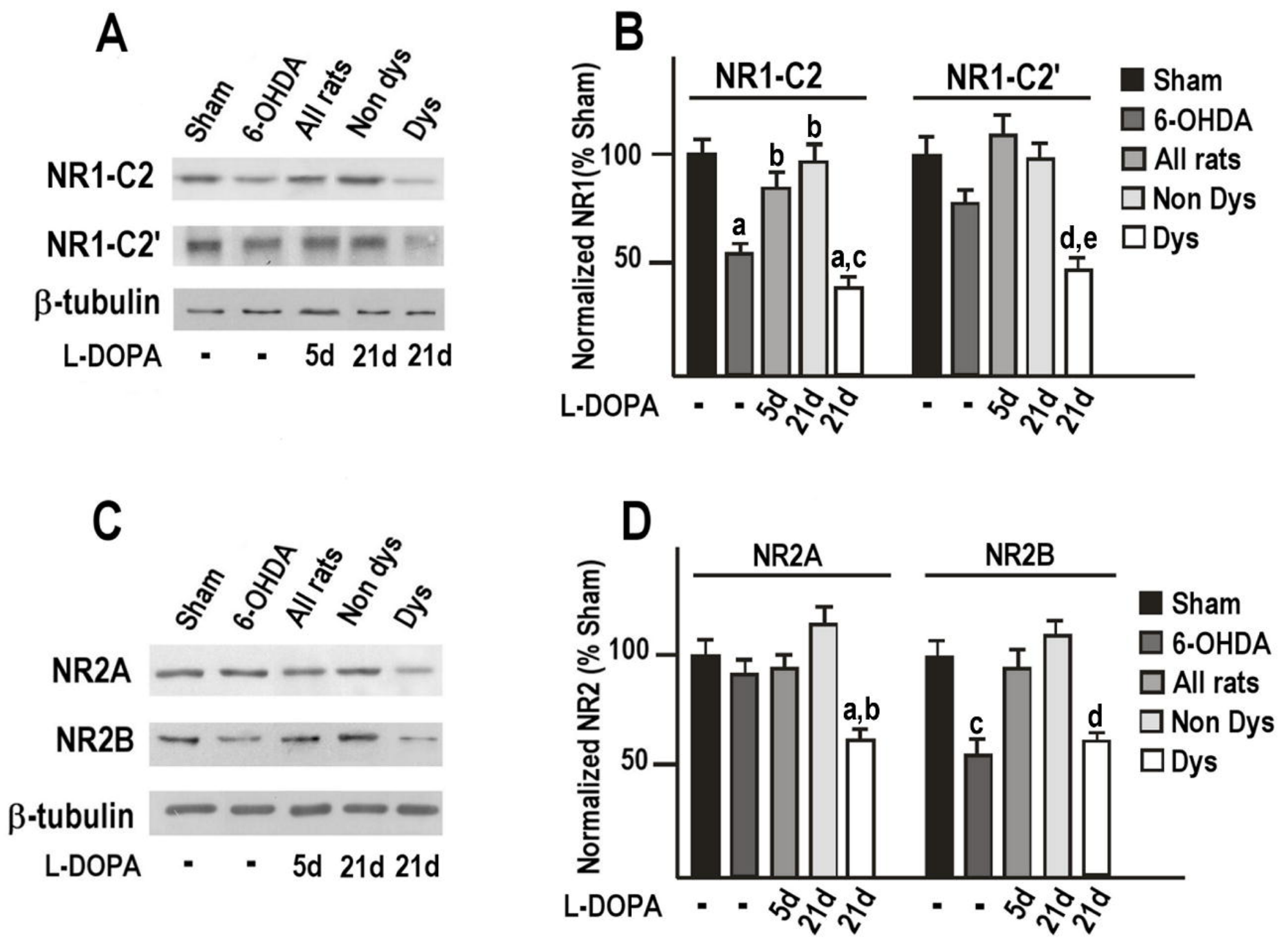
A
Sham
6-OHDA
All rats
Non dys
Dys
NR1-C2
NR1-C2'
β-tubulin
L-DOPA - - 5d 21d 21d
B
NR1-C2
NR1-C2'
Sham
6-OHDA
All rats
Non Dys
Dys
Normalized NR1(% Sham)
100
50
a
b
b
a,c
d,e
L-DOPA - - 5d 21d 21d - - 5d 21d 21d
C
Sham
6-OHDA
All rats
Non dys
Dys
NR2A
NR2B
β-tubulin
L-DOPA - - 5d 21d 21d
D
NR2A
NR2B
Sham
6-OHDA
All rats
Non Dys
Dys
Normalized NR2 (% Sham)
100
50
a,b
c
d
L-DOPA - - 5d 21d 21d - - 5d 21d 21d

Figure 2

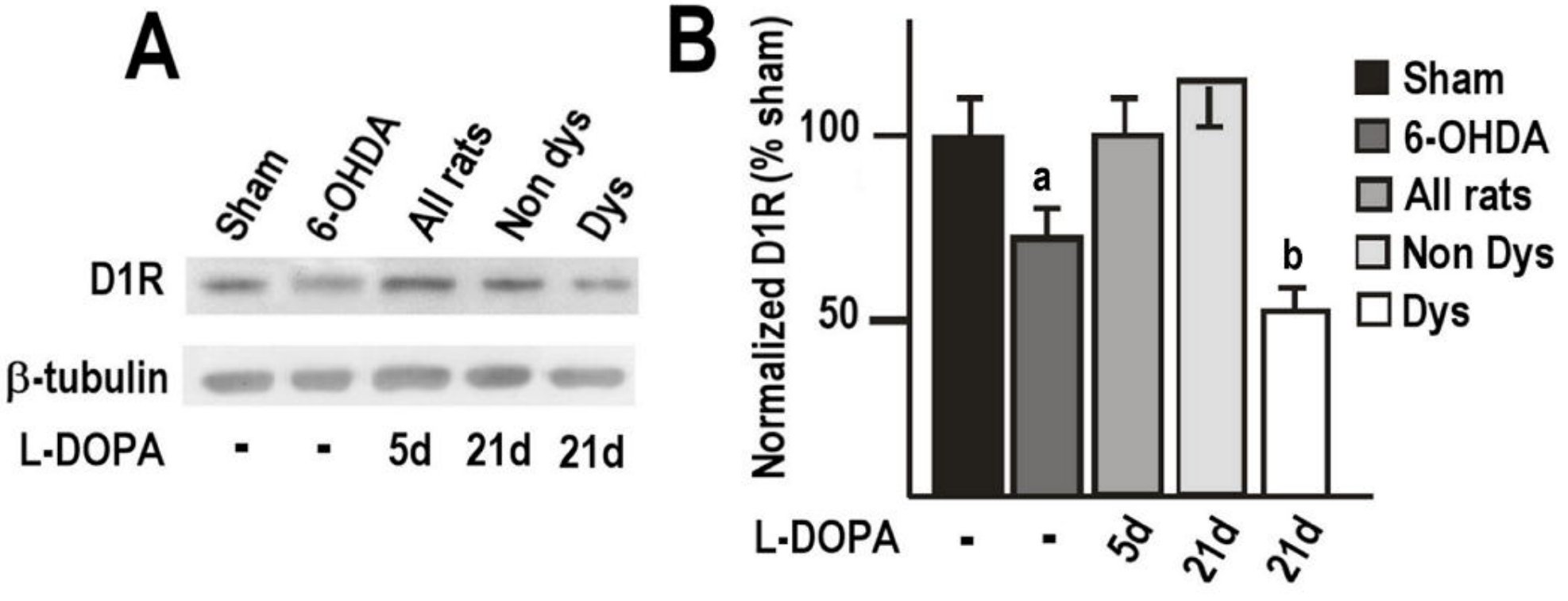
A
Sham
6-OHDA
All rats
Non dys
Dys
D1R
β-tubulin
L-DOPA
-
-
5d
21d
21d
B
Normalized D1R (% sham)
100
50
a
b
Sham
6-OHDA
All rats
Non Dys
Dys
L-DOPA
-
-
5d
21d
21d

Figure 3

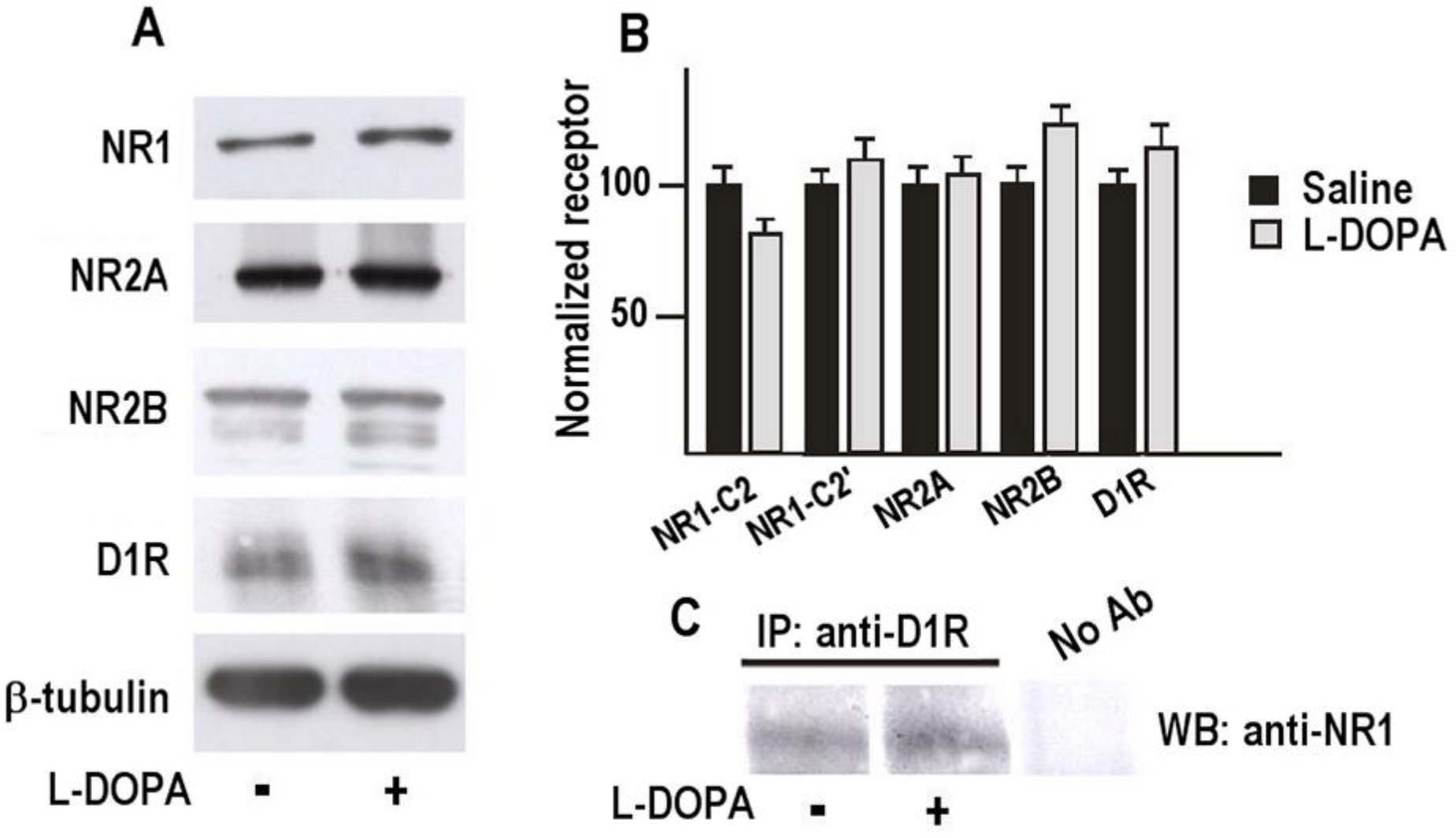
A
NR1
NR2A
NR2B
D1R
β-tubulin
L-DOPA - +
B
Normalized receptor
100
50
Saline
L-DOPA
NR1-C2
NR1-C2'
NR2A
NR2B
D1R
C
IP: anti-D1R
No Ab
WB: anti-NR1
L-DOPA - +

Figure 4

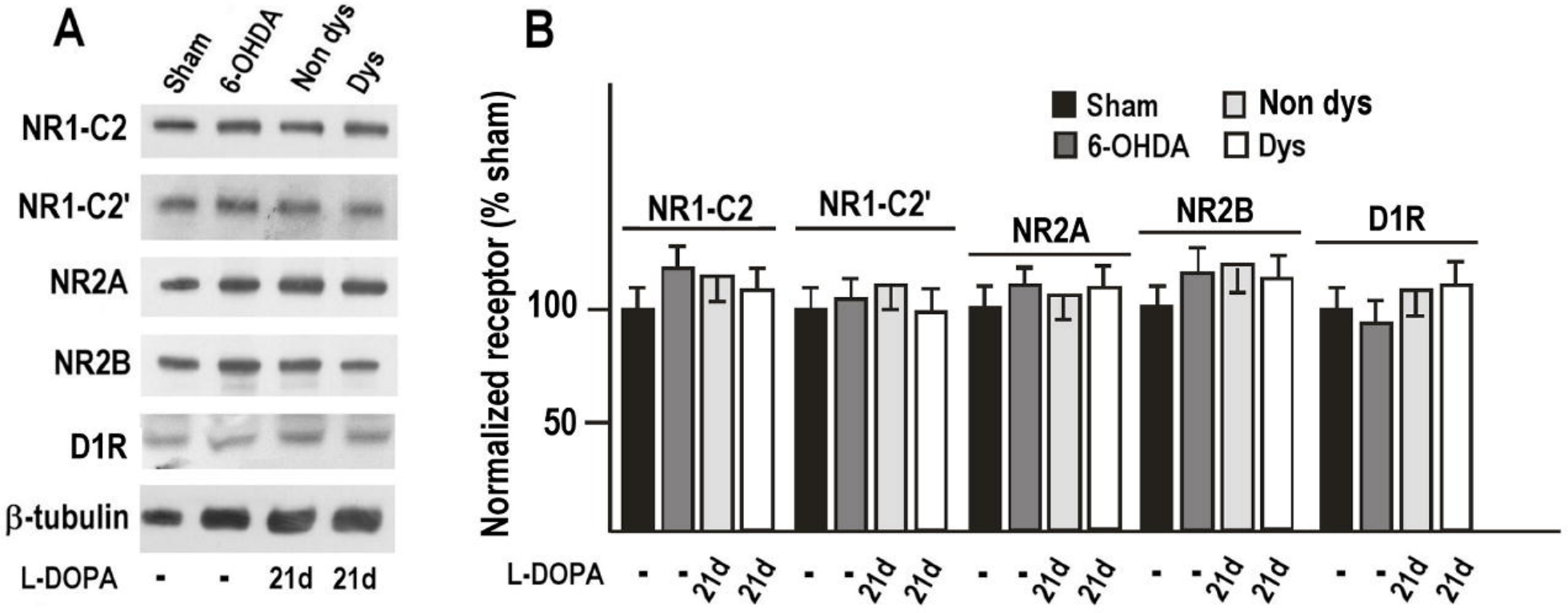
A
Sham
6-OHDA
Non dys
Dys
NR1-C2
NR1-C2'
NR2A
NR2B
D1R
β-tubulin
L-DOPA - - 21d 21d
B
Sham
6-OHDA
Non dys
Dys
Normalized receptor (% sham)
100
50
NR1-C2
NR1-C2'
NR2A
NR2B
D1R
L-DOPA - - 21d 21d

Figure 5

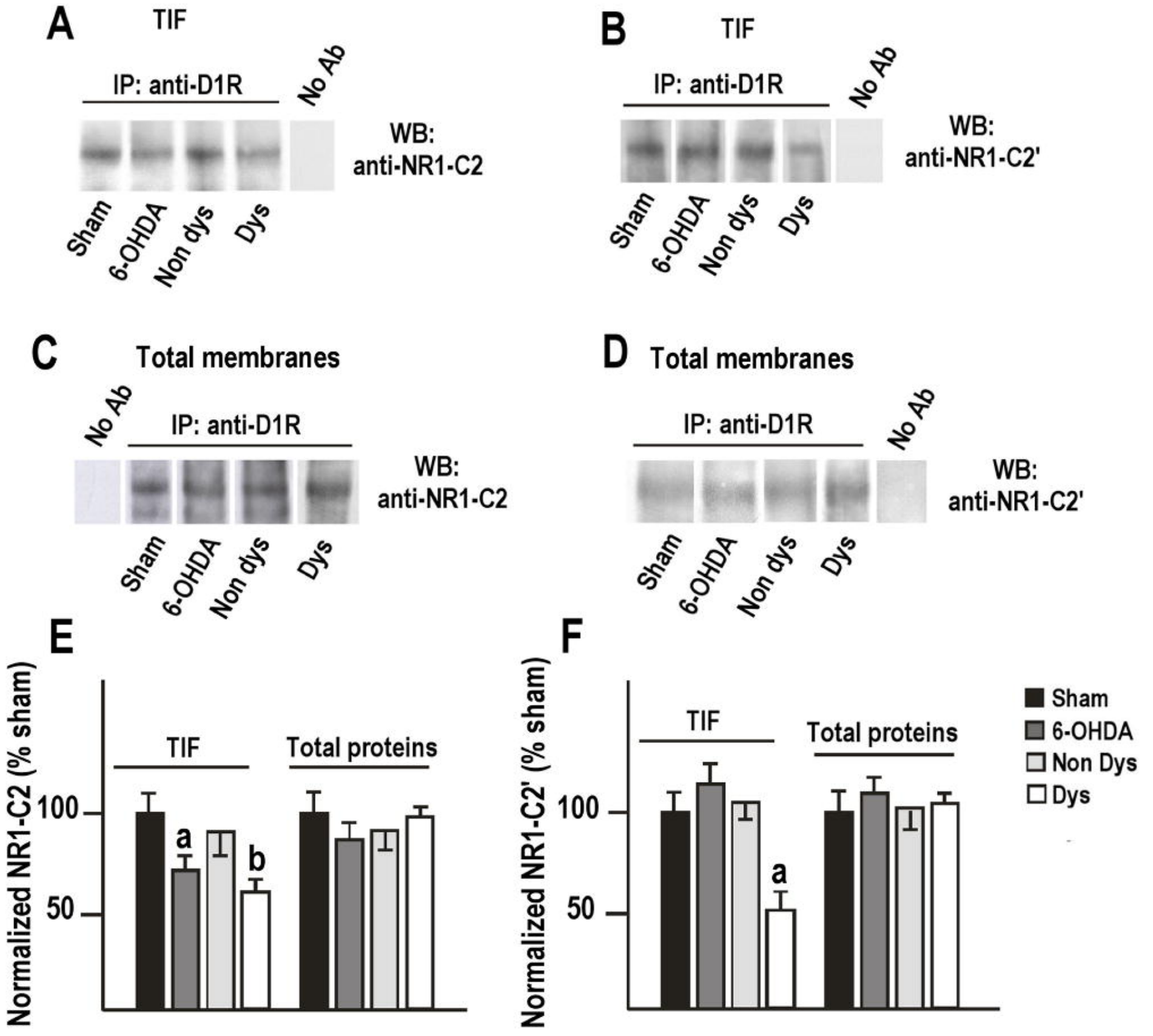
A
TIF
IP: anti-D1R
No Ab
WB:
anti-NR1-C2
Sham
6-OHDA
Non dys
Dys
B
TIF
IP: anti-D1R
No Ab
WB:
anti-NR1-C2'
Sham
6-OHDA
Non dys
Dys
C
Total membranes
No Ab
IP: anti-D1R
WB:
anti-NR1-C2
Sham
6-OHDA
Non dys
Dys
D
Total membranes
IP: anti-D1R
No Ab
WB:
anti-NR1-C2'
Sham
6-OHDA
Non dys
Dys
E
Normalized NR1-C2 (% sham)
100
50
TIF
Total proteins
a
b
F
Normalized NR1-C2' (% sham)
100
50
TIF
Total proteins
a
Sham
6-OHDA
Non Dys
Dys

Figure 6

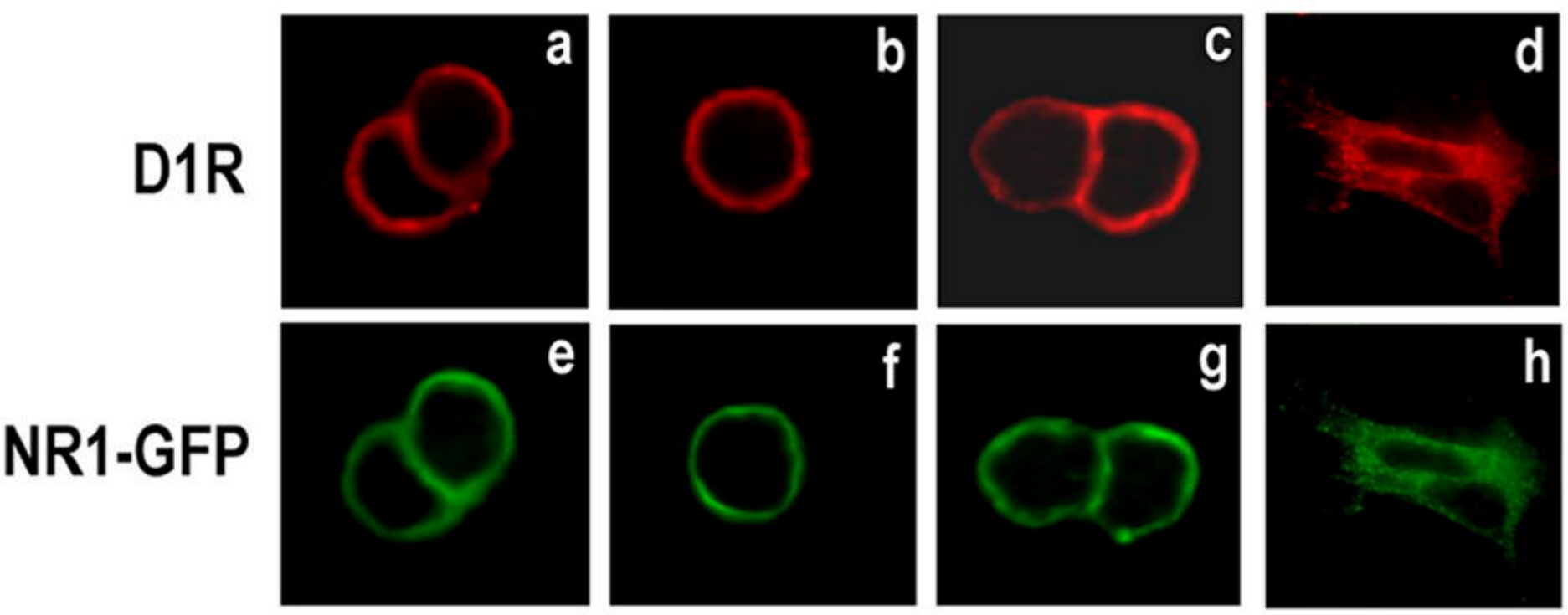
D1R
NR1-GFP
a
b
c
d
e
f
g
h
Untreated
SKF81297
Glu/Gly
SKF81297
+
Glu/Gly

Figure 7