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L-DOPA Impairs Proteasome Activity in Parkinsonism through D₁ Dopamine Receptor

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Aberrant membrane localization of dopamine D₁ receptor (D1R) is associated with L-DOPA-induced dyskinesia (LID), a major complication of L-DOPA treatment in Parkinson's disease (PD). Since the proteasome plays a central role in modulating neuronal response through regulation of neurotransmitter receptor intraneuronal fate, we hypothesized that the ubiquitin-proteasome proteolytic pathway could be impaired in LID. Those LIDs are actually associated with a striatum-specific decrease in proteasome catalytic activity and accumulation of polyubiquitinated proteins in experimental rodent and monkey parkinsonism. We then demonstrated that such decreased proteasome catalytic activity (1) results from D1R activation and (2) feed-back the D1R abnormal trafficking, i.e., its exaggerated cell surface abundance. We further showed that the genetic invalidation of the E3 ubiquitin-protein ligase parkin PD gene leads to exaggerated abnormal involuntary movements compared with wild-type mice. We thus established in an unprecedented series of experimental models that impairment of the ubiquitin-proteasome system at specific nodes (E3 ligase parkin, polyubiquitination, proteasome catalytic activity) leads to the same phenomenon, i.e., aberrant behavioral response to dopamine replacement therapy in PD, highlighting the intimate interplay between dopamine receptor and proteasome activity in a nondegenerative context.

Introduction

In Parkinson's disease (PD), dyskinesia occurs as a debilitating effect of long-term treatment by L-3,4-dihydroxyphenylalanine (L-DOPA) (Cotzias et al., 1969). Experimental evidence demonstrates that dyskinesia is due to complex alterations in dopamine signaling in neurons of dopamine-deprived striatum (Bezard et al., 2001a; Jenner, 2008), especially through dysregulation of dopamine D₁ receptor (D1R) intraneuronal trafficking, subcellular localization, and desensitization (Aubert et al., 2005; Guigoni et al., 2007; Berthet et al., 2009).

Recent studies have demonstrated that modulation of proteasome activity dramatically influences the neuronal response to neurotransmitters and related drugs (DiAntonio and Hicke, 2004; Bingol and Schuman, 2006) through interactions with neu-

rotransmitter receptor biosynthesis and activity (Patrick et al., 2003; Bingol and Schuman, 2006). Impaired proteasome activity has especially been demonstrated to contribute to pathological conditions involving chronic stimulation of neurotransmitter receptors such as opiate dependence and chronic pain. Interestingly, L-DOPA-induced dyskinesia (LID) in PD, associated with chronic stimulation of dopamine receptors, correlates with an increased membranous localization of D1R (Guigoni et al., 2007; Berthet et al., 2009). Since dopamine, the natural agonist of the receptor, is present in large amounts after L-DOPA administration (Meissner et al., 2006), the D1R should internalize as would any G-protein-coupled receptor in the condition of overstimulation. Such impairment in receptor fate therefore suggests the contribution of an altered proteasome function, a key feature that has yet to be explored.

We sought to study whether interactions between dopamine signaling and proteasome may be part of the neuronal events that lead to dyskinesia. We used *in vitro* and *in vivo* models to demonstrate impaired proteasome activity under the control of dopamine signaling in animal models of dyskinesia, specifically in the striatal medium spiny neurons. Our findings demonstrate a hitherto unknown pathological mechanism linking dopamine receptor and proteasome catalytic activity.

Materials and Methods

Ethic statement

All experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for care

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of laboratory animals (1) in an AAALAC-accredited facility following acceptance of study design by the Institute of Laboratory Animal Science (Chinese Academy of Science, Beijing, China) Institutional Animal Care and Use Committee for nonhuman primate experiments and (2) in a government-approved facility following acceptance of study design by the Ethical Committee of Centre National de la Recherche Scientifique, Région Aquitaine for rodent experiments. Skilled veterinarians supervised animal care and maintenance.

Drugs

Unless otherwise noted, drugs were obtained from Sigma. Toxins used for modeling PD were the 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine hydrochloride (MPTP). The animals were treated with L-DOPA, i.e., either a combination of L-DOPA methyl ester and benserazide in rodents or the human oral formulation Modopar (Roche, L-DOPA/carbidopa, ratio 4:1). Dopamine receptor agonists were the D1R agonist SKF-82958 and the D2R agonist Quinpirole. The dopamine D1R antagonist was the SCH-23390. Deep anesthesia was obtained with pentobarbital (Sanofi-Aventis). Two proteasome inhibitors were used, namely the bortezomib (Janssen-Cilag) and the MG132 (Enzo Life Sciences).

Monkey experiments

Forty-five female rhesus monkeys (*Macaca mulatta*, Xierxin; mean age = 5 ± 1 years; mean weight = 5.3 ± 0.8 kg) were used. Animals were singly housed under controlled conditions of humidity ($50 \pm 5\%$), temperature ($24 \pm 1^\circ\text{C}$), and light (12 h light/12 h dark cycle, lights on at 8:00 A.M.); food and water were available *ad libitum*. Experiments were executed according to previously published procedures (Bezard et al., 2001b; Bézard et al., 2003; Gold et al., 2007; Ahmed et al., 2010). As tissues from these animals have been used in former studies, the experimental design, the behavioral characteristics, and the extent of lesions have been previously published (Fernagut et al., 2010; Santini et al., 2010). Briefly, animals were randomly assigned to a particular treatment group. Six animals were kept as untreated controls (control group), six monkeys received a single dose of 20 mg/kg, p.o. L-DOPA (control acute L-DOPA), and six monkeys received 20 mg/kg twice daily for 3 months (control chronic L-DOPA). The remaining 27 animals were treated daily (9:00 A.M.) with 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) hydrochloride (0.2 mg/kg, i.v., Sigma) dissolved in saline according to a previously described protocol (Bezard et al., 2001b). Following stabilization of the MPTP-induced syndrome, animals received twice daily either saline (MPTP: 6 monkeys), or a single dose of L-DOPA (MPTP acute L-DOPA: 6 monkeys) for 3 months (MPTP chronic L-DOPA: 15 monkeys) at a tailored dose designed to fully reverse the parkinsonian features and develop dyskinesia (20 mg/kg L-DOPA p.o.). Nine of the 15 monkeys developed severe and reproducible dyskinesia (MPTP intoxicated, dyskinetic monkey group), whereas six did not (MPTP intoxicated, nondyskinetic monkey group).

Animal behavior was assessed using videotape recordings of monkeys in their home cages as previously described (Bézard et al., 2003; Gold et al., 2007; Ahmed et al., 2010). All observers were blinded with regard to the experimental protocol. During each session, two examiners evaluated the level of motor performance of each animal, coaxing them to perform various tasks by offering appetizing fruits. Animals received supplemental feeding from day 7 onwards to maintain their body weight as constant as possible. The degree of parkinsonism was scored daily using a validated parkinsonian macaque clinical scale (Bézard et al., 2003; Gold et al., 2007; Ahmed et al., 2010), while the severity of dyskinesia was rated using the validated dyskinesia disability scale (Bézard et al., 2003; Gold et al., 2007; Ahmed et al., 2010).

At the end of the experiment, all animals were killed by sodium pentobarbital overdose (150 mg/kg, i.v.), 60 min after the last vehicle or L-DOPA/carbidopa dose, a time at which dyskinesia was maximal in the dyskinetic group. Brains were removed quickly after death. Each brain was bisected along the midline and the two hemispheres were immediately frozen by immersion in isopentane (-45°C) and then stored at -80°C . Tissue of one hemisphere was sectioned coronally at $20 \mu\text{m}$ in a cryostat at -17°C , thaw-mounted onto gelatin-coated slides, dried on

a slide warmer, and stored at -80°C for dopamine transporter binding experiments used to estimate the extent of the lesion in the MPTP-treated groups (Fernagut et al., 2010; Santini et al., 2010). For the other hemisphere, dorsal striatum and frontal cortex were dissected from $300\text{-}\mu\text{m}$ -thick sections for the proteasome activity assays and Western blotting experiments.

Rodent experiments

DAT knock-out mice. The DAT mutant mice were generated by *in vivo* homologous recombination as previously described (Giros et al., 1996). Fifteen female mice between the ages of 2–4 months were used for proteasome activity assays: five wild-type mice (DAT^{+/+}), five heterozygous mice (DAT^{+/-}), and five homozygous mice (DAT^{-/-}). Mice were killed by cervical dislocation followed by decapitation; their brains were removed quickly and immediately frozen by immersion in isopentane (-45°C) and stored at -80°C . Striatal coronal sections ($300 \mu\text{m}$ thick) were cryostat cut and used for the proteasome activity assays.

Ub^{G76V}-GFP transgenic mice. The Ub^{G76V}-GFP transgenic mice were used for *ex vivo* and *in vivo* brain experiments [B6.Cg-Tg(CAG-Ub*G76V/GFP)2Dant/J (The Jackson Laboratory)]. This transgenic mouse strain carries a green fluorescent protein Ub^{G76V}-GFP reporter with a constitutively active degradation signal (Lindsten et al., 2003). *Ex vivo*: 8 heterozygous 8 d postnatal mice were used to check whether proteasome inhibitor leads to GFP accumulation in striatal brain slices. Brain slices were performed as previously described (Baufreton and Bevan, 2008). The effect of proteasomal inhibition on the GFP-tagged peptide was controlled by incubation of slices with bortezomib (10 nM, 20 nM, Janssen-Cilag) (Crawford et al., 2006) for 6 h. *In vivo*: 12 heterozygous male mice (20–33 g) were used to study the effect of D1R stimulation on proteasomal activity in dopamine-depleted striatum by detecting the accumulation of the GFP-tagged peptide reporter of proteasomal activity. The 6-OHDA lesion was performed as described previously (Cenci and Lundblad, 2007; Fasano et al., 2010). Mice underwent stereotaxic surgery under 1.2–1.5% isoflurane. Each mouse received a unilateral injection of 6-OHDA (1 μl /injection; 3 $\mu\text{g}/\mu\text{l}$) into the right medial forebrain bundle at the following coordinates according to the mouse brain atlas: anteroposterior (AP) -0.7 mm, mediolateral (ML) -1.2 mm, dorsoventral (DV) -4.7 mm, relative to bregma and the dural surface. Animals were allowed to recover for 2 weeks before benserazide only (15 mg/kg, i.p.; $n = 8$) and L-DOPA/benserazide (6 mg/kg/15 mg/kg, i.p.; $n = 4$) treatment for 7 d. In such conditions, L-DOPA induces the gradual development of dyskinetic-like abnormal involuntary movements (AIMs). On the penultimate day, for the benserazide group, a subset of the above animals ($n = 4$) received three additional acute challenges of the D1R agonist SKF-82958 (2 mg/kg, i.p.) every 12 h. All mice were killed 60 min after the last benserazide or L-DOPA injection or 6 h after the last SKF-82958 injection. Severity of nigrostriatal denervation was assessed using tyrosine hydroxylase (TH) immunohistochemistry (Fasano et al., 2010).

Reserpine-treated mice. Twenty-four male 3-month-old C57BL/6 mice were injected with saline ($n = 8$) or a combination of reserpine (5 mg/kg, i.p.) and α -methyl-p-tyrosine (a tyrosine hydroxylase inhibitor; 250 mg/kg, i.p.) at 20 and 4 h, respectively (Garcia et al., 2003), before a further pharmacological challenge with saline ($n = 8$) or the D1R agonist SKF-82958 (2 mg/kg, i.p.; $n = 8$). Mice were killed 1 h later by cervical dislocation followed by decapitation; their brains were removed quickly and immediately frozen by immersion in isopentane (-45°C) and stored at -80°C . Striatal coronal sections ($300 \mu\text{m}$ thick) were cryostat cut and used for the proteasome activity assays as well as Western blotting experiments.

Parkin^{-/-} mice. Parkin^{-/-} mice [B6.129S4-Park2^{tm1Shn}/J (The Jackson Laboratory)] bearing a germline disruption of exon 3 were generated by *in vivo* homologous recombination as previously described (Goldberg et al., 2003). Male mutant ($n = 6$) and wild-type controls ($n = 18$) mice were used for the 6-OHDA lesion at the age of 2–4 months (Fasano et al., 2010). Animals were allowed to recover for 2 weeks before benserazide (15 mg/kg, i.p.) and L-DOPA (1.5 and 3 mg/kg, i.p.). Starting from day 16, mice were treated for nine consecutive days with an escalating L-DOPA dosing regimen (1.5 and 3 mg/kg) plus benserazide (15 mg/kg;

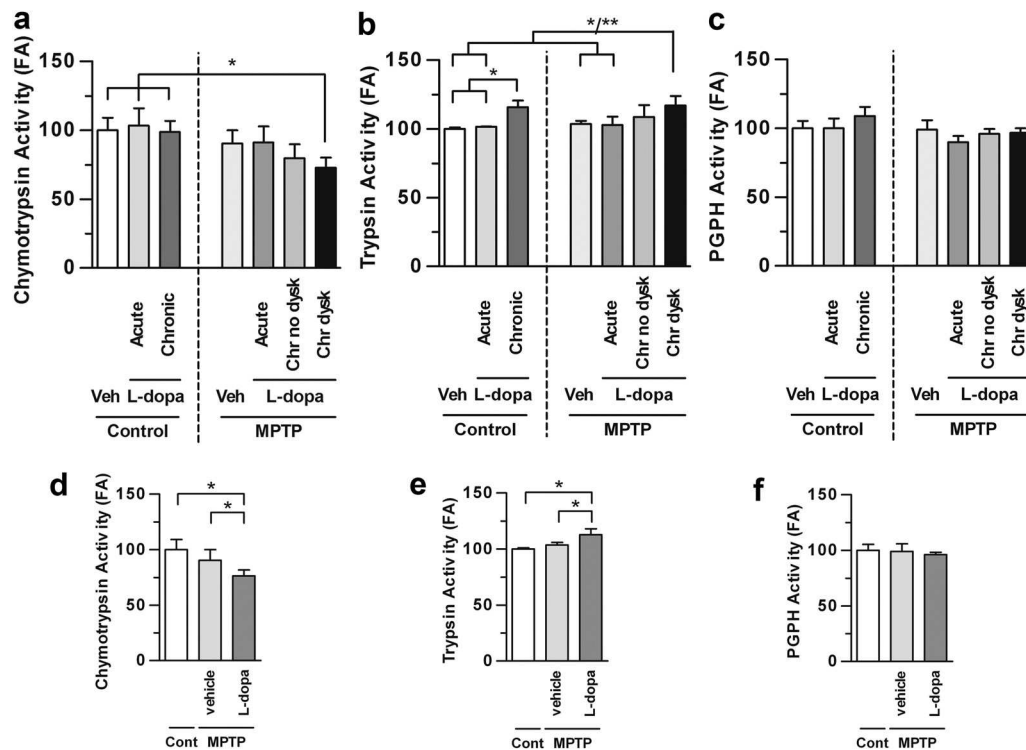


Figure 1. Decreased chymotrypsin catalytic activity in striatum of the dyskinetic MPTP-lesioned monkeys. *a–c*, Striatal homogenates of normal and MPTP-treated macaque monkey that were administered vehicle or L-DOPA, acutely or chronically, were processed to evaluate 20S proteasomal chymotrypsin-like (one-way ANOVA followed by Tukey–Kramer multiple *post hoc* test; $F_{(6,14)} = 3.74, p < 0.01$), trypsin-like ($F_{(6,14)} = 5.323, p < 0.001$), and PGPH activities ($F_{(6,14)} = 3.197, p < 0.05$), using fluorogenic substrates: Suc-LLVY-AMC, Boc-LSTR-AMC, and Z-LLE- β -NA, respectively. Reaction rates are expressed as fluorescence arbitrary units (FA) and standardized by comparison to normal animal values. Data are mean of triplicate measurements \pm SEM ($n = 6$ animals for each group). *d–f*, Subset analysis in which dyskinetic and nondyskinetic L-DOPA-treated MPTP-lesioned monkeys are pooled to focus upon effect of pharmacological treatment in dopamine-depleted striatum without considering the differential behavioral outcome. Chymotrypsin-like (one-way ANOVA followed by Tukey–Kramer multiple *post hoc* test; $F_{(2,7)} = 11.102, p < 0.0001$), trypsin-like ($F_{(2,7)} = 10.776, p < 0.0001$), and PGPH activities ($F_{(2,7)} = 0.9237$). * indicates a significant difference between connected groups.

wild-type, $n = 10$; parkin^{-/-}, $n = 6$) or with benserazide (WT, $n = 8$). AIMs were scored using a 0–4 rating scale according to validated mouse model of LID (Lundblad et al., 2005). Every morning, mice were placed individually in large transparent boxes and observed for 1 min every 20 min during 120 min after L-DOPA injection. On day 10, all mice were killed 60 min after the last benserazide or L-DOPA injection (pentobarbital 30 mg/kg, i.p.). Severity of nigrostriatal denervation was assessed using (TH) immunohistochemistry (see Fig. 3*b*) (Fasano et al., 2010).

Wild-type rats. 30 adult male Sprague Dawley rats (Charles River Laboratories) weighing 175–200 g at the beginning of the experiment were used. On day 0 of the protocol, unilateral dopamine deprivation of the striatum was obtained by 6-OHDA (3 μ g/ μ l) injection in the right medial forebrain bundle (2.5 μ l at AP = -3.7 mm, ML = +1.7 mm, and DV = -8 mm, relative to bregma) as previously described (Meissner et al., 2006; Schuster et al., 2008; Berthet et al., 2009). Animals displaying a loss of TH-immunopositive fibers in the striatum >95% (Bezard et al., 2001b; Guigoni et al., 2005b), as assessed after completion of all experiments, were retained for final analysis. From day 21 postsurgery till day 30, rats were treated once daily with benserazide (15 mg/kg, i.p.) and either vehicle ($n = 20$) or L-DOPA (6 mg/kg, i.p.) ($n = 10$). In such conditions, L-DOPA induces a gradual development of AIMs. On day 29, 10 rats were scored as dyskinetic (score = 12) after observation by a trained investigator as previously described (Meissner et al., 2006; Schuster et al., 2008; Schuster et al., 2009), using a validated rating scale (Cenci et al., 1998; Lundblad et al., 2002). On day 30, all animals received the last vehicle injection +/- L-DOPA. A subset of the above benserazide-treated animals received an additional acute challenge of the D1R agonist SKF-82958 (2 mg/kg i.p.; 15 min after benserazide) 45 min before termination. All animals were killed 60 min after the last benserazide or L-DOPA administration. A total of three experimental groups were thus tested. Four animals were randomly selected in each group for measuring proteasome catalytic activity. Primary medium spiny neuron

culture: Sprague Dawley rat striatal cultures were prepared as previously described (Kowalski and Giraud, 1993; Martin-Negrier et al., 2000; Martin-Negrier et al., 2006). All the reagents for the culture were purchased from Sigma and Invitrogen. For immunohistochemistry, proteasome inhibition was achieved with bortezomib (Raab et al., 2009), prepared as concentrated stock solution of 10 μ M in dimethylsulfoxide (DMSO) and diluted to the final concentration of 10 nM, 0.1% DMSO (Crawford et al., 2006). In all other conditions, incubation media contained 0.1% of DMSO. For the agonist-induced internalization of D1R, cells were incubated with 10 μ M of the full D1R agonist SKF-82958 (Martin-Negrier et al., 2000; Martin-Negrier et al., 2006). To test the effect of the inhibition of proteasome activity on D1R trafficking, neurons were incubated with bortezomib for 60 min before 60 min incubation with SKF-82958. Throughout the incubation, cells were maintained at 37°C in a 5% CO₂ incubator. At the end of incubation, the medium was rapidly removed and replaced by fixative agent. For proteasomal activity assays, cells cultured for 14 d were incubated at 37°C in a 5% CO₂ incubator with either H₂O, dopamine (10 μ M) and acid ascorbic, the full D1R agonist SKF-82958 (10 μ M) (Martin-Negrier et al., 2000, 2006), the D1R antagonist SCH-23390 (2 μ M), the D2R agonist Quinpirole (10 μ M) (Brami-Cherrier et al., 2002), or both agonists, diluted in culture medium for 60 min. At the end of incubation, the medium was rapidly removed and cells were rinsed three times with PBS, detached mechanically, and collected. After centrifugation of 10 min at 800 rpm, PBS was removed and cells were resuspended in homogenization buffer.

Immunohistochemistry

D1R immunofluorescence detection. D1R was detected by immunofluorescence according to previously described and validated procedures (Martin-Negrier et al., 2000, 2006), using a monoclonal antibody raised in mouse against a 126 aa sequence corresponding to the C terminus of the rat D1A receptor (Invitrogen) (Lee et al., 2004). Neurons were viewed

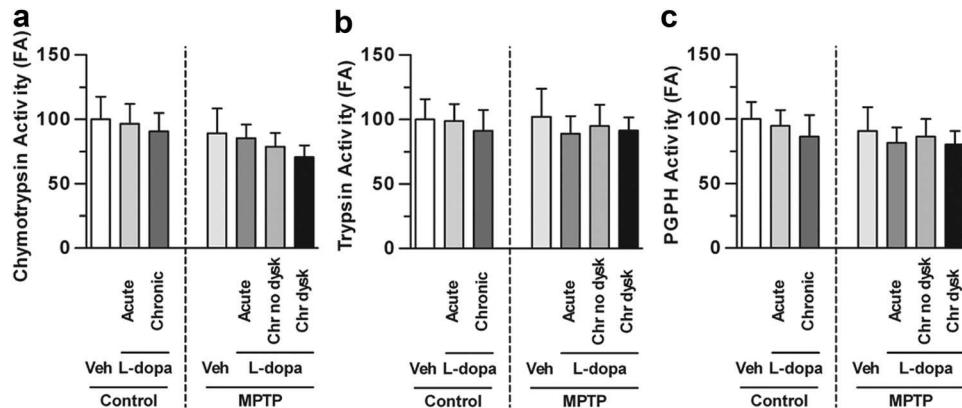


Figure 2. Dysfunctions in catalytic activities are striatum-specific. Superior frontal gyrus (SFG) homogenates of normal and MPTP-treated macaque monkeys that were administered vehicle or L-DOPA, acutely or chronically, were processed to evaluate 20S proteasomal chymotrypsin-like [one-way ANOVA; ($F_{(6,14)} = 0.9912$, not significant (ns)), trypsin-like ($F_{(6,14)} = 0.2084$, ns), and PGPH activities ($F_{(6,14)} = 0.15074$, ns), using fluorogenic substrates: Suc-LLVY-AMC, Boc-LSTR-AMC, and Z-LLE- β -NA, respectively. No change in 20S proteasomal activities was observed in SFG of the dyskinesic MPTP-lesioned monkeys. Reaction rates are expressed as fluorescence arbitrary units (FA) and standardized by comparison to normal animal values. Data are mean of triplicate measurements \pm SEM ($n = 6$ animals for each group).

and randomly selected by the investigator with a Zeiss Axioplan 2 fluorescence microscope with a $\times 100$, 1.4 N.A. lens and captured on a Micromax-cooled CCD camera (Princeton Instruments) using Fluo Up imaging V1.00 software (Explora Nova).

Quantitative analysis was performed in four different conditions: after 60 min of treatment with SKF-82958, after 60 min of treatment with bortezomib, with bortezomib for 60 min followed by 60 min of treatment with SKF-82958, and in control. Data are the results of counting three sets of independent experiments for the four conditions. In each experiment, the neurons were captured from at least three different coverslips for each condition. A total of 61, 54, 45, and 71 neurons, respectively, were analyzed for each condition described above. The analysis was performed using Morpho expert V1.00 software (Explora Nova). Images were background subtracted and thresholded. Threshold value was determined for each image as two-fold above background. For each labeled neurite, the number of D1R labeled puncta per micrometer and the mean surface value of the D1R puncta were determined.

Immunohistochemical detection of GFP. The green fluorescent protein (GFP) was detected using a rabbit polyclonal antibody raised against GFP that was isolated directly from *A. victoria* and purified by ion-exchange to remove nonspecific Ig (Invitrogen) (Franciosi et al., 2007). Immunoperoxidase experiments: The GFP immunodetection was performed as previously described (Ahmed et al., 2010). Immunofluorescence experiments: For GFP immunodetection on slices, a rabbit primary antibody was used at the concentration of 1:1500. After one night of incubation at 4°C, the secondary antibody Alexa 488-conjugated goat anti-rabbit IgG (1:400 in PBS, Invitrogen) concentration was applied for 2 h.

Proteasomal activity assay

Brain structures or cells were placed on ice and homogenized in extraction buffer (20 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM ATP, 10% glycerol, 0.5 mM EDTA, 0.5% Igepal, and 5 mM MgCl₂) (Kisselev and Goldberg, 2005). The lysates were centrifuged at 14,000 \times g at 4°C for 15 min. The resulting supernatants were placed on ice and assayed for protein concentrations by the Lowry method (Bio-Rad DC Protein Assay). The three activities of the 20S proteasomes [chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase (PGPH)-like activities] were analyzed using fluorogenic substrates: Suc-LLVY-MCA, Boc-

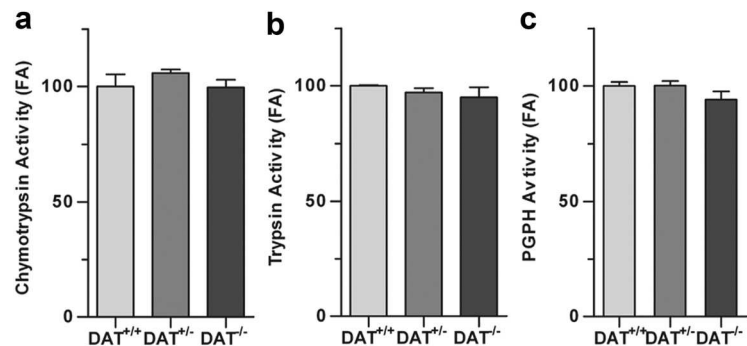


Figure 3. Dysfunctions in catalytic activities are due to the association of chronic L-DOPA treatment with dopamine depletion. Striatal homogenates of a mouse model of hyperdopaminergia without degeneration of the nigrostriatal pathway, the dopamine transport knock-out mice (DAT^{-/-}), normal (DAT^{+/+}), and heterozygous (DAT^{+/-}) mice, were processed to evaluate 20S proteasomal chymotrypsin-like [one-way ANOVA; ($F_{(2,6)} = 1.731$, not significant (ns)), trypsin-like ($F_{(2,6)} = 2.174$, ns), and PGPH activities ($F_{(2,6)} = 4.838$, $p < 0.05$), using fluorogenic substrates: Suc-LLVY-AMC, Boc-LSTR-AMC, and Z-LLE- β -NA, respectively. No change in 20S proteasomal activities was observed in the striatum of DAT^{-/-} mice compared with both DAT^{+/+} and DAT^{+/-}. Reaction rates are expressed as fluorescence arbitrary units (FA) and standardized by comparison to normal animal values. Data are mean of triplicate measurements \pm SEM ($n = 4$ animals for each group).

LSTR-MCA, and Z-LLE- β -naphthylamide (Sigma). Five, 10, 15, 40, or 50 μ g of protein (depending on structures or cells) and the fluorogenic substrate (final concentration, 50 μ M) were incubated together in a buffer containing 20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 1 mM DTT, and 1 mM ATP. All assays were done in triplicate. The incubation took place at 37°C for 60 min in a 96-well plate. Fluorescence was determined at 355 nm excitation/460 nm emission in a Microplate Spectrofluorometer (Twinkle LB970, Berthold). Proteasomal activity was expressed as arbitrary units. The specificity of the proteasomal assay was confirmed by the ability of 50 μ M MG132 (Enzo Life Sciences), a selective inhibitor of proteasomal function, to nearly totally inhibit fluorescence change.

Western blot analysis of ubiquitinated proteins and parkin

Western blot analysis was performed on monkey and reserpine-treated mice striatum. Monkey tissue patches were homogenized in RIPA buffer (Sigma) with a mixture of protease and phosphatase inhibitors (Pierce), while mice samples homogenized in the same buffer have already been used for the measure of the activity of the proteasome. Blots were run in all conditions from 50 μ g of protein separated by SDS-PAGE. For detection of ubiquitinated proteins, proteins were transferred on polyvinylidene fluoride membranes (Millipore) and subjected to Western blot analysis using a rabbit anti-Ubiquitine 1:1000 (Sigma). Signals were revealed with horseradish peroxidase-conjugated secondary antibodies

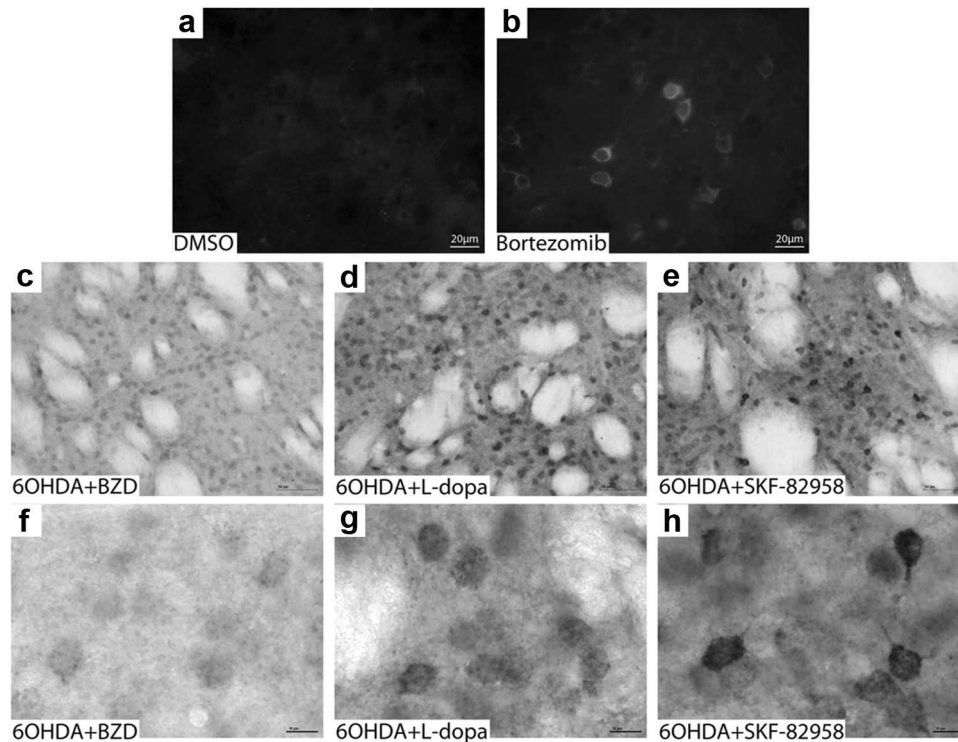


Figure 4. *In vivo* GFP reporter accumulation in 6-OHDA Ub^{G76V}-GFP transgenic mice demonstrates functional proteasome catalytic activity impairment in medium spiny neurons after L-DOPA. **a**, **b**, Immunofluorescent labeling of striatal sections of Ub^{G76V}-GFP mice demonstrates Ub^{G76V}-GFP positive neurons after bortezomib treatment (**b**) as compared with DMSO control (**a**). **c–h**, Immunoperoxidase detection of GFP immunoreactivity (Avidin–biotin DAB Nickel technique) in striatal medium spiny neurons of unilateral 6-OHDA-lesioned mice (scale bars, 50 μm). Control mice (benserazide only) (**c**, **f**); L-DOPA-treated 6-OHDA-lesioned mice with AIMs (**d**, **g**); SKF-82958-treated 6-OHDA-lesioned mice (**e**, **h**). Medium spiny neurons of L-DOPA-treated 6-OHDA-lesioned mice (**d**, **g**) display a strong accumulation of GFP compared with benzerazide group (**c**, **f**). More intense accumulation was observed when mice were treated with D1R full agonist SKF-82958 (**e**, **h**).

and chemiluminescence. Quantification of Western blots was performed using Image J.

For detection of parkin (and the actin reference), proteins were transferred on Nitrocellulose 0.2 μm Membranes (Biorad) and subjected to Western blot analysis using a mouse anti-Parkin 1:1000 (Cell Signaling Technology) and mouse anti-Actin 1:2000 (Sigma). Signals were revealed with IRdye-680-conjugated and IRdye-800CW-conjugated secondary antibodies and fluorescence, respectively. Quantification of Western blots was performed using the odyssey software.

Results

Striatum-specific impairment of proteasome activity in dyskinetic parkinsonian monkeys

Our central hypothesis posits that catalytic activity might be impaired in the L-DOPA-treated dopamine-depleted striatum. We therefore measured the chymotrypsin-like, trypsin-like, and PGPH-like activities of 20S proteasome, which cleave proteins at hydrophobic, basic, and acidic residues, respectively, in the gold standard model of PD and LID. Catalytic activities were measured in striatal homogenates of normal and MPTP-treated macaque monkeys that received vehicle or L-DOPA either acutely or chronically. All assays were performed in the absence and presence of the proteasome inhibitor, MG132, to distinguish proteolysis by the proteasome from alterations by other copurifying proteases. When considering all groups, chymotrypsin-like activity was decreased only in the dyskinetic MPTP-lesioned monkeys ($p < 0.05$; Fig. 1*a*) compared with control animals, being exposed or not to L-DOPA ($p < 0.05$ vs vehicle-treated, acute L-DOPA treated, chronic L-DOPA treated). As data suggested a trend for a decrease in nondyskinetic MPTP-lesioned monkeys, we analyzed the data considering only their exposure to L-DOPA (the nondyskinetic and dyskinetic animals were merged). Interestingly,

L-DOPA-treated MPTP-lesioned animals displayed a decreased chymotrypsin-like activity compared with both the untreated MPTP-lesioned and the vehicle-treated control animals ($p < 0.05$; Fig. 1*d*). This suggests a feature of L-DOPA chronic exposure in dopamine-depleted animals that culminates in dyskinetic animals. By contrast, trypsin-like activity showed a modest but significant increase after chronic L-DOPA exposure in both control and MPTP-lesioned monkeys ($p < 0.05$; Fig. 1*b,e*). The fact that chronic L-DOPA administration in control monkeys also led to an increase in trypsin-like activity rules out a possible involvement of this increase in dyskinesia pathophysiology, as neither normal monkeys nor normal humans exhibit abnormal involuntary movements even after chronic administration. Finally, PGPH-like activity was not affected (Fig. 1*c,f*).

We wondered whether such impairments were striatum-specific or whether they could extend to other brain areas. The same assays performed in the very same monkey population demonstrated that this effect was restricted to the striatum since no change in chymotrypsin-like, trypsin-like, or PGPH activities was observed in other dopamine-innervated brain regions, such as the superior frontal gyrus or prefrontal cortex (Fig. 2).

These data suggest that the dysfunctions in catalytic activities are due to the association of chronic L-DOPA treatment with dopamine depletion. Would this hold true, a hyperdopaminergia superimposed on a nondopamine-depleted striatum would not affect catalytic activity. Proteasome catalytic activities were thus measured in a mouse model of hyperdopaminergia without degeneration of the nigrostriatal pathway, namely the dopamine transport knock-out mouse (DAT^{-/-}). Indeed, DAT^{-/-} mice are known to exhibit a 500% increase in extracellular dopamine content compared with DAT^{+/+} mice (Jones et al., 1998; Benoit-

Marand et al., 2000). No change in proteasomal activity was observed in the striatum of DAT^{-/-} mice compared with both DAT^{+/+} and DAT^{+/-} (Fig. 3). As we have previously shown a strong internalization of D1R in these DAT^{-/-} mice (Dumartin et al., 2000), we concluded that the alterations observed in the monkey model of PD and LID were (1) striatum-specific and (2) the consequence of the chronic L-DOPA exposure superimposed upon dopamine depletion.

Dopamine receptor stimulation decreases proteasome catalytic activity in medium spiny neurons of hemiparkinsonian Ub^{G76V}-GFP transgenic mice

To confirm and localize chymotrypsin-like changes as a marker of proteasome impairment, we developed experiments to measure and visualize the functional index of proteasome activity. We used a proteasome activity reporter mouse line, the Ub^{G76V}-GFP reporter (Lindsten et al., 2003), to address this question. The Ub^{G76V}-GFP transgene was designed with a chicken β -actin promoter upstream of a GFP fused to a mutant ubiquitin moiety (Ub^{G76V}) (Lindsten et al., 2003). Although transcripts of the Ub^{G76V}-GFP fusion gene could be detected at baseline, the G76V substitution prevents removal of the ubiquitin moiety leading to efficient ubiquitination and proteasomal degradation of the fusion protein in these tissues. Administration of proteasome inhibitors or any impairment of proteasome activity thus leads to GFP accumulation in the cells (Lindsten et al., 2003). We first checked that such impairment could be detected in the striatum. Control brain slices from newborn Ub^{G76V}-GFP mice were “*ex vivo*” treated with DMSO or bortezomib, a potent proteasome inhibitor in clinic use for multiple myeloma (Raab et al., 2009). While DMSO treatment (Fig. 4*a*) showed no GFP accumulation, bortezomib treatment led to strong GFP accumulation in striatal medium spiny neurons (Fig. 4*b*).

Adult Ub^{G76V}-GFP mice were then made hemiparkinsonian by 6-OHDA injection in the medial forebrain bundle. Chronically L-DOPA-treated 6-OHDA-lesioned Ub^{G76V}-GFP mice displayed severe abnormal involuntary movements, the rodent analog of dyskinesia (Lundblad et al., 2005). These dyskinetic mice showed a strong accumulation of GFP in the medium spiny neurons of their dopamine-depleted striatum (Fig. 4*d,g*), while benserazide-treated 6-OHDA-lesioned Ub^{G76V}-GFP mice did not present any alteration of proteasome activity (Fig. 4*c,f*). This dataset clearly indicates that L-DOPA administration upon a dopamine-depleted striatum leads to a functional inhibition of proteasome catalytic activity in medium spiny neurons.

L-DOPA-induced decrease in striatal proteasomal activity is mimicked by D1R agonist

As our working hypothesis involved an intricate relationship between dopamine D1R and impairment of proteasomal activity,

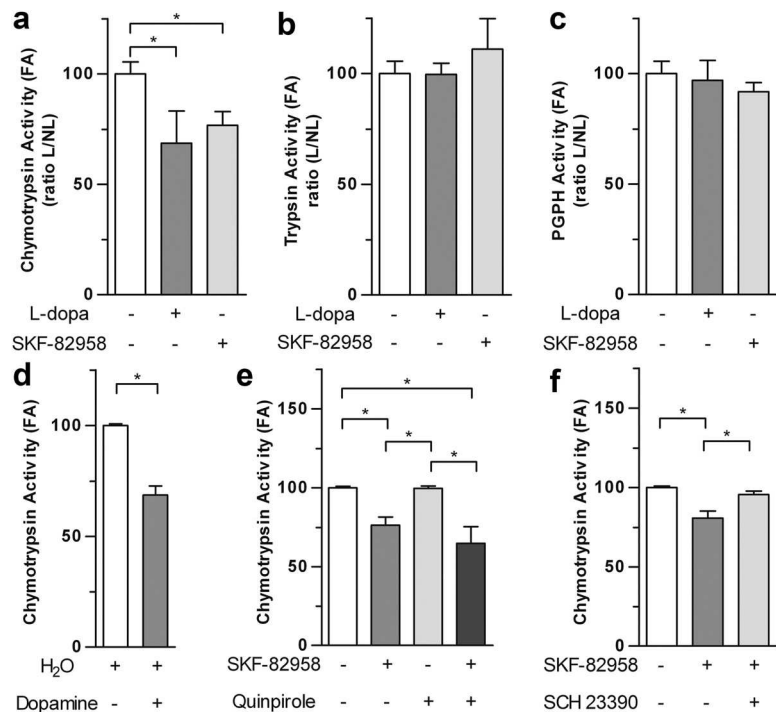


Figure 5. Impairment of proteasome catalytic activity is consequent to D₁ receptor stimulation. *a–c*, L-DOPA-induced decrease in striatal proteasomal activity is mimicked by D1R agonist. 20S proteasomal chymotrypsin-like (*a*), trypsin-like (*b*), and PGPH (*c*) activities were evaluated using fluorogenic substrates in striatal homogenates of 6-OHDA-lesioned rats that were administered vehicle or chronic L-DOPA treatment or acute SKF-82958 injection. Reaction rates are expressed as fluorescence arbitrary units (FA) and standardized by comparison to normal animal values. Data are mean of triplicate measurements \pm SEM ($n = 4$ for each group). Ratio L/NL (mean \pm SEM) represents the FA on lesioned side (L) versus nonlesioned (NL) side. Chronic L-DOPA or acute SKF-82958 treatments show a dramatic decrease only in chymotrypsin-like activity (one-way non-parametric ANOVA, Kruskal–Wallis (KW), followed by Mann–Whitney *post hoc* test; KW = 8.766, $p < 0.05$) (*a*). No changes in 20S proteasomal trypsin-like (KW = 0.2413) (*b*) and PGPH (KW = 3.136) (*c*) activities in striatum were observed. *d–f*, D1R agonist but not D2R decreases proteasomal activity. *d*, 20S proteasomal chymotrypsin-like activity is decreased in rat medium spiny primary culture after dopamine treatment (*t* test; $*p < 0.001$). This decrease is specifically induced by the D1R agonist SKF-82958 since it is (*e*) not achieved by the D2R agonist quinpirole (*t* test; $*p < 0.05$) and (*f*) blocked by the D1R antagonist SCH-23390.

we further investigated this question in the 6-OHDA-lesioned Ub^{G76V}-GFP mice used above, in which GFP accumulation in medium spiny neurons was even more intense when 6-OHDA-lesioned Ub^{G76V}-GFP mice were treated with the D1R full agonist SKF-82958 (Fig. 4*e,h*).

We then used the L-DOPA-treated 6-OHDA-lesioned rat model of PD and LID, with the biochemical approach used in the monkey and DAT mouse models, to assay the chymotrypsin-like, trypsin-like, and PGPH activities of 20S proteasome in striatal homogenates of vehicle-treated 6-OHDA-lesioned rats and L-DOPA-treated 6-OHDA-lesioned rats. Interestingly, only the chymotrypsin-like activity was decreased in L-DOPA-treated 6-OHDA-lesioned rats ($p < 0.05$; Fig. 5*a*) but not the trypsin-like (Fig. 5*b*) and PGPH activities (Fig. 5*c*), thereby confirming the data collected in the Ub^{G76V}-GFP mice. A third group of 6-OHDA-lesioned rats was treated with the D1R full agonist SKF-82958. These SKF-82958-treated 6-OHDA-lesioned rats displayed the exact same impairment of catalytic activities with a significant decrease in chymotrypsin-like activity ($p < 0.05$; Fig. 5*a*) compared with vehicle-treated 6-OHDA-lesioned animals, but not in trypsin-like (Fig. 5*b*) and PGPH (Fig. 5*c*) activities.

In our animal experiments, we primarily used L-DOPA and not dopamine, as dopamine does not cross the blood–brain barrier. Using rat primary striatal cell cultures (Martin-Negrier et al., 2000, 2006), we now show that chymotrypsin-

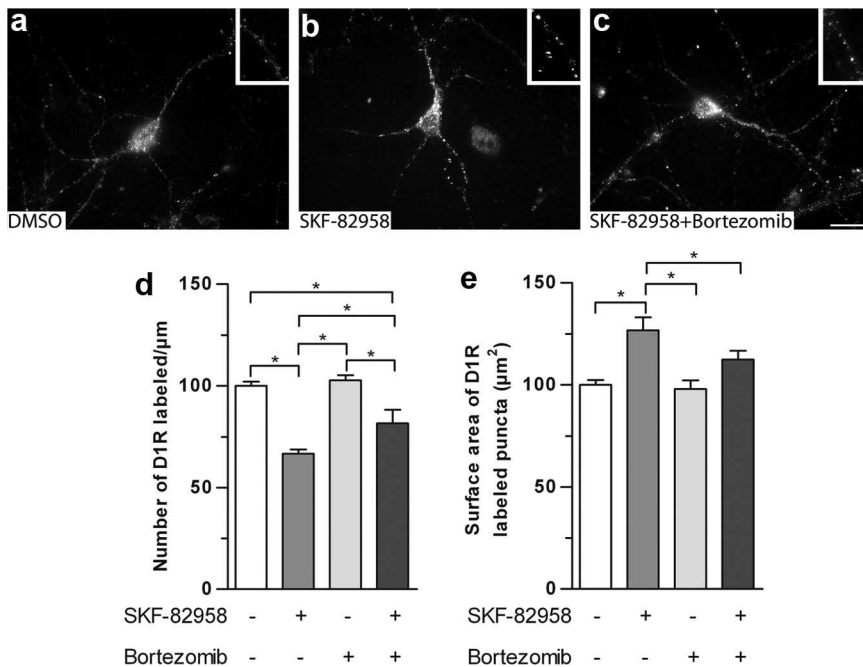


Figure 6. Proteasome inhibition reduces D1R internalization in neurites in 15 d rat striatal cultured neurons. *a*, In control condition, D1R immunofluorescent staining is punctated at the surface of the neurites. *b*, SKF-82958 treatment (60 min) reveals larger and brighter fluorescent spots in the neurites in a compartment corresponding to endosome (Martin-Negrier et al., 2006). *c*, Proteasome inhibition impairs this agonist-induced endocytosis, with reduced modifications of the density and the size of fluorescent puncta. Scale bar, 15 μm. *d–e*, D1R immunolabeling was quantified in the different experimental conditions as (*d*) the mean number of D1R labeled puncta per 100 μm neurites ± SEM and (*e*) the mean value of the surface of D1R labeled puncta ± SEM. Number of D1R puncta decreases (one-way ANOVA followed by Tukey–Kramer *post hoc* test; $F_{(3,226)} = 59.15, p < 0.0001$), while the area of D1R puncta in neurites increases ($F_{(3,185)} = 7.12, p < 0.001$) after 60 min of D1R agonist stimulation in neurites compared with control, as previously described (Martin-Negrier et al., 2006). By contrast, there was no change when neurons were treated with bortezomib (60 min). Combined SKF-82958/bortezomib treatments reduced the decrease in density in D1R labeled in neurites as compared with SKF-82958 treatment alone. * indicates a significant difference between connected groups.

like activity is significantly impaired after dopamine treatment (Fig. 5*d*; $p < 0.0001$). Interestingly, while application of the D1R agonist SKF-82958 mimicked the dopamine-induced decrease in chymotrypsin-like activity (Fig. 5*e*; $p < 0.05$), application of the dopamine D₂ receptor (D2R) agonist quinpirole failed to inhibit this activity (Fig. 5*e*) as well as the trypsin-like and PGPH-like activities. The D1R antagonist SCH-23390, however, prevented the D1R-induced decrease in chymotrypsin-like activity (Fig. 5*f*; $p < 0.05$). Altogether, these data therefore assign a key role to D1R (among dopamine receptors) in mediating the impairment of proteasome activity.

Proteasome inhibition dramatically limits D1R internalization

The hypothesis that proteasomal activity might be impaired arises from the observation that D1R is anchored at the membrane in dyskinetic L-DOPA-treated 6-OHDA-lesioned rats (Berthet et al., 2009) and MPTP-treated monkeys (Guigoni et al., 2007). In the same rat primary striatal cell culture, D1R stimulation by the D1R full agonist SKF-82958 induces a dramatic redistribution of D1R immunolabeling corresponding to an internalization of the receptor in early endosomes, as expected and previously reported (Martin-Negrier et al., 2000, 2006) (Fig. 6*b,d,e*). Interestingly, however, such agonist-induced endocytosis was significantly impaired when proteasome activity was simultaneously inhibited by coapplication of bortezomib, as evidenced by the reduced decrease in D1R immunopositive puncta density in neurites of striatal neurons (Fig. 6*c–e*). Proteasome inhibition alone, however,

had no effect on the D1R distribution as compared with the control situation (Fig. 6*d,e*). These data therefore suggest that D1R stimulation is the “*primum movens*” leading to the inhibition of the chymotrypsin-like catalytic activity of the proteasome. This may in turn contribute to a membrane localization of D1R through reduction of its degradation in the course of the homologous desensitization process.

D1R-induced decrease in striatal proteasomal activity is accompanied by accumulation of polyubiquitinated proteins

A direct consequence of the D1R-mediated inhibition of the chymotrypsin-like catalytic activity of the proteasome should be a relative accumulation of polyubiquitinated proteins. We therefore measured the amount of polyubiquitinated proteins in striatal homogenates of normal and MPTP-treated macaque monkeys that received vehicle or chronic L-DOPA. In keeping with the impairment in proteasome catalytic activity reported in the very same animals (Fig. 1*d*), chronic L-DOPA treated MPTP-lesioned monkeys displayed increased levels of ubiquitinated proteins compared with control animals ($p < 0.05$; Fig. 7*a,b*). That the accumulation of polyubiquitinated proteins is mediated by activation of D1R was confirmed in the reserpine-treated mouse model of PD. D1R full agonist SKF-82958/reserpine-treated mice displayed accumulation of

polyubiquitinated proteins compared with saline/reserpine-treated mice (Fig. 7*c,d*) that showed a significant impairment in chymotrypsin-like activity (Fig. 7*e*). Such a comprehensive dataset strongly relates D1R stimulation in the parkinsonian brain with functional impairment of proteasome-mediated protein catabolism and with occurrence of dyskinesia. Since we used highly translational experimental models, this is likely to reflect what happens in idiopathic PD.

The E3 ligase Parkin^{-/-} mice develop more severe abnormal involuntary movements

Interestingly, the familial PD gene product parkin is an E3 ubiquitin-protein ligase (Shimura et al., 2000), whose function is to ubiquitinate its partners for later degradation by the proteasome. Besides the specific processes causing cell loss in the nigra, these patients also develop an early appearance of LID. While the above dataset points out a key role for the impaired proteasome activity itself in the dysregulated response to chronic L-DOPA treatment in idiopathic PD, this familial form of PD might involve a further possible mechanism leading to the dysregulation of protein degradation. Parkin levels are not affected by nigrostriatal denervation or depletion in both the MPTP-lesioned macaque and reserpine-treated models of PD and LID (Fig. 7*f,g*). In the parkin mutants, however, the sole functional loss of parkin might offer an explanation for the early development of LID in these patients. We therefore tested the kinetics of AIMs severity, the rodent analog of LID, in wild-type and Parkin^{-/-} mice. Daily scoring of AIMs revealed a gradual development of dyskinesia in

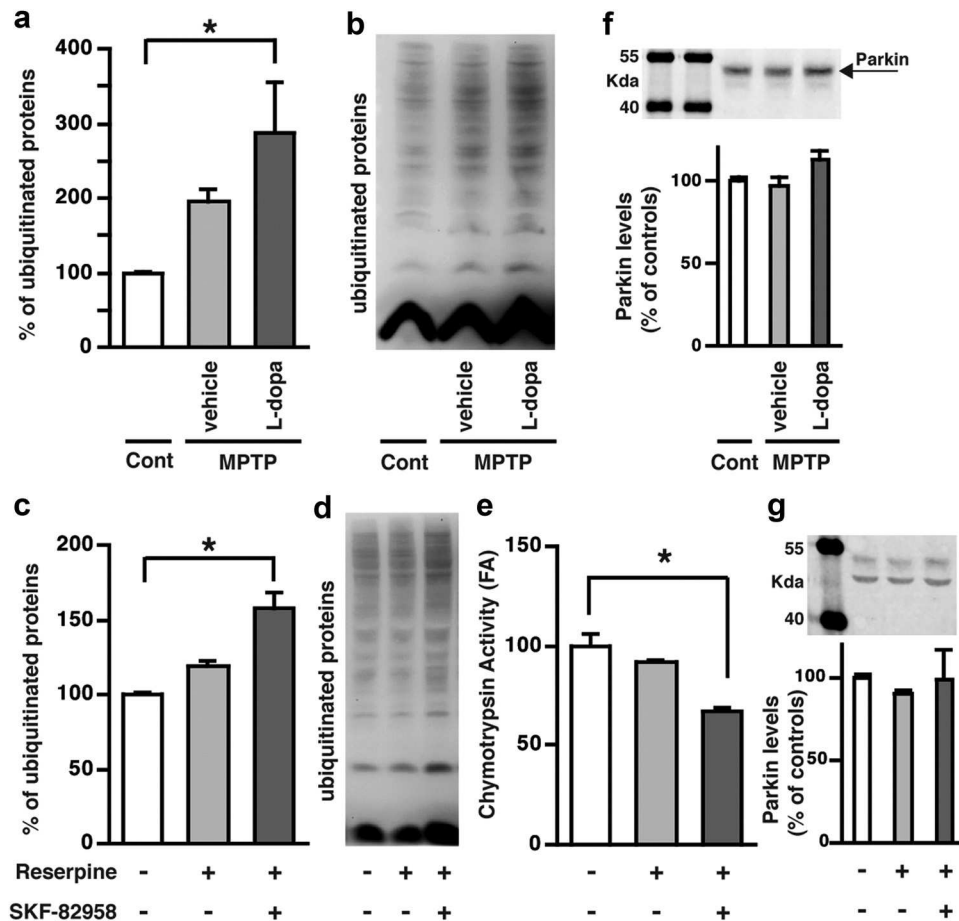


Figure 7. Accumulation of ubiquitinated proteins consequent to D_1 receptor stimulation. **a, b**, Striatal homogenates of normal and MPTP-treated macaque monkey that were administered vehicle or chronic L-DOPA (same animals as those used in Fig. 1*d*) were processed to evaluate the levels of ubiquitinated proteins (one-way ANOVA followed by Tukey–Kramer multiple *post hoc* test; $F_{(2,7)} = 8.36, p < 0.05$). **c, d**, Striatal homogenates of saline and reserpine-treated mice that were administered vehicle or and the D_1R agonist SKF-82958 were processed to evaluate the levels of ubiquitinated proteins (one-way ANOVA followed by Tukey–Kramer multiple *post hoc* test; $F_{(2,15)} = 7.62, p < 0.01$). **f**, Striatal homogenates of normal and MPTP-treated macaque monkey that were administered vehicle or chronic L-DOPA were processed to evaluate the levels of parkin ($F_{(2,7)} = 0.38$). **g**, Striatal homogenates of saline and reserpine-treated mice that were administered vehicle or and the D_1R agonist SKF-82958 were processed to evaluate the levels of parkin ($F_{(2,15)} = 0.16$). Doublet can be seen as previously reported (Pawlyk et al., 2003). * indicates a significant difference between connected groups.

both 6-OHDA-lesioned groups in response to L-DOPA (Fig. 8*a*). Whatever the doses used, the AIMs scores were significantly higher in Parkin^{-/-} mice than in wild-type (Fig. 8*a,c*; $p < 0.05$). Mutant mice also displayed a faster onset of AIMs than in the control group (Fig. 8*d*; $p < 0.05$), further grounding a higher incapacitation. Altogether, these data suggest that the loss of an E3 ubiquitin-protein ligase leads to more severe LID.

Discussion

We show here that L-DOPA-induced dyskinesia associated with chronic L-DOPA treatment in rodent and monkey experimental parkinsonism is associated with a striatum-specific decrease in proteasome chymotrypsin-like catalytic activity. We demonstrate that such decreased proteasome catalytic activity (1) results from D_1R activation and (2) feeds back the D_1R abnormal trafficking, i.e., its exaggerated cell surface abundance and signaling (Aubert et al., 2005; Guigoni et al., 2007; Berthet et al., 2009). We establish in an unprecedented series of experimental models that impairment of the ubiquitin-proteasome system at specific nodes (E3 ligase parkin, polyubiquitination, proteasome catalytic activity) leads to the same phenomenon, i.e., aberrant behavioral response to dopamine replacement therapy further grounding our hypothesis of a role for ubiquitin-proteasome impairment in LID.

Only a few studies have documented the contributions of proteasome activity in neurotransmission control. The mechanisms of regulation between proteasome and neurotransmission are highly complex involving multiple targets that may change upon the given neurotransmitter, and are still poorly understood. For example, proteasome function affects synaptic plasticity such as LTP and LTD (DiAntonio and Hicke, 2004) and regulates endocytosis, signaling, and downregulation of various neurotransmitter receptors (Patrick et al., 2003; Guo and Wang, 2007; Citri et al., 2009). It contributes to directly controlling neuronal electrical activity through regulation of the degradation of the presynaptic proteins involved in neurotransmitter release (Jiang et al., 2010). In our model, proteasome impairment resulted from D_1R stimulation and internalization and thereby contributes to dopamine signaling. Interestingly, proteasome involvement in neuronal dysfunction after chronic stimulation by neurotransmitters or related drugs has been demonstrated in several models not only at the cellular level (Sun and Wolf, 2009) but also at the network and behavioral levels (Citri et al., 2009; Jiang et al., 2010). For instance, in chronic pain, which is known to be maintained in part by long-lasting neuroplastic changes in synapses, several proteins critical for synaptic plasticity are degraded by the ubiquitin-pro-

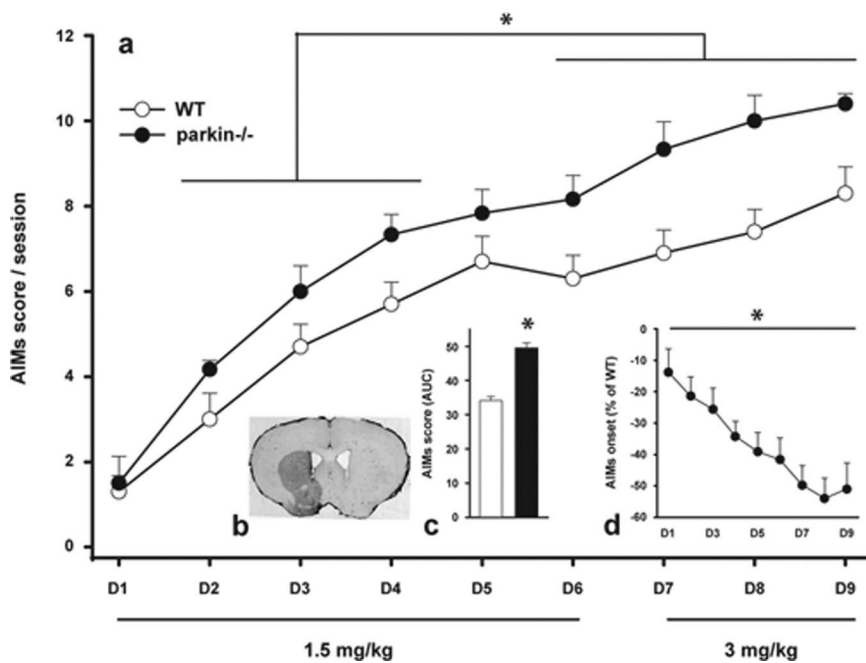


Figure 8. Increased AIMs in *Parkin*^{-/-} mice after L-DOPA treatment. **a**, Time course of axial, limb, and orolingual AIMs induced by an increasing L-DOPA regimen (1.5 and 3 mg/kg, i.p.) administered for 10 consecutive days. The AIMs scores were significantly increased in *parkin*^{-/-} mice (closed circles, $n = 6$) in comparison with the wild-type controls (WT, open circles, $n = 10$) (Mann–Whitney test, $*p < 0.05$ from day 2 till day 9—except at day 5). Vehicle treatment did not induce involuntary movements ($n = 8$, data not shown). **b**, Representative example of striatal TH immunostaining of 6-OHDA-lesioned mice. **c**, Sum of axial, limb, and orolingual scores after 10 d of escalating doses of L-DOPA indicating an increase of dyskinesia in *parkin*^{-/-} animals treated with L-DOPA (Mann–Whitney test, $*p < 0.05$). **d**, Time course of the onset of AIMs in WT and *parkin*^{-/-} mice treated with L-DOPA showing that abnormal movements came earlier in *parkin*^{-/-} than in WT animals (unpaired t test, $*p < 0.05$ from day 1 till day 9).

teasome system. In this condition, proteasome inhibitors prevented the development of nerve injury-induced pain behavior and reversed it, notably through abolishment of the enhanced capsaicin-evoked calcitonin gene-related peptide (CGRP) release and dynorphin A upregulation, which are both triggered by nerve injury (Ossipov et al., 2007).

Particularly relevant to this work is the study by Sun and Wolf (2009), where, in an attempt to broadly recapitulate the conditions of chronic cocaine use in a culture system, they administered chronic dopamine treatments [which is known to enhance surface expression of AMPA receptors (Wolf et al., 2004)] before performing synaptic scaling experiments. Intriguingly, they showed that the dopamine-induced increase in AMPA receptor expression occluded the increased expression more classically induced by scaling challenge. This was mimicked by proteasome inhibition, providing the first indirect evidence of a putative interplay between dopamine receptors and proteasome catalytic activity (Sun and Wolf, 2009). As evidenced for glutamate receptors (Citri et al., 2009; Sun and Wolf, 2009), it may well be that in LID, proteasome activity is reduced after chronic dopamine receptor stimulation as part of a feedback mechanism that may directly or indirectly control dopamine receptor internalization, trafficking, and/or metabolic half-life. One should stress the requirement of previous dopamine denervation for the impairment to take place as hyperdopaminergic *DAT*^{-/-} mice do internalize D1R (Dumartin et al., 2000). Nevertheless, the targets of dopamine-dependent proteasome activity in striatal neurons remain to be identified. They involve D1R itself as evidenced here (1) by the limitation of the agonist-induced D1R endocytosis while proteasome was blocked, (2) by the demonstration that only D1R stimulation affects proteasome catalytic activity while D2R does not (Fig. 5e), and (3) by

the striking resulting accumulation of ubiquitinated proteins both after chronic L-DOPA administration and acute D1R agonist treatment (Fig. 7).

Since L-DOPA is the treatment of choice for PD (Cotzias et al., 1969) and since D1R intraneuronal trafficking, subcellular localization, and desensitization is dysregulated in dyskinesia, with an abnormal abundance of D1R at the plasma membrane of striatal neurons (but not D2R) (Guigoni et al., 2005a, 2007; Berthet et al., 2009), we started the elucidation of the proteasome-neurotransmitter receptor relationship by the dopamine receptors. Whether proteasome may also act on companion molecules involved in dopamine signaling such as arrestins, or on other receptors including D3R (Fiorentini et al., 2008) or glutamate receptors that are associated with D1R (Lee et al., 2002) in striatal neurons, remains to be established. Strikingly, by being specifically addressed, glutamate receptors such as the NMDA (Hallett et al., 2005) and AMPA (Silverdale et al., 2010) receptors are comparably affected with a specific addressing at membrane in LID. In particular, NR2A NMDA subunit and AMPA GluR2/3 subunits are preferentially recruited at synaptic membrane in LID (Hallett et al., 2005; Silverdale et al., 2010). This is highly reminiscent of AMPA recruitment at membrane after cocaine treatment (Sun and Wolf, 2009).

That key receptors participating in the corticostriatal transmission are wrongly maintained or addressed at membrane (Guigoni et al., 2005a, 2007; Hallett et al., 2005; Berthet et al., 2009; Silverdale et al., 2010) is thus a feature of LID. One can thus hypothesize the predictable involvement of other neurotransmitter receptors in experiencing such complex relationship with proteasome catalytic activity, a hypothesis that does not diminish the importance of D1R involvement because of the nature of the pharmacological treatment, i.e., L-DOPA.

Our data collected in experimental models of sporadic PD point out the impairment of proteasome catalytic activity and the resulting abnormal accumulation of ubiquitinated proteins in the striatum. Interestingly, the recessive form of parkinsonism due to *parkin* mutation presents clinical features of PD but with early onset and early appearance of disabling LID (Kitada et al., 1998). Since *parkin* is an E3 ubiquitin-protein ligase (Shimura et al., 2000), its functional loss would precede a putative dopamine-dependent impairment in proteasome activity in striatal neurons, offering an explanation for the early appearance of LID in these patients. Interestingly, our data show that the E3 ligase *Parkin*^{-/-} mice developed more severe AIMs than wild-type mice did. The lack of E3 ligase *Parkin* therefore prevents the degradation of proteins key to LID genesis by the proteasome. In this familial form of PD, accelerated occurrence of LID would thus result from lack of addressing dopamine receptor-related key proteins to proteasome; while in sporadic PD, LID would be linked to a D1R-mediated decreased proteasome catalytic activity. Altogether, we propose a unified view of LID occurrence in both sporadic and one familial form of PD through the impairment of the ubiquitine-proteasome system at specific nodes (E3 ligase

parkin, polyubiquitination, proteasome catalytic activity) leading to aberrant behavioral response to dopamine replacement therapy.

Proteasome deregulation has now been demonstrated to play a key role in the initiation and progression of several human diseases. The control of its activity, almost exclusively through inhibitors, is an emerging challenge in clinical practice in humans, as demonstrated for multiple myeloma and related diseases (Genin et al., 2010). However, in our study, we challenge the idea that in LID, the proteasome activity should instead be increased. Interestingly, the ubiquitin–proteasome system displays an increased activity in animals chronically exposed to nicotine (Kane et al., 2004), a drug that displays anti-dyskinetic activity (Quik et al., 2007; Bordia et al., 2008). Further exploration is now needed to fully understand the relationships between dopamine signaling, the mechanisms of proteasome regulation and proteasome targets in medium spiny striatal neurons. This may lead to new opportunities for controlling and preventing the severity of the disabling dyskinesia in PD.

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