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To cite this version:

HAL Id: hal-01053479
https://hal.archives-ouvertes.fr/hal-01053479
Submitted on 31 Jul 2014

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Neurobiological Disease

1-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 1-DOPA-induced dyskinesia (LID), a major complication of 1-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 ubiquitine-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 ubiquitine-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 1,3,4-dihydroxyphenylalanine (1-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 neurotransmitter 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, 1-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 1-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
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 benzerazide in rodents or the human oral formulation Modopar (Roche, t-DOPA/carbidopa, ratio 4:1). Dopamine receptor agonists were the D1R agonist SKF-82958 and the D2R agonist Quinipilo. 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 Laboratories) 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 ± 5%), temperature (24 ± 1°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; Bezard 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 (group control), six monkeys received a single dose of 20 mg/kg, p.o. t-DOPA (control acute t-DOPA), and six monkeys received 20 mg/kg twice daily for 3 months (control chronic t-DOPA). The remaining 27 animals were treated (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 t-DOPA (MPTP acute t-DOPA: 6 monkeys) for 3 months (MPTP chronic t-DOPA: 15 monkeys) at a tailored dose designed to fully reverse the parkinsonian features and develop dyskiniesia (20 mg/kg t-DOPA p.o.). Nine of the 15 monkeys developed severe and reproducible dyskiniesia (MPTP intoxicated, dyskinetic monkey group), whereas six did not (MPTP intoxicated, non-dyskinetic monkey group).

Animal behavior was assessed using videotape recordings of monkeys in their home cages as previously described (Bezard 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 dyskiniesia was rated using the validated dyskiniesia 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 t-DOPA/carbidopa dose, a time at which dyskiniesia 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 (−80°C) and then stored at −80°C. Tissue of one hemisphere was sectioned coronally at 20 µm 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-µm-thick sections for the protease 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 protease 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. Strialtional coronal sections (300 µm thick) were cryostat cut and used for the protease activity assays.

UbG76V·GFP transgenic mice. The UbG76V·GFP transgenic mice were used for ex vivo and in vivo brain experiments [B6.Cg-Tg(CAG·Ub★G76V·GFP2Dant/J] (The Jackson Laboratory)]. This transgenic mouse strain carries a green fluorescent protein UbG76V·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 protease inhibitor leads to GFP accumulation in striatal brain slices. Brain slices were performed as previously described (Baufret 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) and the MG132 (Enzo Life Sciences) and developed by immunofluorescence microscopy. Mice underwent stereotactic surgery under 1.2–1.5% isoflurane. Each mouse received a unilateral injection of 6-OHDA (1 µl/injection; 3 µg/µl) 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 benzerazide only (15 mg/kg, i.p.; n = 8) and t-DOPA/benzerazide (6 mg/kg/15 mg/kg, i.p.; n = 4) treatment for 7 d. In such conditions, t-DOPA induces the gradual development of dyskinetic-like abnormal involuntary movements (AIMs). On the penultimate day, for the benzerazide 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 benzerazide or t-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 C57BL6 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. Strialtal coronal sections (300 µm thick) were cryostat cut and used for the protease activity assays as well as Western blotting experiments.

Parkin−/− mice. Parkin−/− mice [B6.129S4-Park2tm1Shn/J] (The Jackson Laboratory) bearing a germine disruption of exon 5 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 benzerazide (15 mg/kg, i.p.) and t-DOPA (1.5 and 3 mg/kg, i.p.). Starting from day 16, mice were treated for nine consecutive days with an escalating t-DOPA dosing regimen (1.5 and 3 mg/kg) plus benzerazide (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(6,16) = 3.74, p < 0.01), trypsin-like (F(6,16) = 5.323, p < 0.001), and PGPH activities (F(6,16) = 3.197, p < 0.05), using fluorogenic substrates: Suc-LLY-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 ± 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(2,9) = 11.102, p < 0.0001), trypsin-like (F(2,9) = 10.776, p < 0.0001), and PGPH activities (F(2,9) = 0.9237), * indicates a significant difference between connected groups.

Wild-type rats. In vivo: 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 μg/μl) injection in the right medial forebrain bundle (2.5 μl 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., 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-/− 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 μM 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 μM 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 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 μM) and acid ascorbic, the full D1R agonist SKF-82958 (10 μM) (Martin-Negrier et al., 2000, 2006), the D1R antagonist SCH-23390 (2 μM), the D2R agonist Quinpirole (10 μM) (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...
Dysfunctions in catalytic activities are striatum-specific. Superior frontal gyrus (SFG) homogenates of normal and MPTP-treated macaque monkeys that were administered vehicle or i-DOPA, acutely or chronically, were processed to evaluate 20S proteasomal chymotrypsin-like [one-way ANOVA; ($F_{1,14}$ = 0.9912, not significant (ns)), trypsin-like ($F_{1,14}$ = 2.0844, ns), and PGPH activities ($F_{1,14}$ = 1.5074, 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 dyskinetic 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 ± SEM (n = 6 animals for each group).

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 μg of protein separated on polyvinylidene fluoride membranes (Millipore) and subjected to Western blot analysis using a rabbit anti-Ubiquitine 1:1000 (Sigma). Signals were recovered by horseradish peroxidase-conjugated secondary antibodies.
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 monkeys that received vehicle or l-DOPA (Fig. 1a–d). More intense accumulation was observed when mice were treated with D1R full agonist SKF-82958 (Fig. 1e, h). These data suggest that the dysfunctions in catalytic activities are due to the association of 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. 1e, 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 hyperdopaminergic 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<sup>−/−</sup> mice compared with both DAT<sup>+/+</sup> and DAT<sup>+/−</sup> (Fig. 3). As we have previously shown a strong internalization of D1R in these DAT<sup>−/−</sup> 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<sup>G76V</sup>-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<sup>G76V</sup>-GFP reporter (Lindsten et al., 2003), to address this question. The Ub<sup>G76V</sup>-GFP transgene was designed with a chicken β-actin promoter upstream of a GFP fused to a mutant ubiquitin moiety (UbG76V) (Lindsten et al., 2003). Although transcripts of the Ub<sup>G76V</sup>-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<sup>G76V</sup>-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 treated with DMSO or bortezomib, a potent proteasome inhibitor, we developed experiments to measure these changes as a marker of proteasome impair-
like activity is significantly impaired after dopamine treatment (Fig. 5d; \( p < 0.0001 \)). Interestingly, while application of the D1R agonist SKF-82958 mimicked the dopamine-induced decrease in chymotrypsin-like activity (Fig. 5c; \( p < 0.05 \)), application of the dopamine D2 receptor (D2R) agonist quinpirole failed to inhibit this activity (Fig. 5e) 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. 5f; \( p < 0.05 \)). 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. 6b–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. 6c–e). Proteasome inhibition alone, however, had no effect on the D1R distribution as compared with the control situation (Fig. 6d,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. 1d), chronic l-DOPA treated MPTP-lesioned monkeys displayed increased levels of ubiquitinated proteins compared with control animals (\( p < 0.05 \); Fig. 7a,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. 7c,d) that showed a significant impairment in chymotrypsin-like activity (Fig. 7e). 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. 7g). 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...
both 6-OHDA-lesioned groups in response to L-DOPA (Fig. 8).

Whatever the doses used, the AIMs scores were significantly higher in Parkin/H11002 mice than in wild-type (Fig. 8a, c; p < 0.05). Mutant mice also displayed a faster onset of AIMs than in the control group (Fig. 8d; 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 D1R activation and (2) feeds back the D1R 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 ubiquitine-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 ubiquitine-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 D1R 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-
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 expression occluded the increased expression more classically 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).

Figure 6. Increased AIMs in Parkin−/− mice after 1-DOPA treatment. a. Time course of axial, limb, and orolingual AIMs induced by an increasing 1-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). 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 1-DOPA indicating an increase of dyskinesia in parkin−/− animals treated with 1-DOPA (Mann–Whitney test, *p < 0.05). d. Time course of the onset of AIMs in WT and parkin−/− mice treated with 1-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).

The striking resulting accumulation of ubiquitinated proteins both after chronic 1-DOPA administration and acute D1R agonist treatment (Fig. 7).

Since 1-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., 1-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., 2002), 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

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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.

References


