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Microtubule Alterations Occur Early in Experimental Parkinsonism and The Microtubule Stabilizer Epothilone D Is Neuroprotective

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The role of microtubule (MT) dysfunction in Parkinson's disease is emerging. It is still unknown whether it is a cause or a consequence of neurodegeneration. Our objective was to assess whether alterations of MT stability precede or follow axonal transport impairment and neurite degeneration in experimental parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57Bl mice. MPTP induced a time- and dose-dependent increase in fibres with altered mitochondria distribution, and early changes in cytoskeletal proteins and MT stability. Indeed, we observed significant increases in neuron-specific βIII tubulin and enrichment of deTyr tubulin in dopaminergic neurons. Finally, we showed that repeated daily administrations of the MT stabilizer Epothilone D rescued MT defects and attenuated nigrostriatal degeneration induced by MPTP. These data suggest that alteration of MTs is an early event specifically associated with dopaminergic neuron degeneration. Pharmacological stabilization of MTs may be a viable strategy for the management of parkinsonism.

Axonogenesis and dendritogenesis, which are essential for the normal development of neurons, rely on the coordinated organization and dynamics of the actin and microtubule (MT) cytoskeleton. MTs are polymers built up by α/β tubulin heterodimers, characterized by an intrinsic resistance to bending and compression[1]. They are capable of switching between phases of slow growth and of rapid depolymerization[2], features implicated in generating pushing and pulling forces within cells[3]. During neuronal differentiation, MTs are highly dynamic and ensure outgrowth and branching[4]. In mature neurons, MT stability increases[5]. The proper control of MT dynamics is essential for many neuronal activities, such as synaptic remodelling[6], and both MT stability and neuronal functions are regulated through tubulin posttranslational modifications (PTMs)[7].

Axon degeneration is a hallmark of neurodegenerative disorders and often precedes the onset of symptoms. Axonal destruction is an active process rather than a passive event[8]. Although little is known about the mechanisms involved, a growing body of evidence suggests a primary role of the MT system. In fact, MT fragmentation is the first detectable event during Wallerian degeneration[9], and disorganized and bent MTs accompany the formation of retraction bulbs[10] and axonal retraction[11].

Parkinson's disease (PD) is the most common motor neurodegenerative disorder and each symptom depends on the reduction in dopamine (DA) levels in the striatum. Degeneration of nigrostriatal dopaminergic synaptic terminals precedes the death of dopaminergic neurons in the substantia nigra[12]. Many PD-linked proteins, such as α-synuclein[13], parkin[14], and leucine rich repeat kinase 2[15], modulate the stability of MTs, highlighting the crucial role of MTs during PD progression. Furthermore, PD is the only neurodegenerative disorder that is clearly related to environmental toxins, such as 1-methyl-4-phenylpiridinium (MPP^+) or rotenone[12], which both destabilize MTs[16,17]. Alterations in MT stability precede axonal transport impairment and neurite degeneration in MPP^+-exposed PC12 cells[18], suggesting that MT dysfunction plays an important role in mediating the toxicity of these compounds. It is noteworthy that both prophylactic and interventional treatments with the MT-stabilizer Epothilone D (EpoD), which is able to pass the blood-brain barrier, improve axonal MT density, reduce axonal

dystrophy and alleviate cognitive deficits in transgenic mouse models of tauopathies[19,20]. It has also been shown that the dynamicity of MTs is increased in tau transgenic mice and that treatment with EpoD restores MT dynamics to baseline levels and exerts beneficial effects on behavior, tau pathology and neurodegeneration[21].

We now show that C57Bl mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) express early alterations of α-tubulin PTMs specifically in dopaminergic neurons, and that systemic injections of EpoD rescue MT defects and attenuate nigrostriatal degeneration in mice with parkinsonian symptoms produced by exposure to the toxin MPTP.

Results

Treatment paradigm underlines early alterations. One single dose of MPTP (30 mg/kg, i.p.) induced about 50–60% reduction in striatal DA levels 7 days later in C57Bl mice, as expected[22]. To highlight very early alterations, mice were treated either with a single dose (30 mg/kg, i.p.) or with a cumulative dose (60 mg/kg) of MPTP, and sacrificed 12 or 72 h later. Biochemical analyses showed that MPTP induced a significant reduction in striatal DA and its metabolites, a sign of ongoing neurodegeneration (Fig. 1a). Western Blotting analysis showed that TH, the rate-limiting enzyme of DA biosynthesis, was decreased in the striatum and substantia nigra only with the highest dose of MPTP (Fig. 1b,c). Ara and colleagues[23] showed that MPTP induces early inactivation of the enzyme, followed by reduction in TH levels; therefore, mice treated with the low dose of MPTP (30 mg/kg), which do not show any changes in TH levels, can be considered to be in an early phase of neurodegeneration, and may be used to uncover very early alterations in experimental parkinsonism.

MPTP causes axonal transport impairment in dopaminergic fibres. Mitochondria, as well the other organelles, accumulate into varicosities when axonal transport is impaired[24], and there is evidence that MPP^+ interferes with this process[18,25,26]. Therefore, to assess the status of axonal transport in MPTP-treated mice, we evaluated mitochondria distribution within dopaminergic fibres by performing a double immunohistochemical analysis in sagittal sections of TH and voltage-dependent anion channel (VDAC)-porin, a structural protein of the mitochondrial pore. We observed dopaminergic fibres with a homogeneous distribution of mitochondria and fibres showing sparse or accumulated mitochondria (Fig. 2a, arrowheads). It is noteworthy that only mice treated with MPTP and killed 72 h later showed empty fibres (Fig. 2a, arrow), as the mitochondria had been kept out of neuronal processes. Quantification of the different types of fibres (Fig. 2b), showed a time- and dose-dependent increase in fibres with altered mitochondria distribution in treated mice. No significant differences were observed between control mice and mice treated for 12 h with 30 mg/kg MPTP. These data show that MPTP affects axonal transport *in vivo*.

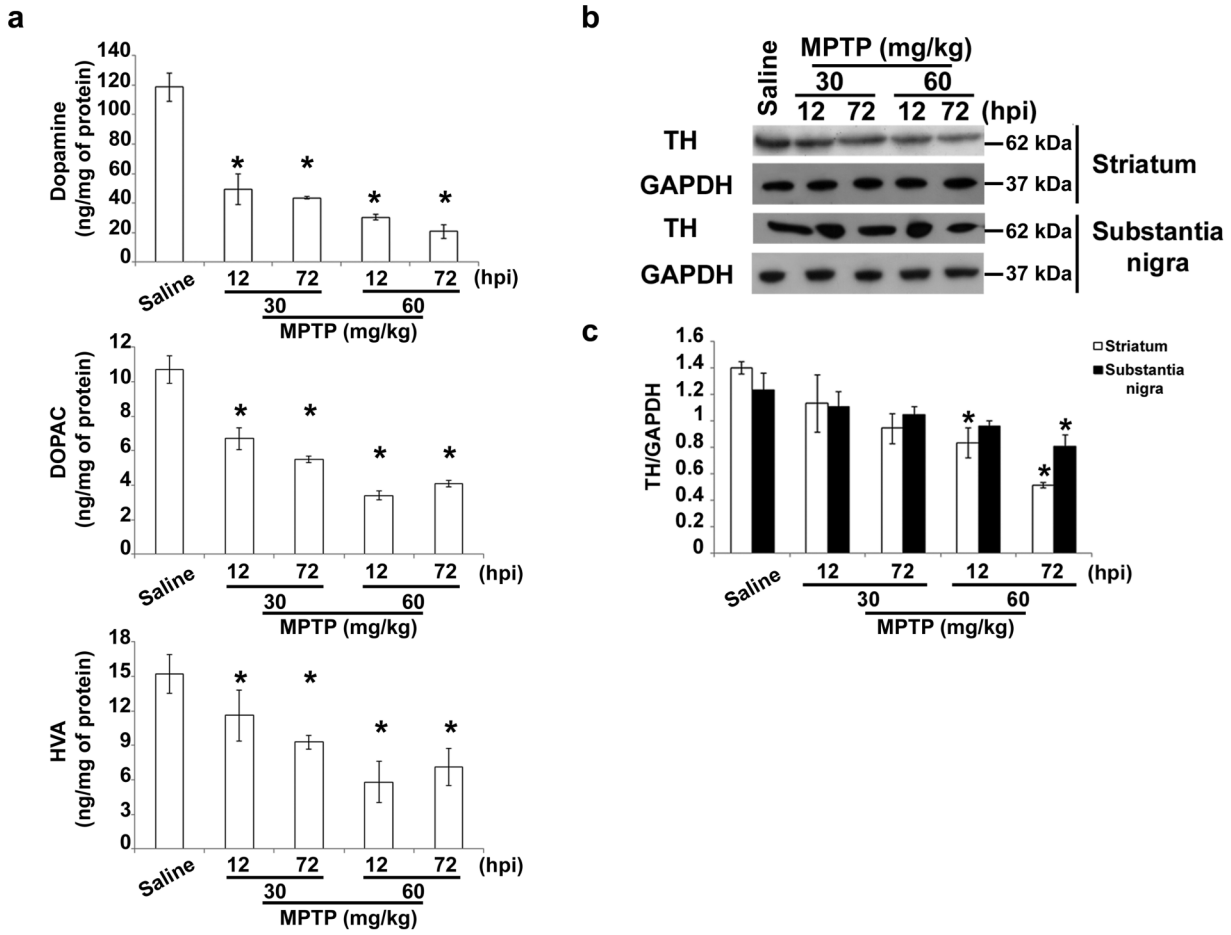

Figure 1 | Treatment paradigm underlines early alterations. (a) Biochemical analyses of striatal dopamine, DOPAC and HVA levels in C57Bl mice injected with saline or MPTP (30 mg/kg, i.p., single injection or 20 mg/kg, i.p., × 3, 2 h apart) and killed 12 or 72 hours later (mean ± S.E.M., n = 8–10 mice per group). *$P < 0.05$; one-way ANOVA, Dunnett *post hoc* versus saline-injected mice. (b) Immunoblot of TH levels in lysates of the corpus striatum and substantia nigra of mice injected with saline or MPTP as in a. (c) Densitometric analyses of immunoblot reported in b (mean ± S.E.M., n = 8–10 mice per group). *$P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. hpi = hours post last injection of MPTP.

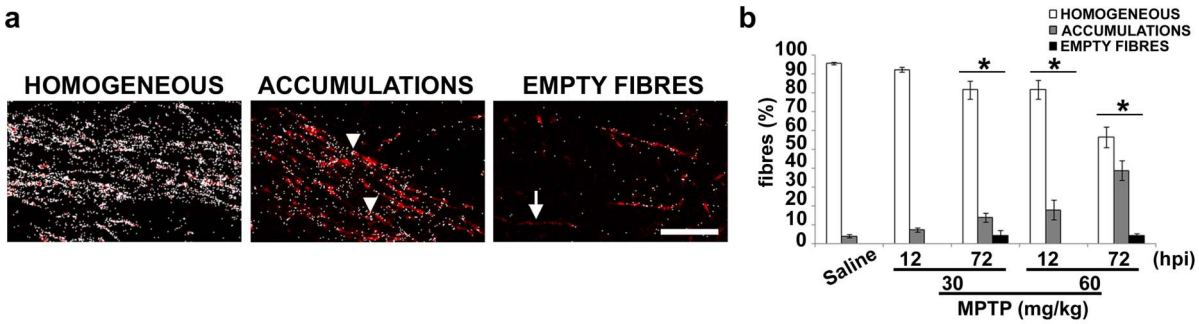

Figure 2 | MPTP causes axonal transport impairment. (a) Mask projections of sagittal sections of the nigrostriatal pathway in mice injected with saline or MPTP as in Fig. 1, showing the distribution of mitochondria (white spots) inside dopaminergic fibres (red). Arrowheads indicate mitochondria accumulations and arrow highlights an empty fibre. Scale bar = 20 μm. (b) Percentage of fibres displaying a homogeneous distribution of mitochondria, fibres showing dispersed or accumulated mitochondria or empty fibres. hpi = hours post last injection of MPTP (mean ± S.E.M., n = 9 sections from 3 different mice per group). $*P < 0.05$; χ^2 test versus saline-injected mice.

MPTP increases the neuron-specific βIII tubulin isotype. Axonal transport defects could be related to alterations of cytoskeletal proteins and molecular motors. Thus, we evaluated levels of actin, α- and β-tubulin, and the neuron-specific βIII tubulin isotype. Western blotting and densitometric analyses showed that levels of actin and α/β tubulin were unchanged in the striatum (Fig. 3a,b) and substantia nigra (Fig. 3c,d) of MPTP-treated mice. β-tubulin was significantly reduced in the striatum of mice treated with MPTP (30 mg/kg) and killed 72 h later (Fig. 3a,b). The specific change in β-tubulin is not surprising, because Chung and colleagues[27] already showed differential variations in α- and β-tubulin monomers in a rat model of synucleinopathy, and we have recently found significant enrichment of β-tubulin in the fibroblasts of PD patients carrying parkin mutations[28]. On the other hand, the neuron-specific βIII tubulin isotype was significantly increased by MPTP treatment in both striatum and substantia nigra, although at different time points

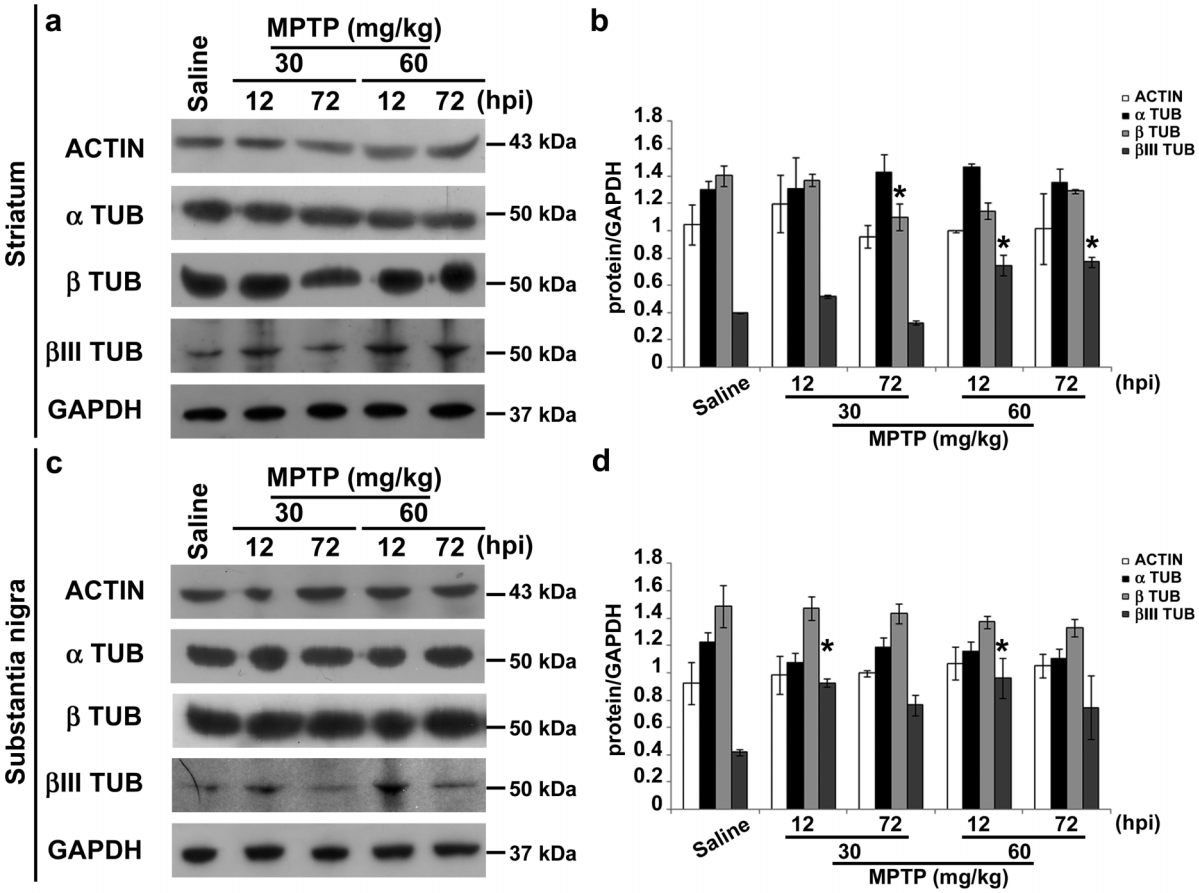

Figure 3 | MPTP treatment increases the βIII tubulin isotype. (a) Immunoblots of actin, α-tubulin, β-tubulin and βIII tubulin in lysates of striatum of mice treated as in Fig. 1. (b) Densitometric analyses of immunoblot reported in a (mean ± S.E.M., n = 4–6 individuals per group). $*P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. (c) Immunoblot of actin, α-tubulin, β-tubulin and βIII tubulin in lysates of substantia nigra of mice treated as in Fig. 1. (d) Densitometric analyses of immunoblot reported in c (mean ± S.E.M., n = 4–6 individuals per group). $*P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. hpi = hours post last injection of MPTP.

(Fig. 3). Levels of kinesin and dynein, responsible for MT-dependent anterograde and retrograde axonal transport, respectively[29], were not affected by MPTP treatment (Supplementary Fig. S1), suggesting that axonal transport defects may be due to alternative factors.

MPTP specifically affects MT stability in dopaminergic neurons. α-tubulin PTMs are usually used as markers of MTs with different stability, being tyrosinated (Tyr) MTs the most dynamic, and acetylated (Ac) or detyrosinated (deTyr) MTs the most stable pools[7]. Recently, it has been shown that α-tubulin PTMs are directly linked to neurodegeneration in mice[30] and MPP^+ affects α-tubulin PTMs in PC12 cells[18]. Therefore, we evaluated MT stability in the striatum (Fig. 4) and substantia nigra (Fig. 5) of MPTP-treated mice, as well as along their nigrostriatal pathway (Supplementary Fig. S2). Biochemical analyses showed the enrichment of deTyr tubulin in striatum (Fig. 4a,b), which was detectable already 12 h after the injection of the lowest dose of MPTP, and a later increase in both Tyr and Ac tubulins. Confocal analyses and the evaluation of Manders' coefficients (M1 and M2, reported in Table 1 and Supplementary Table S1, respectively), which are good indicators of relative signal distribution[31], showed that changes in the levels of tubulin PTMs were located in dopaminergic terminals. In fact, we observed an increased co-localization between deTyr or Ac Tub and TH signals (Fig. 4c) and significant elevation in M1 parameter (Table 1). On the other hand, the significant decrease in the M1 parameter (Table 1) demonstrated that the enrichment of Tyr tubulin was associated with neurons residing in the striatum.

Western blotting analysis performed on substantia nigra lysates (Fig. 5a, b), revealed a significant decrease in Tyr tubulin in MPTP-treated mice, suggesting that the dynamic MT pool was reduced, as we already showed in cultured cells[18]. On the other hand, MPTP induced a significant enrichment of deTyr tubulin in mice treated with MPTP (60 mg/kg) and killed 12 h later, suggesting an increase in stable MTs, which mirrors the changes observed in the striatum. Confocal analyses showed that α-tubulin PTM changes occurred in dopaminergic cell soma (Fig. 5c), and the quantification of fluorescence intensity within single cells (Supplementary Table S2) showed that alterations of MT stability are higher in dopaminergic neurons. Indeed, we observed a significant reduction in Tyr tubulin and an early increase in deTyr and Ac tubulins that resulted in the decrease of stable MTs.

Finally, as α-tubulin PTMs influence axonal transport[32,33], we evaluated potential changes within dopaminergic fibres by confocal microscopy (Supplementary Fig. S2). Besides the slight changes observed in Tyr and Ac tubulin content, the overall result we obtained is an early increase in deTyr tubulin, in line with the modifications of this subset of stable MTs in the striatum and substantia nigra of MPTP-treated mice, which could be responsible for the impairment of axonal transport, as already suggested[18]. Taken together, these results show that MPTP affects MT stability *in vivo*, and that the alteration of α-tubulin PTMs is an early event specifically associated with dopaminergic neurons.

Stabilization of MT attenuates MPTP-induced neurodegeneration. To test the hypothesis that stabilization of MT is able to exert a neuroprotective effect in experimental parkinsonism, we used the classical model induced by MPTP. C57 Black mice were challenged with a single dose of MPTP (30 mg/kg, i.p.), which led, 7 days later, to about 50% reduction in striatal DA levels. Similar reductions were found in the striatal levels of DOPAC and HVA, although changes in DOPAC and HVA levels were variable in different experiments (Fig. 6a). Reductions in DA were not affected in mice systemically injected with 1 or 3 mg/kg, i.p., of EpoD 30 min prior to MPTP (Fig. 6a). Repeated treatment with EpoD (1 mg/kg, i.p.), injected 30 min prior to MPTP and then for the following 4 days once a day, induced substantial attenuation of MPTP-induced striatal DA reduction (Fig. 6b). Note that, for unknown reasons, MPTP toxicity was greater in control animals injected with DMSO alone. Neuroprotection by repeated injections of EpoD was confirmed by stereological counts of TH-positive neurons in the pars compacta of substantia nigra (Fig. 6c,d). To assess the effects of EpoD on motor activity, mice were treated i.p. with EpoD alone (3 mg/kg, every day for four days) or 30 min prior MPTP and then every day for four days. EpoD did not affect the

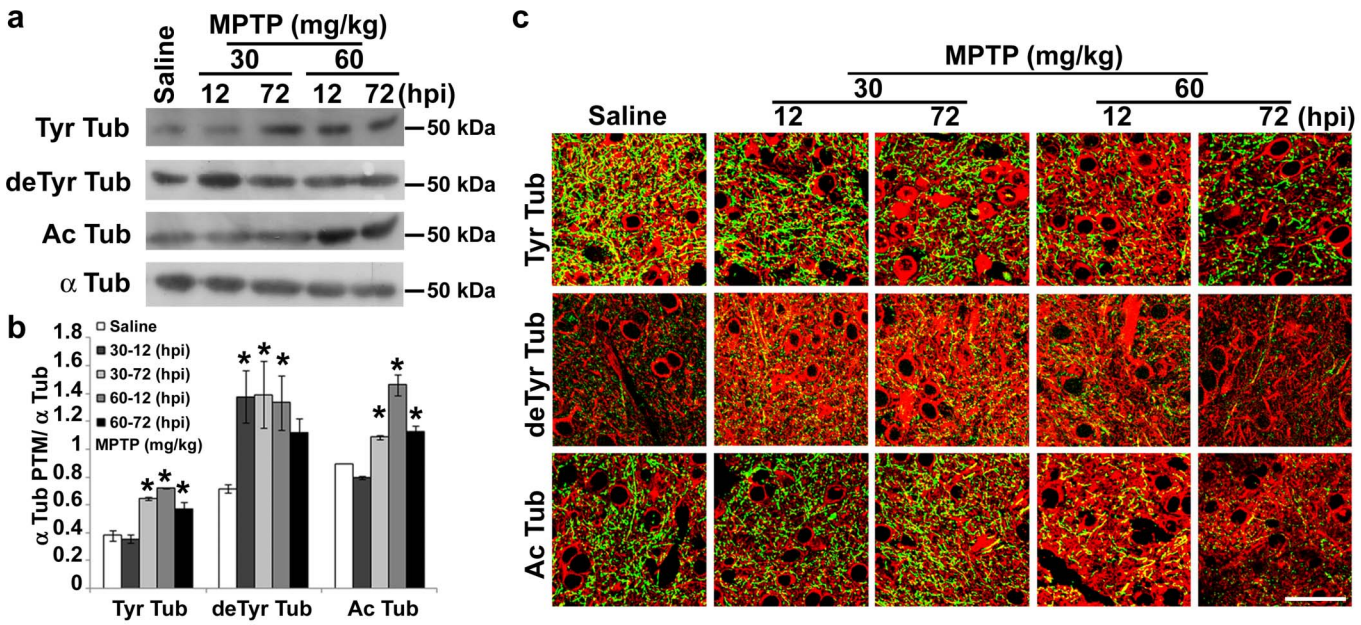

Figure 4 | MPTP affects MT stability in dopaminergic terminals. (a) Immunoblot of levels of tyrosinated tubulin (Tyr Tub), detyrosinated tubulin (deTyr Tub) and acetylated tubulin (Ac Tub) in lysates of striatum of mice treated as in Fig. 1. (b) Densitometric analyses of immunoblot reported in a (mean ± S.E.M., n = 4–6 mice per group). For the quantitation, values of each α-tubulin PTM were normalized on the level of α-tubulin (α Tub) of the relative sample. *$P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. (c) Confocal images of striatum of mice treated as in Fig. 1. Green represents TH staining and red signals the various tubulin PTMs. Scale bar = 50 μm. hpi = hours post last injection of MPTP.

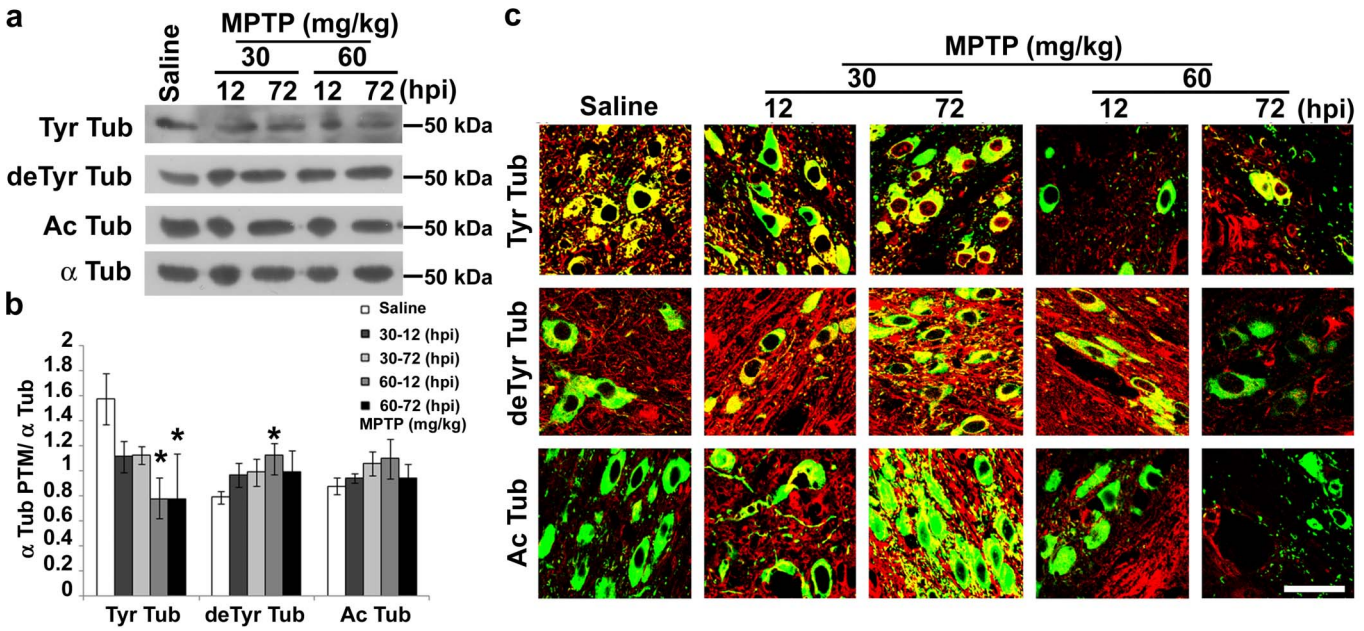

Figure 5 | MPTP affects MT stability in dopaminergic neurons. (a) Immunoblot of levels of tyrosinated tubulin (Tyr Tub), detyrosinated tubulin (deTyr Tub) and acetylated tubulin (Ac Tub) in lysates of substantia nigra of mice treated as in Fig. 1. (b) Densitometric analyses of immunoblot reported in a (mean ± S.E.M., n = 4–6 mice per group). For the quantitation, values of each α-tubulin PTM were normalized on the level of α-tubulin (α Tub) of the relative sample. $*P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. (c) confocal images of substantia nigra of mice treated as in Fig. 1. Green represents TH staining and red signals the various tubulin PTMs. Scale bar = 50 μm. hpi = hours post last injection of MPTP.

motor performance on the rotarod test by itself (not shown). At the doses of MPTP used, we did not observe any modification of motor performance and EpoD treatment did not modify the motor coordination at the rotarod test (not shown). We also monitored general health conditions of mice treated with EpoD and MPTP+EpoD. Both fur and body weight were not affected by EpoD or MPTP+EpoD treatments.

To verify that the neuprotective effect of EpoD was due to its action on MT system, we analyzed α-tubulin PTMs in the corpus striatum and substantia nigra of mice challenged with MPTP and chronically treated with EpoD. We observed significant increase of both Tyr and deTyr tubulin in the striatum of MPTP-treated mice (Fig. 7a,b), whereas only deTyr tubulin was increased by MPTP in the substantia nigra (Fig. 7c,d). Furthermore, our data show the ability of EpoD to restore deTyr tubulin baseline levels, both in striatum (Fig. 7a,b) and substantia nigra (Fig. 7c,d). The reduction in a marker of stable MTs, as deTyr tubulin is, induced by a stabilizer drug, as EpoD is, could seem surprising. However, this is perfectly consistent with the hypothesis that the increase of stable MTs in MPTP-treated mice may be an attempt to counteract the drug-induced MT destabilization[18]. MPP^+ is known to promote MT catastrophes[16] and, in respect to dynamic ones, deTyr MTs are more resistant to induced MT depolymerization; hence, tubulin detyrosination could reduce MPP^+-provoked MT catastrophes. On the other side, as many other MT-stabilizing agent, EpoD directly induces MT polymerization under conditions in which tubulin is not more able to assemble, shifting the equilibrium of tubulin toward the polymeric state. To verify our hypothesis, we measured the amount of tubulin associated to cytosolic dimers and to polymeric MTs (Fig. 7e,f), both in striatum and substantia nigra. Our data reveal, for the first time, that MPTP is able to specifically destabilize MTs in the substantia nigra of treated mice and that chronic treatment with EpoD prevents the MPTP-induced destabilization (Fig. 7e,f).

Discussion

Cytoskeletal alterations have been described in many central nervous system disorders, but it is unclear whether they are a cause or simply a by-product of neurodegeneration. Here, we show that systemic injection of MPTP in mice induces MT dysfunction that occurs very early, before axonal transport impairment, TH depletion, and, ultimately, dopaminergic neuron degeneration. Noteworthy is that chronic administration of the MT stabilizer Epo D rescues MT defects and is neuroprotective, supplying reliable proof that MT dysfunction may contribute to actually cause neurodegeneration.

Axonal transport impairment in the MPTP model of PD was first suggested by Morfini et al. (2007), who proposed that it was an early event in neurodegeneration, based on the outcome of a study on giant squid axons. Here we demonstrate that axonal transport impairment occurs in MPTP-treated mice, showing changes in mitochondria distribution in dopaminergic fibres. In addition, the earlier decrease

Table 1 | Analysis of M1 parameter (Tubulins vs. TH) in striatal sections

	Tyr Tubulin	deTyr Tubulin	Ac Tubulin
Saline	0.41 ± 0.032	0.30 ± 0.025	0.16 ± 0.013
MPTP, 30 mg/kg (12 h)	0.27 ± 0.021 (*)	0.39 ± 0.013	0.28 ± 0.028 (*)
MPTP, 30 mg/kg (72 h)	0.27 ± 0.048 (*)	0.51 ± 0.027 (*)	0.33 ± 0.021 (*)
MPTP, 60 mg/kg (12 h)	0.17 ± 0.029 (*)	0.34 ± 0.034	0.36 ± 0.019 (*)
MPTP, 60 mg/kg (72 h)	0.24 ± 0.043 (*)	0.32 ± 0.030	0.16 ± 0.023

Data are expressed as mean ± S.E.M., n = 4 sections for each mouse from 4–6 mice per group. $*P < 0.05$; one-way ANOVA, Dunnett *post hoc*: *versus* saline-injected mice.

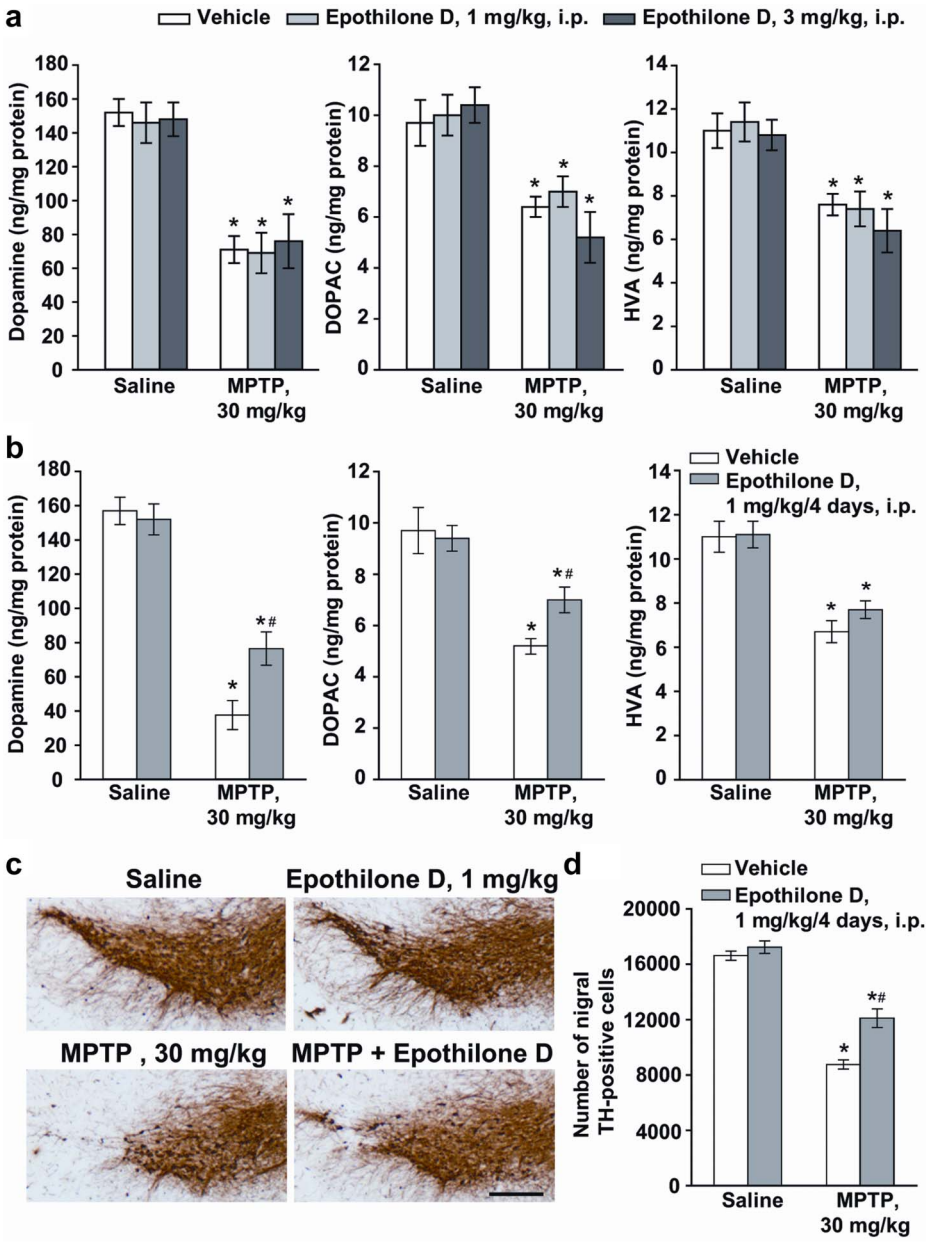

Figure 6 | Repeated systemic injections of EpoD attenuate MPTP toxicity in mice. (a) Biochemical analyses of striatal dopamine, DOPAC, and HVA levels in C57Bl mice injected with MPTP (30 mg/kg, i.p., single injection) alone or in combination with EpoD (1 or 3 mg/kg, i.p.) dissolved in DMSO and injected 30 min before MPTP. Mice were killed 7 days later (mean ± S.E.M., n = 8–10 mice per group). *$P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice. (b) Biochemical analyses of striatal dopamine, DOPAC, and HVA levels in C57Bl mice injected with MPTP (30 mg/kg, i.p., single injection) alone or in combination with EpoD (1 mg/kg, i.p.) dissolved in DMSO and injected 30 min before MPTP and then for the following 4 days once a day. Mice were killed 7 days later (mean ± S.E.M., n = 8–10 mice per group). *, # $P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice (*) or versus mice injected with MPTP alone (#). (c) Immunohistochemical analysis of TH in the pars compacta of substantia nigra of mice injected with a single i.p. injection of 30 mg/kg of MPTP, alone or combined with EpoD (1 mg/kg, i.p., 30 min prior to MPTP and then for the following 4 days, once a day). Scale bar = 250 μm. (d) Stereological counts of TH-positive cell in the substantia nigra pars compacta (mean ± S.E.M., n = 5 mice per group). *, # $P < 0.05$; one-way ANOVA, Dunnett *post hoc* test versus saline-injected mice (*) or versus mice injected with MPTP alone (#).

in TH in striatum, followed by TH depletion in substantia nigra, can easily be explained by impairment of axonal transport, other than by the dying back mechanism of degeneration, typical of PD[12] and mice exposed to MPTP[34]. Looking for the mechanisms underlying axonal transport impairment, we found that MPTP does not impact motor protein levels. However, it affects the levels of deTyr MTs, suggesting that altered MT stability is responsible for the alterations of axonal transport. Indeed, Kinesin 1, which is particularly abundant in neurons, is preferentially recruited, but moves slowly along highly modified MTs[33]. Furthermore, MPP^+ induces increases in deTyr tubulin content that precede and therefore may cause the reduction in mitochondrial transport in PC12 cells[18]. Consequently, we supposed that a similar scenario is likely to occur in MPTP-treated mice. Nevertheless, another paper by O'Malley's group[26] reported that MPP^+ specifically impairs mitochondria transport in dopaminergic neurons, an event that precedes autophagy and MT defects. However, they looked at gross, i.e. Ac-MT fragmentation and α-tubulin content reduction, rather than at subtle alterations of MTs, such as

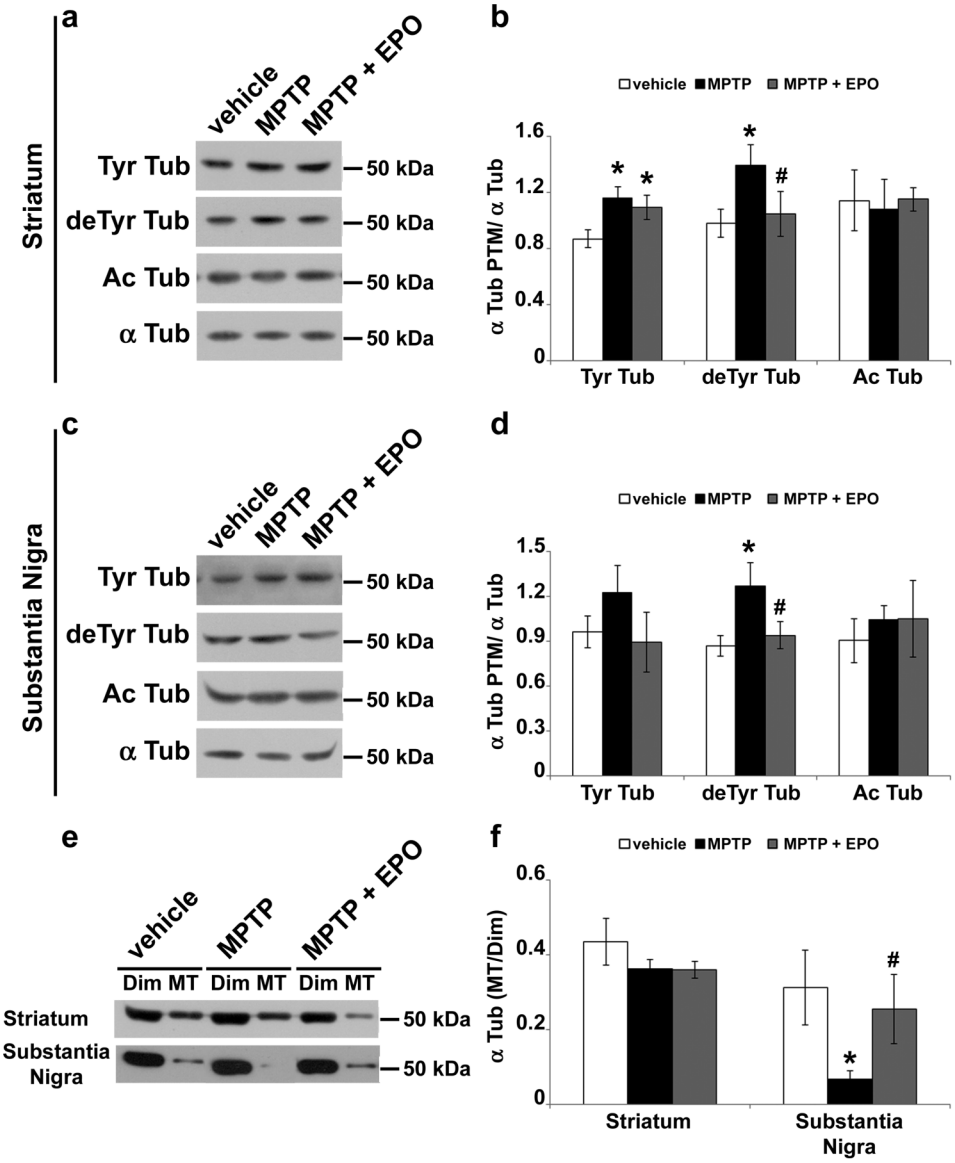

Figure 7 | EpoD rescues MT system in MPTP-treated mice. (a) Immunoblot of levels of tyrosinated tubulin (Tyr Tub), detyrosinated tubulin (deTyr Tub) and acetylated tubulin (Ac Tub) in lysates of striatum of mice treated as in Fig. 6. (b) Densitometric analyses of immunoblot reported in a (mean ± S.E.M., n = 5 mice per group). For the quantitation, values of each α-tubulin PTM were normalized on the level of α-tubulin (α Tub) of the relative sample. *, # $P < 0.05$; one-way ANOVA, Fischer LSD *post hoc* test versus saline-injected mice (*) or versus mice injected with MPTP alone (#). (c) Immunoblot of levels of tyrosinated tubulin (Tyr Tub), detyrosinated tubulin (deTyr Tub) and acetylated tubulin (Ac Tub) in lysates of substantia nigra of mice treated as in Fig. 6. (d) Densitometric analyses of immunoblot reported in c (mean ± S.E.M., n = 5 mice per group). For the quantitation, values of each α-tubulin PTM were normalized on the level of α-tubulin (α Tub) of the relative sample. *, # $P < 0.05$; one-way ANOVA, Fischer LSD *post hoc* test versus saline-injected mice (*) or versus mice injected with MPTP alone (#). Tubulin dimers (Dim) and MT polymers (MT) of corpus striatum and substantia nigra were analyzed by (e) immunoblot and (f) densitometric analyses and are shown as ratio (mean ± S.E.M., n = 3 mice per group). *, # $P < 0.05$; one-way ANOVA, Fischer LSD *post hoc* test versus saline-injected mice (*) or versus mice injected with MPTP alone (#).

changes in PTMs. A further possible cause of the axonal transport block is MT reorientation, which is induced either by MPP^+ [18] or by human mutant Tau expression[35] leading to traffic jams. Therefore, the observed impairment of axonal transport is likely mediated by alterations of MT stability and organization, which, in turn, lead to distal axon degeneration, as dying back is, and to the accumulation of DA loaded vesicles in cell soma. This could really be detrimental to the neurons since DA oxidation produces large quantities of reactive oxygen species (ROS) triggering dopaminergic neuron death[36].

The observation that levels of the neuron-specific βIII tubulin, which is the most dynamic among the β-tubulin isotypes[37], are increased points out the importance of MT dynamics and its tight regulation in MPTP-mediated neurodegeneration, as we have already shown[16,18]. βIII tubulin enrichment could be explained as an adaptive mechanism to counteract the MPTP-induced reduction in Tyr α−tubulin, usually associated with highly dynamic MTs. Moreover, βIII tubulin expression is primarily restricted to the nervous system[38], and it has been suggested that different β-tubulin isotypes could serve specific and unique roles[39], such as neuronal elongation and axon guidance. Banarjee and colleagues[40] showed that in the presence of Tau, a neuron-specific MT binding protein, βIII tubulin was more prone to polymerize than the other isotypes. Worthy of note is the recent suggestion that the Tau-MTs interaction may be important not only in the pathogenesis of Alzheimer's disease, but also of PD. Indeed, both MPTP and α-synuclein mutations promote Tau phosphorylation, causing MT instability, which leads

to loss of dopaminergic neurons in PD brain[41]. Nevertheless, trying to counteract the ongoing axonal destruction, the dopaminergic neurons could promote MT polymerization through increase in the βIII tubulin content or in MT stability, as suggested by the enrichment in deTyr and Ac α-tubulin.

Mitochondria are largely considered crucial players in the pathogenesis of PD. Indeed, both MPP^{+} [42] and rotenone[43] inhibit mitochondrial complex I, reducing ATP synthesis and increasing ROS production. However, the lack of complex I does not protect dopaminergic neurons from toxin administration[44], suggesting the existence of alternative mechanisms of action. It is well known that tubulin interacts with VDAC, the most abundant protein in the mitochondrial outer membrane, and mitochondria-associated tubulin is enriched in βIII isotype[45]. Recently, it has been reported that tubulin decreases the respiration rate of isolated mitochondria[46] and that the increase in tubulin dimers induces mitochondrial depolarization in human cancer cells[47]. Furthermore, the administration of tubulin-targeted drugs induces mitochondrial depolarization and Ca^{++} release[48]. This body of evidence clearly shows that interfering with MT system impairs mitochondria activity. Therefore, enrichment in free tubulin dimers in PC12 cells[18] and MPTP-induced increase in βIII tubulin (present data) could induce adverse effects, such as mitochondrial dysfunction. Being life a matter of balance, when the equilibrium shifts from a beneficial event, such as MT polymerization induced by βIII tubulin increases, toward a detrimental one, such as mitochondrial dysfunction caused by the same factor, dopaminergic neurons may die leading to PD. In this context, it is essential to consider parkin, an E3 ligase promoting degradation of tubulin and other proteins, known to interact with MTs and to play a central role in the regulation of mitophagy. Thanks to its particular position, parkin may quarantine damaged mitochondria, by severing their connection to the MT network, before promoting their clearance[49]. If MT-parkin interaction was impaired, as in the case of MT destabilization, proper regulation of mitophagy would fail, leading to dopaminergic neuron loss. Thus, tubulin partitioning between dimer and polymer pools regulates multiple steps in mitochondrial metabolism and, therefore, in the control of neuronal health and death.

A first step designed to block the progression of the disease would be the regeneration of collapsing axons. MT stabilization could be useful to physically counteract axon disruption, reinforcing the pillars that support the structures, and to prevent mitochondrial damage, reducing the level of free tubulin dimers. In fact, the activation of signal cascades that converge on MT stability modulation has trophic effects on axon formation *in vivo*[50,51]. It has already been shown that the MT-stabilizer Taxol protects cultured dopaminergic neurons against rotenone toxicity[17,52], reduces scarring formation and promotes regeneration of central nervous system axons[53,54]. Unfortunately, the blood-brain barrier penetration of Taxol is very poor[55]. EpoD, another MT-stabilizing compound, penetrates through the blood-brain barrier and has resulted to be neuroprotective in mouse models of schizophrenia[56] and tauopathy[19]. Moreover, it improves axonal MT density, reduces axonal dystrophy and alleviates cognitive deficits in transgenic mouse models of tauopathies[19,20]. What is more, the dynamicity of MTs is increased in tau transgenic mice and treatment with EpoD restored MT dynamics. MT stabilization had beneficial effects on behavior, tau pathology and neurodegeneration[21]. Here, we used the classical model of experimental parkinsonism induced by MPTP to test the hypothesis that MT stabilization is also able to counteract degeneration in another model of neurodegeneration. Acute injections of EpoD did not affect the toxic effect induced by MPTP, whereas repeated injections of EpoD restored tubulin PTMs and MT mass in the substantia nigra and exerted neuroprotective effects in the dopaminergic nigrostriatal system of MPTP-treated mice. Therefore, doses of EpoD, which were chosen according to Barten and colleagues[21], are also effective in the MPTP model of dopaminergic neurotoxicity. These data pinpoint that alterations of MTs are very early events, which specifically occur in dopaminergic neurons in experimental parkinsonism, and reinforce the idea that the cytoskeleton of dopaminergic neurons is particularly vulnerable but is also highly responsive to MT-targeting agents.

Taken together with our recent findings that MT stability is impaired in human fibroblasts deriving from PD patients and that MT stabilization rescues control phenotype[28], the present work suggests that MTs are a potential target for pharmacological therapy designed to block the axonal disruption leading to PD. Thus, chronic pharmacological stabilization of MT may be a viable strategy for the management of the disease.

Methods

Materials. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was purchased from Sigma (St. Louis, MO). Epothilone D was purchased from Acme Bioscience (Palo Alto, CA).

Animals. Male C57 Black mice (22–24 g, b.w., 8–9 week old) were purchased from Charles River (Calco, Italy) and used for all experiments. Mice were kept under environmentally controlled conditions (ambient temperature = 22°C, humidity = 40%) on a 12-h light/dark cycle with food and water *ad libitum*. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Italian Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the I.R.C.C.S. Neuromed Institute. Permit Number 432007/A was issued by the Italian Ministry of Health. All efforts were made to minimize suffering.

Treatments. Mice were treated with one single i.p. injection of 36 mg/kg of MPTP (corresponding to 30 mg/kg of free MPTP) or with three doses of 24 mg/kg of MPTP hydrochloride (corresponding to 20 mg/kg of free MPTP), injected i.p. at 2 h intervals (cumulative dose = 60 mg/kg of free MPTP). Twelve or 72 h after last injection of MPTP, mice were killed by decapitation or by intracardiac perfusion, to perform biochemical or immunohistochemical analysis, respectively. One cerebral hemisphere of mice used for biochemical analysis, was used to evaluate levels of striatal DA and its metabolites, 3,5-dihydroxyphenylactic acid (DOPAC) and homovanillic acid (HVA). For EpoD experiments, mice were injected with a single i.p. dose of 36 mg/kg of MPTP and then acutely (1 or 3 mg/kg, i.p., 30 min prior to MPTP) or chronically treated (1 mg/kg, i.p., 30 min prior to MPTP and then every day for 4 days) with EpoD. Mice were killed 7 days later for biochemical analysis of MTs and biochemical and immunohistochemical assessment of nigro-striatal damage. EpoD was dissolved in DMSO and injected i.p. (in a volume of 50 μl/mouse). Control mice were injected with the vehicle alone.

Motor activity assessment. Motor coordination was assessed by the rotarod test. The rotarod apparatus consisted of a motor driver control unit (Ugo Basile, Varese, Italy) and a rotating horizontal cylinder (30 mm), divided into five separate rotating compartments and fully enclosed to ensure that the mice did not jump out of their area. Automatic timers recorded the time (in seconds) the mice remained on the rod, which was rotating at an accelerating speed from 5 to 15 rpm. Mice were tested for 10 min on the rotarod every day, starting 2 days after MPTP or EpoD injection (as above). General health conditions (fur, body weight and mortality) were also monitored by an experimenter unaware of treatments.

Monoamine assay. The corpus striatum was immediately dissected out homogenized by sonication in 0.6 ml of ice-cold 0.1 M PCA. Fifty μl of the homogenate were used for protein determination[57]. The remaining aliquot was centrifuged at 8,000 *g* for 10 min, and 20 μl of the supernatant was injected into an HPLC equipped with autosampler 507 (Beckman Instruments, Fullerton, CA), a programmable solvent module 126 (Beckman), an analytical C-18 reverse-phase column kept at 30°C (Ultrasphere ODS 5 μm, 80 Å pore, 250 × 4.6 mm (Beckman), and a Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA). The holding potentials were set at +350 and −350 mV for the detection of DA, DOPAC and HVA. The mobile phase consisted of 80 mM sodium phosphate, 40 mM citric acid, 0.4 mM EDTA, 3 mM 1-heptansulphonic acid and 8.5% methanol, brought to pH 2.75 with phosphoric acid (run under isocratic conditions, at 1 ml/min).

Western blot analysis. Western blot analysis was performed on protein extracts or cytoskeletal fractions obtained from mouse brain regions. To get total proteins, corpus striatum and substantia nigra were immediately dissected out on ice, mechanically homogenized and, subsequently, sonicated in SDS-PAGE sample buffer. Separation of cytosolic tubulin dimers from MT polymers was performed accordingly to Fanara and colleagues[58]. Briefly, corpus striatum and substantia nigra were gently homogenized in MT-stabilizing buffer and postnuclear supernatants were centrifuged at 200,000 *g* at 20°C for 20 min; the supernatant (containing the soluble dimeric tubulin) and the pellet (containing the MT fraction) were separated and stored at −20°C. Western blots were made as previously described[18] using the

following antibodies: α-tubulin mouse IgG (clone B-5-1-2, Sigma-Aldrich); deTyr tubulin rabbit IgG (Chemicon, Temecula, CA); Tyr tubulin mouse IgG (clone TUB-1A2, Sigma-Aldrich); Ac tubulin mouse IgG (clone 6-11B-1, Sigma-Aldrich); β-tubulin mouse IgG (clone Tub 2.1, Sigma-Aldrich); βIII tubulin mouse IgG (clone TU-20, kindly provided by Dr. Pavel Dràber, Prague, Czec Republic); actin mouse IgM (N350, Amersham, Little Chalfont, UK); tyrosine hydroxylase (TH) mouse IgG (clone 6dt, Abcam, Cambride, UK) 1 : 600; kinesin rabbit IgG (Abcam) 1 : 1000; Dynein mouse IgG (clone 74.1, Millipore) 1 : 500, GAPDH (Abcam). Membranes were washed for 30 min with 3 changes and incubated for 1 h at room temperature with HRP donkey anti-mouse IgG (Pierce), HRP goat anti-mouse IgM (Sigma-Aldrich), or HRP goat anti-rabbit IgG (Pierce). Immunostaining was revealed by enhanced chemiluminescence (Super-Signal West Pico Chemiluminescent, Pierce). Quantification was performed by ImageJ software (NIH, Bethesda, MD).

Confocal analysis. Mice were anesthetized with chloralium hydrate (320 mg/kg, i.p.) and transcardially perfused with 4% PFA in 0.1 M phosphate buffer, pH 7.4. Brains were removed, postfixed overnight in 4% PFA, and then transferred in 30% sucrose for cryoprotection. Sagittal sections (50 μm thick) were cut with a Vibratome (VT1000S, Leica). Sections were stained with porin rabbit IgG (VDAC1/porin, Abcam, Cambride, UK) and the following antibodies previously used for immmunoblotting: deTyr tubulin rabbit IgG; Tyr tubulin mouse IgG; Ac tubulin mouse IgG; porin rabbit IgG (VDAC1/porin, Abcam). To identify dopaminergic neurons and fibres, each section was concurrently stained with anti-TH antibody, made in mice (clone LCN1, Millipore) or rabbits (Millipore) as appropriate. As secondary antibodies we used Alexa Fluor™ 568 donkey anti-mouse IgG, and Alexa Fluor™ 488 goat anti-rabbit IgG (Invitrogen). Coverslips were mounted in PBS-glycerol and examined with a confocal laser scan microscope imaging system (TCS SP2 AOBS, Leica Microsystems, Heidelberg, Germany) equipped with an Ar/Ar-Kr 488 nm, 561 nm and 405 nm diode lasers. Photomultiplier gain for each channel was adjusted to minimize background noise and saturated pixels and, once defined for control conditions, parameters were kept constant for all acquisitions. To estimate the overlapping area between red and green signals, analyses were carried out on single-plane raw images and Manders' coefficients were calculated applying the JACoP plug-in (developed and reviewed by 31) for ImageJ software. To evaluate the mitochondria distribution, the porin signal was superimposed on dopaminergic fibres, using the Mask tool of the Leica Confocal Software (Leica); mitochondria accumulations were identified as white pixels-containing area, as thick as long, clearly separated from other white pixels. TH-positive signal longer than 5 μm was considered as dopaminergic fibre, and signals separated by more than 10 μm were counted as two distinct fibres.

Immunohistochemical analysis of tyrosine hydroxylase. Brains were removed, fixed in ethanol (60%), acetic acid (10%), and chloroform (30%), and included in paraffin. Tissue sections (30 μm) were incubated overnight with monoclonal mouse anti-TH (1 : 200; Sigma-Aldrich) and then for 1 h with secondary biotin-coupled anti-mouse antibodies (1 : 200; Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine tetrachloride (Sigma) was used for detection.

Stereological cell counting of tyrosine hydroxylase-positive cells in the substantia nigra pars compacta. The number of TH-positive cells in the substantia nigra pars compacta was assessed by stereological technique and an optical fractionator using a Zeiss Axio Imager M1 microscope equipped with a motorized stage and focus control system (Zeta axis), and with a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc., Bethesda, MD) equipped with a Macro was used for the analysis of digital images. The Macro was obtained by "Immagini e Computer" (Bareggio, Italy). The characteristics of this Macro are published[59]. The analysis was performed on 6 sections of 20 μm, sampled every 200 μm on the rostro-caudal extension, in which the substantia nigra was identified and outlined at 2.5× magnification. TH-positive cells were counted at 100× magnification as described[60]. For stereological analysis, we used a grid of disectors (counting frame of 100 × 100 μm; grid size 50 × 50 μm), with 1.3 as numerical aperture of the lens. The total number of TH-positive cells in the substantia nigra was computed from the formula: $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where n is the total number of cells counted on each disector; SSF (fraction of sections sampled) the number of regularly spaced sections used for counts divided by the total number of sections across the substantia nigra pars compacta; ASF (area sampling frequency) the disector area divided by the area between disectors (7500 μm^2 × disector number/region area); and TSF (thickness sampling frequency) the disector thickness divided by the section thickness (20 μm).

Statistical analysis and data managing. The statistical significance of treatment was assessed by one-way ANOVA with Dunnett 2-sided or Fischer LSD post-hoc testing or χ^2 test when appropriate. Analyses were performed using STATISTICA (StatSoft Inc., Tulsa, OK).

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Author contributions

D.C. performed protein analysis by Western blotting and confocal microscopy analysis; F.C. performed immunohistochemistry; C.L.B. and G.M. performed in vivo experiments and prepared tissue for protein analysis; D.B. performed immunohistochemistry and stereological counts of tyrosine hydroxylase-positive cells; A.T. performed biochemical analysis of striatal dopamine and its metabolite levels; D.P. and E.G. designed experiments; D.C., G.P., G.B. and C.G. designed the experiments, analysed the data and wrote the paper.

Additional information

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