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Review

The Proteasome Inhibition Model of Parkinson's Disease

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Abstract. The pathological hallmarks of Parkinson's disease are the progressive loss of nigral dopaminergic neurons and the formation of intracellular inclusion bodies, termed Lewy bodies, in surviving neurons. Accumulation of proteins in large insoluble cytoplasmic aggregates has been proposed to result, partly, from a failure in the function of intracellular protein degradation pathways. Evidence in support for such a hypothesis emerged in the beginning of the years 2000 with studies demonstrating structural and functional deficits in the ubiquitin-proteasome pathway in post-mortem nigral tissue of patients with Parkinson's disease. These fundamental findings have inspired the development of a new generation of animal models based on the use of proteasome inhibitors to disturb protein homeostasis and trigger nigral dopaminergic neurodegeneration. In this review, we provide an updated overview of the current approaches in employing proteasome inhibitors to model Parkinson's disease, with particular emphasis on rodent studies. In addition, the mechanisms underlying proteasome inhibition-induced cell death and the validity criteria (construct, face and predictive validity) of the model will be critically discussed. Due to its distinct, but highly relevant mechanism of inducing neuronal death, the proteasome inhibition model represents a useful addition to the repertoire of toxin-based models of Parkinson's disease that might provide novel clues to unravel the complex pathogenesis of this disorder.

Keywords: Proteasome, lactacystin, neurodegeneration, animal model, Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative condition, in which nigral dopamine (DA) producing neurons become dysfunctional and are progressively lost during a protracted period of disease development. The gradual degeneration of the nigrostriatal DA-ergic pathway leads to the manifestation of motor-related symptoms, such as resting tremor, difficulty in initiating movements (akinesia), slowness of movements (bradykinesia), muscular rigidity and loss of balance [1]. The main pathological hallmarks of PD are the depigmentation of the substantia nigra pars compacta (SNc) caused by the progressive loss of nigral DA-ergic neurons, and the presence of intracellular inclusion bodies, termed Lewy bodies (LBs), in surviving neurons [2]. These LBs are proteinaceous aggregates highly enriched in the pre-synaptic protein α-synuclein [3] and their formation indicates that disturbances in protein homeostasis (i.e. proteostasis) are part of the pathomechanisms underlying DA neuron loss in PD. Accordingly, similar to other neurodegenerative disorders such as multiple system atrophy and dementia with Lewy bodies (DLB), PD is classified as a synucleinopathy [4].

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Although the precise cause of PD has remained elusive, various pathogenic mechanisms have been associated with nigral cell death, including oxidative stress, mitochondrial dysfunction, neuroinflammation, glutamate excitotoxicity and impairment in protein degradation pathways, such as the ubiquitin-proteasome system [5]. These fundamental discoveries regarding the pathogenesis of PD have been instrumental in our attempts for modeling the disease *in vitro* and *in vivo*. Introduced during the early 2000s, following the discovery of structural and functional deficits in the proteasome system in patients with PD, the proteasome inhibition model relies on application of proteasome inhibitors to induce disturbances in proteostasis and trigger DA neurodegeneration. The model has attracted attention from the research community for being able to trigger accumulation and aggregation of endogenous proteins, such as α-synuclein, leading to neurotoxicity and thereby replicating one of the central pathogenic pathways in PD. However, in spite of its contemporary use, certain aspects related to the model are still under research and need to be further defined, such as the validity in replicating PD related changes, the similarities / dissimilarities with other toxin-based or genetic models of PD, the mechanisms of DA neuron death in conditions of proteasome inhibition, and the interaction of this pathway with PD related genetic factors. In this review, we will discuss these issues and provide an updated overview on the proteasome inhibition model of PD, highlighting defining features with regards to molecular and cellular changes, and the implementation of the model both in cell culture and *in vivo*. In order to aid prospective research groups that would like to implement this model in their laboratories, we also provide a comprehensive guide with the different sites of administration of proteasome inhibitors in rats and mice, and their effects on the nigrostriatal DA-ergic pathway and motor behavior.

THE UBIQUITIN-PROTEASOME SYSTEM AND ITS DYSFUNCTION IN PARKINSON'S DISEASE

The ubiquitin-proteasome system is a major intracellular protein degradation pathway that ensures proteostasis in eukaryotic cells (for review see [6, 7]). The cycle of protein degradation via the proteasome system begins with the attachment of a polyubiquitin chain (typically containing at least four ubiquitin molecules, linked to each other via Lys48-linkages) to the target protein. This process requires ATP and is accomplished in a series of reactions catalyzed by ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes. During the first step, E1 activates ubiquitin molecules in an ATP-dependent reaction, followed by the transfer of activated ubiquitin from E1 to E2. Next, ubiquitin is successively conjugated to the target protein by an E3 ubiquitin-ligase enzyme, leading to the covalent attachment of a polyubiquitin chain to the substrate. The tagged protein is subsequently recognized by the 26S proteasome, a large multimeric complex formed by a catalytic core (20S) and a regulatory particle (19S, also known as PA700) that caps the 20S proteasome at either end (Fig. 1). The 19S regulatory particle binds the polyubiquitin chain, cleaves it from the substrate, and unfolds the substrate, allowing it to enter 20S, a core particle that contains 28 subunits organized in two outer rings (α) and two inner rings (β) (Fig. 1). Within the 20S core particle, the substrate is digested into peptide fragments by three types of enzymatic activities (chymotrypsin-like, trypsin-like and caspase-like protease activities) provided by the β subunits. In addition to 26S proteasomes, free (i.e. uncapped) 20S proteasomes also exist in mammalian cells and mediate ubiquitin-independent and ATP-independent protein degradation of partially unfolded proteins (natively unfolded or damaged proteins) (Fig. 1) [8]. The 20S proteasome can become a major degradation pathway in certain conditions (e.g. oxidative stress) [9], and the expression of the 20S proteasome can increase via disassembly of the 26S proteasome into its 20S and 19S components in such situations [10]. While most proteins require polyubiquitination to be directed to and degraded by the 26S proteasome, α-synuclein is an example of a protein that can be directly degraded by the 20S proteasome without requirement of ubiquitination. Both ubiquitin-dependent [11, 12] and ubiquitin-independent [13, 14] degradation via the 26S/20S proteasome have been described for α-synuclein.

An increasing amount of evidence indicates that the proteasome function is impaired in the substantia nigra (SN) of PD patients (Table 1). Post-mortem data obtained from sporadic PD indicate the presence of structural and functional defects in the 26/20S proteasome, including loss of 20S core α-subunits [15, 16], decreased expression of PA700 [17], as well as a general loss of all three peptidase activities of the 20S proteasome [18, 19]. In addition, indirect proof of a failure of the proteasome system

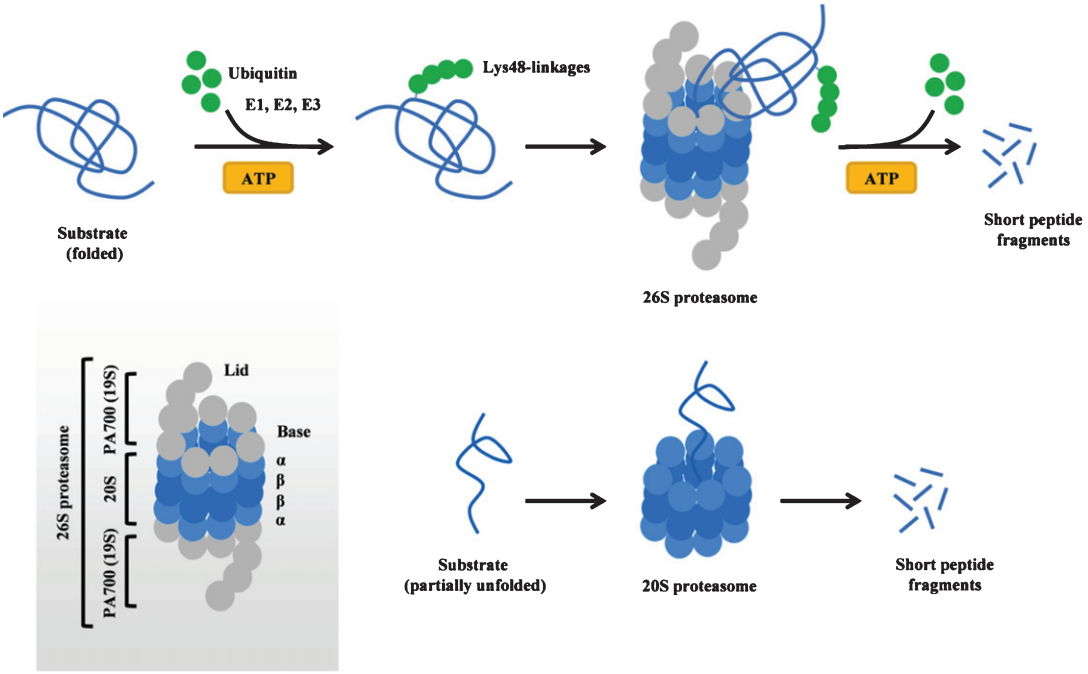


Fig. 1. The 26S/20S proteasome structure and function. Top: Ubiquitin- and ATP-dependent protein degradation of substrates via the 26S proteasome. The 26S proteasome can also degrade proteins in an ubiquitin-independent manner (not shown). Bottom: Ubiquitin- and ATP-independent protein degradation of natively unfolded or damaged and partly unfolded substrates via the 20S proteasome. Inset shows the structural composition of the 26S proteasome. ATP adenosine triphosphate.

Table 1
Functional and structural defects in the proteasome system in sporadic PD

Ref	Origin	Structural and/or functional changes
[15]	*Post-mortem* SN	Decreased immunoreactivity for 20S α-subunits in nigral neurons. No change in the expression of 20S β-subunits.
[213]	PD iPSCs	Decreased 20S chymotrypsin-like activity.
[160]	*Post-mortem* SN	Decreased immunoreactivity for 20S proteasomes in nigral neurons containing α-synuclein inclusions.
[32]	PD cybrids	Decreased 20S trypsin-like and caspase-like activities.
[18]	*Post-mortem* SN	Decreased 20S chymotrypsin-like, trypsin-like, and caspase-like activities.
[16]	*Post-mortem* SN	Decreased expression of 20S α-subunits.
[17]	*Post-mortem* SN	Decreased expression of 20S α-subunits. No change in the expression of 20S β-subunits. Decreased expression of PA700. Decreased 20S chymotrypsin-like, trypsin-like, and caspase-like activities.
[19]	*Post-mortem* SN	Decreased 20S chymotrypsin-like activity.

iPSC induced pluripotent stem cells, SN substantia nigra, PD Parkinson's disease.

in PD was provided from neuropathological studies documenting an accumulation of components of the proteasome system, such as proteasomal subunits and proteasomal activators, heat shock proteins, and ubiquitin and ubiquitinated proteins in LBs [20]. However, more recent evidence seems to suggest that ubiquitin accumulated in LBs is preferentially K63-linked (rather than K48-linked) and that this process may be unrelated to impaired proteasome function [21]. In particular, accumulation of K63-linked ubiquitin in LB disease was found to be partly caused by an increase in the levels of the Usp8, an enzyme that deubiquitinates K63-linked chains on α-synuclein leading to a decrease of its degradation via the lysosomal pathway [21].

The underlying causes of proteasome inhibition in PD have not been elucidated. Interestingly, ageing, the main risk factor for developing PD, has been shown to negatively affect both proteasome structure and function [22–24]. Of note, the SN is particularly

vulnerable to age-related decreases in proteasome activity, evidenced by a simultaneous decrease of all three protease activities of the proteasome in the aged SN of rats and mice [25]. In addition, various disease-relevant factors have been demonstrated to negatively influence the function of the proteasome system, including pesticides such as rotenone [26], paraquat [27], dieldrin [28] and maneb [29], as well as the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [30]. The fact that toxins affecting mitochondrial function also lead to impairment of proteasome degradation is not surprising, given that the proteasome degradation cycle is ATP-dependent. Bioenergetic failure, as occurs in PD, could be a significant contributor to the impairment in proteasome function [31]. A recent study using PD cybrids created by transferring mitochondria of PD patients into recipient mitochondrial DNA-depleted cells (NT2 Rho0 cells), demonstrated that PD-related mitochondrial dysfunction is sufficient to decrease the catalytic activity of the 20S proteasome [32]. Also disease-relevant, α-synuclein, especially in its mutated [33, 34] or aggregated [35, 36] forms, can bind to and inhibit the proteasome. Moreover, the finding that DA [37, 38] or factors intrinsic to nigral DA neurons, such as neuromelanin [39] or the DA metabolite aminochrome [40], can inhibit proteasomal function is intriguing, and might underlie the selective vulnerability of nigral DA neurons to proteasomal impairment in PD.

PROTEASOME INHIBITORS AND THEIR MECHANISM OF ACTION

Proteasome inhibitors can be broadly categorized based on their origin into synthetic or natural compounds. Some of the first synthetic inhibitors designed to target the proteasome were peptide aldehydes that act as substrate analogues and potent transition-state inhibitors, primarily of the chymotrypsin-like activity of the 20S proteasome [41]. These compounds, including carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132), carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (MG115) and carbobenzoxy-L-isoleucyl-L-gamma-t-butyl-L-glut-amyl-L-alanyl-L-leucinal (PSI), are cell-permeable and block the proteolytic activity of the 26S proteasome, in a reversible manner. In spite of their potency, one of the drawbacks of these compounds is their decreased specificity, as they also inhibit certain lysosomal cysteine proteases and calpains [41]. Actinobacteria have been found to naturally produce proteasome inhibitors such as lactacystin and epoxomicin. In contrast to synthetic peptide aldehydes, these structurally distinct natural inhibitors covalently bind to subunits of the proteasome and irreversibly block the proteolytic activity of the proteasome [42]. Previous *in vitro* studies have provided detailed insight into the molecular mechanism of action of lactacystin by demonstrating that in aqueous environments, lactacystin undergoes spontaneous hydrolysis to clasto-lactacystin dihydroxic acid and N-acetylcysteine, with the intermediacy of clasto-lactacystin-β-lactone [43]. Subsequent studies have demonstrated that clasto-lactacystin-β-lactone, but not lactacystin, is cell permeable and can enter cells where it interacts with the 20S proteasome [44]. In particular, clasto-lactacystin-β-lactone was found to form an ester-linked adduct with the amino-terminal threonine of the mammalian proteasome subunit X, a β-subunit of the 20S proteasome [45]. By covalently attaching to subunit X, clasto-lactacystin-β-lactone potently inhibits all three peptidase activities of the 20S proteasome [45]. Early studies indicated that lactacystin (via the intermediacy of the β-lactone) is highly specific for the proteasome and does not inhibit serine and cysteine proteases [45] or lysosomal protein degradation [46]. Subsequent studies, however, have highlighted additional intracellular targets besides the 20S proteasome, including cathepsin A [47] and tripeptidyl peptidase II [48], which should be acknowledged when interpreting the biological effects using this compound. Given the widespread use of the lactacystin model (especially for rodent studies), findings obtained using this neurotoxin will be emphasized and supported by studies using structurally distinct inhibitors.

MODELING PARKINSON'S DISEASE USING PROTEASOME INHIBITORS

In order to better understand the relation between proteasome inhibition and pathogenic processes relevant in PD, various studies have assessed the effects of proteasome inhibitors on the viability of DA-ergic neurons *in vitro* and *in vivo*. In turn, these findings have led to the development and characterization of the proteasome inhibition model of PD.

In this section, we will separately discuss the findings obtained in different model systems, including results from cell culture, *Caenorhabditis elegans*

(*C. elegans*), medaka fish, rodent models and non-human primates. Each of these model systems provides their own unique advantages and limitations. Studies from cell cultures (either DA-ergic cell lines or primary neuronal cultures) have provided fundamental insight with regards to the pathways recruited and the cell type specificity of proteasome inhibition-induced cell death. Application of proteasome inhibitors in small animal models, such as *C. elegans* and medaka fish, provides a unique advantage in modeling this PD-related pathway for high throughput genetic or drug screening. The higher complexity of the nervous system of rodents makes them suited as model species to investigate the DA-ergic neurotoxicity of proteasome inhibitors, in particular as it allows the simultaneous assessment of behavioral features. In addition, the use of genetically engineered mice (such as knock-out mice) can reveal novel mediators of proteasome inhibition-induced neurodegeneration *in vivo*. Finally, non-human primates represent the best approximation of the human central nervous system and their use is indispensable for both investigating pathogenic mechanisms and validating promising therapeutic drugs before they enter clinical trials. As will be discussed, with small exceptions, application of proteasome inhibitors to various model systems leads to death of DA neurons and formation of intracytoplasmic LB-like inclusion bodies (Table 2).

DA-ergic cell lines

Rat adrenal pheochromocytoma PC12 cells have been shown to synthesize, store, release and metabolize DA, and can be differentiated into a neuron-like phenotype upon treatment with nerve growth factor [49]. Treatment of naive and neuronally differentiated PC12 cells with structurally distinct proteasome inhibitors, including lactacystin, epoxomicin, PSI and MG115, was found to lead to dose-dependent cell death [50–54]. Application of proteasome inhibitors caused mitochondrial-mediated apoptotic cell death, as evidenced by increases in mitochondrial membrane permeabilization, mitochondrial cytochrome c release, caspase-3 and -9 activation [51, 53, 55] as well as morphological assessment by electron microscopy [50]. The fact that cell death induced by proteasome inhibitors is apoptotic was confirmed by the protection offered by caspase inhibitor zVAD-fmk against lactacystin- [54] and PSI-induced cell death [53]. Importantly, Fornai and collaborators reported that treatment of PC12 cells with proteasome inhibitors such as lactacystin or epoxomicin leads to the formation of intracellular LB-like aggregates containing α-synuclein, ubiquitin, parkin and ubiquitin-activating enzyme E1 [50]. Consistent with these findings, treatment of neuronally differentiated PC12 cells with lactacystin, epoxomicin or PSI was shown to induce formation of cytoplasmic fibrillar inclusions containing α-synuclein and ubiquitin [54, 56, 57]. Comparable findings were obtained using the mesencephalic DA-ergic cell line N27. Treatment with MG132 was found to lead to dose-dependent death of cultured N27 cells. Similar to PC12 cells, cell death was apoptotic and initiated by a mitochondrial-mediated cascade involving mitochondrial depolarization, cytochrome c release and activation of caspase-2, -3 and -9 [38, 58, 59].

The human neuroblastoma cell line SH-SY5Y has both DA β-hydroxylase and DA transporter (DAT) activity and has been previously used as a model of DA-ergic neurons [60]. Treatment of this cell line with lactacystin has been found to cause dose-dependent apoptotic cell death, involving loss of mitochondrial membrane potential and cytochrome c release [61, 62]. Furthermore, administration of lactacystin to SH-SY5Y cells leads to formation of cytoplasmic aggregates containing ubiquitin and α-synuclein [63].

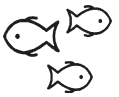
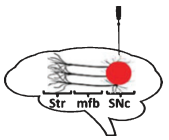
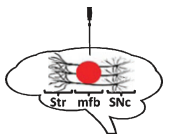
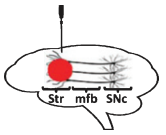
Primary neuronal cultures

In accordance with the experiments performed on DA-ergic cell lines, application of structurally distinct proteasome inhibitors including lactacystin, epoxomicin and MG132 leads to dose-dependent cell death of DA-ergic neurons in primary ventral mesencephalic cultures [55, 58, 64–67]. Apoptotic cell death is probably involved, as lactacystin induces mitochondrial release of cytochrome c and activation of caspase-3 [55], while caspase inhibitor BAF protects DA neurons against lactacystin- or epoxomicin-induced cell death [67]. Importantly, application of proteasome inhibitors to primary ventral mesencephalic cultures leads to generation of α-synuclein oligomers [68] and formation of cytoplasmic inclusion bodies containing ubiquitin and α-synuclein in surviving DA neurons [64, 67].

C. elegans

Exposure of *C. elegans* to proteasome inhibitor MG132 results in impaired ubiquitin-related protein degradation and progressive loss of DA neurons [69]. Interestingly, exposure of *C. elegans* overexpressing

Table 2
Key findings obtained using proteasome inhibitors to model PD *in vitro* and *in vivo*

	Cell cultures
	Dose-dependent apoptotic death of cultured DA-ergic cells and DA-ergic neurons in primary ventral mesencephalic cultures. Formation of aggregates containing α-synuclein in surviving cells.
	Medaka fish
	Loss of DA-ergic neurons with protein accumulation / aggregation. Motor impairment.
	Rodents: intra-nigral administration
	Loss of nigral DA-ergic neurons with α-synuclein accumulation / aggregation in surviving neurons. Motor impairment. Comparable findings in mice and rats. Extensively replicated as model for PD.
	Rodents: intra-mfb administration
	Loss of nigral DA-ergic neurons with α-synuclein accumulation / aggregation in surviving neurons. Motor impairment. Comparable findings in mice and rats. Replicated as model for PD.
	Rodents: intra-striatal administration
	Loss of nigral DA-ergic neurons with α-synuclein accumulation / aggregation in surviving neurons, however not replicated in one study. Approach not attempted in mice. Reproducibility of the model unclear.
	Rodents: systemic administration
	Controversy regarding the effects of systemic administration of proteasome inhibitors in both rats and mice. Not a reliable PD model with the current approach.
	Non-human primates: systemic administration
	Approach not successful in generating a PD model. Possibly linked with the failure of the systemic model in rodents.

DA dopamine, mfb medial forebrain bundle, PD Parkinson's disease.

α-synuclein in DA-ergic neurons to MG132 led to a significant increase in the degree of protein accumulation and neurodegeneration compared to control α-synuclein overexpressing worms [70].

Medaka fish

Recently, Matsui and collaborators reported the induction of PD-like features after proteasome inhibition in medaka, a small fish of the *Actinopterygii* class (to which zebrafish also belong) [71]. The authors found that administration of proteasome inhibitors such as lactacystin or epoxomicin to medaka via the cerebrospinal fluid induces a selective loss of DA-ergic and noradrenergic neurons (with a concomitant decrease in DA and noradrenaline tissue levels), leading to a reduction in spontaneous swimming movements. Interestingly, these effects were accompanied by formation of cytoplasmic inclusions containing ubiquitin in different regions of the brain, including in tyrosine hydroxylase (TH)-positive neurons [71]. The development of a medaka proteasome inhibition model showing DA neuron loss and protein aggregation is of note as it can be amenable to

high throughput genetic or drug screening experiments evaluating PD-related pathogenesis or novel therapeutic targets [72].

Rodent models

The use of proteasome inhibitors to study the link between impaired protein degradation and DA neuron degeneration has been extended to rodent studies. Proteasome inhibitors have been administered to rats and mice either intracerebrally (at different sites of the nigrostriatal DA-ergic pathway) (Table 3) or systemically (Table 4).

Stereotaxic infusion in the substantia nigra pars compacta

Administration of proteasome inhibitor lactacystin to the SNc of rats leads to nigrostriatal DA neurodegeneration and motor impairment [73–81]. Loss of nigral DA neurons is dose-dependent [77–79] and was found to be either progressive [73, 80] or non-progressive [74, 78], and in certain cases to spread bilaterally [78]. DA cell death following lactacystin is most likely apoptotic in nature, as observed by ultrastructural assessment using electron microscopy [80]. In addition to nigral neurons, neighboring regions such as the ventral tegmental area (VTA) and substantia nigra pars reticulata (SNr) were also investigated for their susceptibility to intranigral lactacystin. VTA DA neurons were seen to be damaged by toxin administration, but to a smaller degree than nigral DA neurons [74, 78], in line with the pattern of degeneration observed in human PD. Furthermore, intranigral lactacystin leads to a moderate loss of non-DA neurons in the SNr and loss of γ-aminobutyric acid (GABA) tissue levels [75], reflecting a certain degree of non-specific damage to SNr GABA-ergic neurons. At a pathological level, DA neuron degeneration resulting from toxin administration is accompanied by microglial activation [73, 82] and iron accumulation [81]. Notably, intranigral lactacystin administration leads to cytoplasmic accumulation of α-synuclein and formation of α-synuclein-positive aggregates in surviving nigral DA neurons [79–82], indicating that proteasome impairment leads to formation of intracellular inclusion bodies in this model. Motor dysfunction induced by intranigral lactacystin is reversed by administration of apomorphine [79] and L-DOPA [76], reflecting a good degree of predictive validity.

Besides affecting neuronal cell populations located in (or neighboring the) SNc, intranigral administration of lactacystin to rats further involves extra-nigral brain regions. In particular, nigral infusion of lactacystin was found to damage cholinergic [82] and non-cholinergic [83] neurons located in the pedunculopontine nucleus (PPN). This was associated with significant microgliosis in the PPN and presence of α-synuclein aggregates in the surviving PPN neurons [82, 83]. The spread of the toxic effects of lactacystin to the ipsilateral PPN is intriguing and might result from the transport or diffusion of injected lactacystin (or pathogenic proteins induced by lactacystin).

Despite the extensive characterization of the intranigral proteasome inhibition model in rats, its application in mice has been more limited. Sun and collaborators initially reported that intranigral proteasome inhibition using MG132 leads to loss of nigral DA neurons and striatal DA levels [58]. Subsequently, Subramaniam and collaborators found that intranigral injection of epoxomicin in mice leads to significant loss of nigral DA neurons, which followed a caudo-rostral gradient similar to that observed in PD [84]. Interestingly, despite the significant loss of nigral DA neurons (60%), neighboring VTA DA neurons were unaffected. In line with the increased resistance of VTA DA neurons to proteasome inhibition-induced cell death, the authors reported that direct intra-VTA infusion of epoxomicin failed to affect this neuronal population [84]. Similarly, intranigral infusion of lactacystin in mice in a dose that causes partial loss of SNc DA neurons (40%) does not lead to loss of VTA DA neurons [85]. These findings are at odds with the intranigral rat models, where loss of VTA DA neurons was previously reported [74, 78]. However, variations in the dose of proteasome inhibitor or time point when neurodegeneration was assessed might account for these differences. In particular, loss of VTA DA neurons following intranigral proteasome inhibition in rats was shown to be both dose-dependent [78] and progressive [74]. Furthermore, infusion of the toxin could cause only a local effect on VTA DA neurons surrounding the injection tract [81, 85] and might not be easily detectable when counting the structure in its entirety. Pathologically, the degeneration of the nigrostriatal DA-ergic pathway following lactacystin administration to the SNc of mice occurs acutely and does not show a progressive pattern [85]. Furthermore, the lesion is accompanied by nigral accumulation of α-synuclein and Ser129-phosphorylated α-synuclein, a post-translationally

Table 3
Overview of the local proteasome inhibition model of PD

Site	Species	Inhibitor	Effects on SNc DA-ergic neurons	Effects on Str DA-ergic fibers	Additional effects on the brain	Effects on α-syn	Effects on motor function	Ref
SNc	Rats	LAC 3μg	2d: ↓ TH+ cells (∼20%) 1w: ↓ TH+ cells (∼65%)	NR	NR	NR	NR	[73]
SNc	Rats	LAC 10μg	1w: ↓ TH+ cells (∼45%) 5w: ↓ TH+ cells (∼50%)	NR	5w: ↓ TH+ cells in VTA (∼30%)	NR	NR	[74]
SNc	Rats	LAC 2.5μg	6w: ↓ TH+ cells (∼90%)	6w: ↓ DA levels (∼90%)	6w: ↓ Volume of SN (∼20%). ↓ Non-TH+ cells in SN (∼25%). ↓ GABA levels in SN (∼15%)	NR	4w: ↓ Spontaneous motor activity (open-field test)	[75]
SNc	Rats	LAC 2.5μg	NR	6w: ↓ DA levels (∼90%)	6w: No effect on 5HT levels in Str	NR	4w: ↓ Initiation of movement / akinesia (catalepsy test). ↓ Use of contralateral forelimb (cylinder test)	[76]
SNc	Rats	LAC 0.5μg, 1μg, 2.5μg, 5μg	1w: ↓ TH+ cells (∼80% at 1μg, other doses NR)	1w: ↓ DA levels (∼25% at 0.5μg, ∼70% at 1μg, ∼75% at 2.5μg and ∼85% at 5μg)	NR	1w: ↓ α-Syn expression in SN (∼50% at 1μg, other doses NR)	No changes in motor behavior (qualitative)	[77]
SNc	Rats	LAC 0.5μg, 1μg, 2μg, 10μg, 20μg	2-3 w or 8-10w: ↓ TH+ cells (∼40% at 1μg, ∼70% at 2μg, ∼80% at 10μg and ∼80% at 20μg) *The effects of the lesion spread partially to the contralateral SNc*	2-3 w or 8-10w: ↓ [^{11}C] DTBZ binding to VMAT2 (∼55% at 1μg, ∼75% at 2μg, ∼80% at 10μg and ∼75% at 20μg) (PET)	2-3 w or 8-10w: ↓ TH+ cells in VTA (∼20% at 1μg, ∼25% at 2μg, ∼50% at 10μg and ∼50% at 20μg) *The effects of the lesion spread partially to the contralateral VTA*	NR	2-3 w or 8-10w: ↓ Motor function of contralateral hindlimb (tapered ledged beam task) (at all doses, except 2μg)	[78]
SNc	Rats	LAC 0.2μg, 2μg, 20μg	3w: ↓ TH+ cells (∼75% at 2μg and ∼95% at 20μg)	NR	NR	3w: Accumulation / aggregation of α-syn in TH+ cells in SNc (reported at 2μg)	3w: ↓ Motor function (qualitative)	[79]
SNc	Rats	LAC 10μg	2w: ↓ TH+ cells (∼40%) 3w: ↓ TH+ cells (∼85%)	↓ TH fiber density	NR	Accumulation / aggregation of α-syn in SNc	4w: ↓ Spontaneous motor activity (open-field test)	[80]

SNc	Rats	LAC 10μg	3w: ↓ TH+ cells (∼80%)	3w: ↓ TH fiber density (∼70%)	3w: ↓ Volume of the Str and midbrain (MRI). ↓ TH+ and NeuN+ cells in the VTA, SNr and extra-nigral nuclei close to injection (e.g. red nucleus). No effect on NeuN+ or DARPP-32 + cells in the Str	3w: Accumulation / aggregation of α-syn in SNc	3w: ↑ Median neurological score. ↓ Grip strength of the ipsilateral forelimb (grip strength test)	[81]
SNc	Rats	LAC 10μg	5w: ↓ TH+ cells (∼50%)	NR	5w: ↓ ChAT+ cells in PPN (∼35%)	5w: Accumulation / aggregation of α-syn in TH+ cells in the SNc and ChAT+ cells in PPN	5w: ↓ Use of contralateral forelimb (cylinder test)	[82]
SNc	Rats	LAC 10μg	5w: ↓ TH+ cells (∼50%)	NR	5w: ↓ ChAT+ cells (∼45%) and ↓ non-ChAT+ cells (∼25%) in PPN	5w: Accumulation / aggregation of α-syn in ChAT+ and non-ChAT+ cells in PPN	NR	[83]
SNc	Rats	MG 20μg	8d: ↓ TH+ cells (∼40%)	NR	NR	NR	7d: No effect on spontaneous motor behavior (cage activity monitoring)	[203]
SNc	Mice	MG 0.4μg *Bilateral*	12d: ↓ TH+ cells (∼40%)	12d: ↓ DA levels (∼55%)	NR	NR	NR	[58]
SNc	Mice	EPO 0.02μg	2w: ↓ TH+ cells (∼60%)	NR	2w: No effect on TH+ cells in VTA	NR	2w: No effect on spontaneous motor behavior (open-field test)	[84]
SNc	Mice	LAC 3μg	1 w and 3w: ↓ TH+ cells (∼40%)	1 w and 3w: ↓ DA levels (∼40%)	1 w and 3w: ↓ TH+ cells in the lateral VTA (PBP) at the injection site (∼30%). No effect on TH+ cells in the entire VTA. ↓ Volume of the SN (∼20%). No effect on PV+ cells in the SNr at the injection site	1 w and 3w: Accumulation of α-syn and Ser129-P α-syn in SN	1 w and 3w: ↓ Motor coordination and balance (rotarod test). ↓ Use of contralateral forelimb (cylinder test). ↓ Sensorimotor function (adhesive removal test)	[85]
Mfb	Rats	LAC 3μg	3w: ↓ TH+ cells (∼20%) 4w: ↓ TH+ cells (∼30%)	3w: ↓ DA levels (∼35%) 4w: ↓ DA levels (∼40%)	NR	NR	NR	[55]
Mfb	Rats	LAC 10μg	5w: ↓ TH+ cells (∼85%)	5w: ↓ TH fiber density (∼70%)	5w: ↓ Volume of Str, ventral midbrain and cortex (M1, S1BF) (MRI). No effect on NeuN+ cells in Str or M1/S1BF cortex. Loss of NeuN+ cells in thalamic and ventral midbrain nuclei. Loss of NeuN+ cells in the VTA and SNr	5w: Accumulation / aggregation of α-syn in TH+ cells in SNc	5w: ↑ Median neurological score. ↓ Grip strength of the ipsilateral forelimb (grip strength test). ↓ Motor coordination and balance (rotarod test)	[87]

(*Continued*)

Table 3
(*Continued*)

Site	Species	Inhibitor	Effects on SNc DA-ergic neurons	Effects on Str DA-ergic fibers	Additional effects on the brain	Effects on α-syn	Effects on motor function	Ref
Mfb	Mice	LAC 1.25μg	3w: ↓ TH+ cells (∼35%)	3w: ↓ TH fiber density (∼55%)	NR	3w: Accumulation of α-syn oligomers in SN	NR	[68]
Mfb	Mice	LAC 1.25μg, 5μg	4w: ↓ TH+ cells (∼45% at 1.25μg and ∼60% at 5μg)	NR	4w: No effect on TH+ cells in VTA	2w: Accumulation of α-syn in TH+ cells of SNc	NR	[88]
		PSI 0.4μg	4w: ↓ TH+ cells (∼25%)	4w: ↓ DA levels (∼45%)	4w: No effect on 5HT levels in Str	NR	NR	[88]
		MG 0.4μg	4w: ↓ TH+ cells (∼25%)	4w: ↓ DA levels (∼45%)	4w: No effect on 5HT levels in Str	NR	NR	[88]
Mfb	Mice	LAC 5μg *Bilateral*	1w: ↓ TH+ cells (∼25%) 3w: ↓ TH+ cells (∼30%) 4w: ↓ TH+ cells (∼45%)	1w: ↓ DA levels (∼20%) 2w: ↓ DA levels (∼40%) 4w: ↓ DA levels (∼50%) 12w: ↓ DA levels (∼50%)	NR	2w: Accumulation / aggregation of α-syn in TH+ cells of SNc	4w: ↓ Spontaneous motor activity (open-field test). ↓ Forelimb use and skilled motor function (dowel test, suspended thread test)	[88]
Mfb	Mice	LAC 1μg	4w: ↓ TH+ cells (∼25% in 5 month old mice and ∼50% in 12 month old mice)	4w: ↓ DA levels (∼55% in 5 month old mice and ∼65% in 12 month old mice)	No effect on TH+ cells in VTA	Accumulation / aggregation of α-syn in TH+ cells in SNc (in 12 month old mice)	NR	[89]
Str	Rats	LAC 0.04μg	1w: ↓ TH+ cells (∼40%)	1w: ↓ DA levels (∼30%)	1w: No effect on GAD-67 + cells in Str. No effect on TH+ cells in VTA. No effect on 5HT levels in Str	1w: Accumulation / aggregation of α-syn in SNc	NR	[50]
		EPO 0.05μg	1w: ↓ TH+ cells (∼40%)	1w: ↓ DA levels (∼40%)	NR	1w: Accumulation / aggregation of α-syn in SNc	NR	[50]
Str	Rats	LAC 5μg, 10μg	2w: No effect on TH expression in SN (either dose)	2w: No effect on DA levels (either dose)	NR	2w: No effect on α-syn expression in SN (either dose)	NR	[77]
Str	Rats	LAC 1μg, 10μg	2w: ↓ TH+ cells (∼20% at 1μg and ∼75% at 10μg)	NR	Non-specific toxic effects on striatal cells (CV; observed only at 10μg)	Accumulation /aggregation of α-syn in SNc (reported at 10μg)	NR	[90]

All injections are unilateral unless stated otherwise. 5HT 5-hydroxytryptamine, α-syn α-synuclein, ChAT choline acetyl transferase, CV cresyl violet, DA dopamine, DARPP-32 α-dopamine and cAMP-regulated phosphoprotein-32, DTBZ dihydrotetrabenazine, EPO epoxomicin, GABA gamma-aminobutyric acid, GAD-67 glutamic acid decarboxylase-67, LAC lactacystin, Mfb medial forebrain bundle, MG MG132, MRI magnetic resonance imaging, NR not reported, PBP parabrachial pigmented nucleus, PET positron emission tomography, PPN pedunculopontine nucleus, PV parvalbumin, SN substantia nigra, SNc substantia nigra pars compacta, SNr substantia nigra pars reticulata, Str striatum, TH tyrosine hydroxylase, VMAT2 vesicular monoamine transporter 2, VTA ventral tegmental area.

Table 4
Overview of the systemic proteasome inhibition model of PD

Species	Protocol	UPS activity	Effects on SNc DA-ergic neurons	Effects on Str DA-ergic fibers	Additional effects on the brain	Effects on α-syn	Effects on motor function	Ref
Rats	3 mg/kg PSI s.c.: 6x over 2w	1w: ↑ Activity in ventral midbrain (∼50%)	2w: ↓ TH+ cells (∼55%)	2 w and 6w: ↓ TH+ fiber density	2 w and 6w: VTA TH+ cells relatively spared. SNr neurons spared (H&E)	6w: Accumulation / aggregation of α-syn in SNc, LC and DMN	2 w and 6w: Motor dysfunction (semiquantitative behavioral rating scale). ↓ Spontaneous motor activity (open-field test)	[92]
		2w: ↓ Activity in ventral midbrain (∼50%)	6w: ↓ TH+ cells (∼70%)	17-19w: ↓ [^{11}C] CFT binding to DAT (PET) (∼40%)	6w: ↓ TH+ cells in LC. ↓ Cells in DMNV (H&E). ↓ ChAT+ cells in NMB. No effect on DARPP-32 + and ChAT+ cells in Str			
	1.5 mg/kg EPO i.p.: 6x over 2w	4w: ↓ Activity in ventral midbrain (∼50%)	2w: ↓ TH+ cells (∼60%)	2w: ↓ DA levels (∼70%)	NR	2w: Accumulation / aggregation of α-syn in SNc	2w: Motor dysfunction (semiquantitative behavioral rating scale)	[92]
Rats	8 mg/kg PSI s.c.: 6x over 2w	NR	5w: ↓ FG+ cells (total counts) (∼20%). ↓ FG+ cells (morphologically normal) (∼40%)	NR	NR	NR	5w: ↓ Spontaneous motor activity (cage activity monitoring)	[93]
Rats	8 mg/kg PSI s.c.: 6x over 2w	NR	15 w and 22w: ↓ TH+ cells (∼60%)	NR	15 w and 22w: ↓ 5HT+ cells in RN. ↓ DBH+ cells in LC.↓ ChAT+ cells in DMNV. No effect on ChAT+ cells in NBM	NR	15 w and 22w: ↓ Spontaneous motor activity (open-field test)	[94]
	8 mg/kg PSI p.o.: 6x over 2w	NR	22w: ↓ TH+ cells (∼30%)	NR	22w: No effect on 5HT+ cells in RN or DBH+ cells in LC.↓ ChAT+ cells in DMNV and NBM	NR	22w: ↓ Spontaneous motor activity (open-field test)	[94]
	8 mg/kg PSI i.p.: 6x over 2w	NR	22w: No effect on TH+ cells	NR	22w: No effect on 5HT+ cells in RN, DBH+ cells in LC, ChAT+ cells in DMNV or ChAT+ cells in NBM	NR	22w: No effect on spontaneous motor activity (open-field test)	[94]
	12 mg/kg PSI s.c.: 6x over 2w	NR	15w: ↓ TH+ cells (∼30%)	NR	15w: No effect on 5HT+ cells in RN or DBH+ cells in LC.↓ ChAT+ cells in DMNV and NBM	NR	15w: ↓ Spontaneous motor activity (open-field test)	[94]

(*Continued*)

Table 4
(*Continued*)

Species	Protocol	UPS activity	Effects on SNc DA-ergic neurons	Effects on Str DA-ergic fibers	Additional effects on the brain	Effects on α-syn	Effects on motor function	Ref
Rats	16 mg/kg PSI s.c.: 6x over 2w	NR	15w: ↓ TH+ cells (∼30%)	NR	15w: No effect on 5HT+ cells in RN or DBH+ cells in LC.↓ ChAT+ cells in DMNV. No effect on ChAT+ cells in NBM	NR	15: No effect on spontaneous motor activity (open-field test)	[94]
Rats	3 mg/kg PSI s.c.: 6x over 2w	NR	NR	6w: ↓ DA levels (∼25%) 10w: No effect on DA levels	NR	NR	6w: ↓ Spontaneous motor activity (open-field test) 6 w and 10w: ↓ Motor coordination and balance (rotarod test)	[95]
Rats	3 mg/kg PSI s.c.: 6x over 2w	After last injection: ↓ Activity in midbrain 2w: No effect on activity in midbrain	6w: ↓ TH+ cells (∼40%)	NR	NR	6w: No evidence of α-syn aggregation in SNc	6w: No effect on motor function (SHIRPA scale)	[96]
Rats	5-8 mg/kg PSI s.c.: 6x over 2 w	NR	8w: ↓ TH+ cells (∼50%) 20w: ↓ TH+ cells (∼50%) 40w: ↓ TH+ cells (∼45%)	NR	20 w and 40w: ↓ 5HT+ neurons in RP. ↓ DBH+ cells in LC. ↓ ChAT+ cells in DMNV	8 w, 20 w and 40w: Accumulation / aggregation of α-syn in TH+ cells in SNc	8 w, 20 w and 40w: ↓ Spontaneous motor activity (hole-board apparatus)	[97]
Rats	3 mg/kg or 6 mg/kg PSI s.c.: 6x over 2w	NR	12w: No effect on TH+ cells (either dose)	12w: No effect on TH+ fiber density (either dose)	NR	12w: No evidence of α-syn aggregation in SNc (either dose)	12w: No effect on spontaneous motor activity (either dose)	[98]
GFPu mice	6 mg/kg or 9 mg/kg PSI s.c.: 6x over 2w	Lack of GFPu accumulation in the brain (either dose)	NR	NR	NR	NR	NR	[98]
Rats	3 mg/kg PSI s.c.: 6x over 2w	NR	8w: No effect on TH expression in SN	8w: No effect on TH expression	8w: ↑ TH expression in the glomerular layer of olfactory bulb	8w: No evidence of α-syn aggregation in SNc	8w: No effect on motor function (footprint test)	[99]
Rats	3 mg/kg PSI s.c.: 6x over 2w	NR	8w: No effect on TH+ cells	8w: No effect on TH+ fiber density	8w: No cell loss in LC (CV). No effect on ChAT+ cells in NBM	8w: No evidence of α-syn aggregation in SNc	8w: No effect on spontaneous motor activity (cage activity monitoring)	[101]

Rats	3 mg/kg PSI s.c.: 6x over 2w	NR	6w: No effect on TH+ cells	6w: No effect on DA levels. No effect on TH or DAT expression	NR	6w: No evidence of α-syn aggregation in SNc	6w: No effect on spontaneous motor activity (open-field test). No effect on forelimb stride length (gait analysis)	[102]
Rats	3 mg/kg PSI s.c.: 6x over 2w	2w: No effect on SN activity, despite ↓ brain activity 12w: No effect on SN or brain activity	12w: No effect on TH+ cells	12w: No effect on DA levels. No effect on TH+ fiber density	12w: No effect on norepinephrine or 5HT levels in Str	NR	12w: No effect on spontaneous motor activity (cage activity monitoring)	[103]
Rats	0.5 mg/kg MG i.p.: 7x over 2w	NR	6w: ↓ TH+ cells (∼20%)	6w: ↓ TH+ fiber density in dorsolateral Str. No effect on TH fiber density in dorsomedial or ventral Str	NR	NR	1 w and 6w: No effect on spontaneous motor activity (open-field test). No effect on motor coordination and balance (rotarod test)	[214]
Mice	3 mg/kg PSI s.c.: 6x over 2w	NR	6w: ↓ TH+ cells (∼45%)	NR	NR	NR	NR	[53]
Mice	3 mg/kg PSI s.c.: 3x over 6 h	NR	NR	5d: No effect on DA levels. No effect on TH expression	NR	NR	NR	[100]
Mice	6 mg/kg PSI s.c.: 6x over 2w	NR	8w: No effect on TH+ cells (vs. sham (70% ethanol)-injected mice), although ↓ 35% TH+ cells compared to saline-injected mice	8w: No effect on DA levels (vs. sham (70% ethanol)-injected mice), although ↓ 80% loss of DA compared to saline-injected mice	NR	NR	8w: No effect on spontaneous motor activity (cage activity monitoring), although transient decrease at 2w	[104]
Mice	10 mg/kg/ day PSI: osmotic pump 3w	NR	3w: No effect on TH+ cells	3w: No effect on DA levels	NR	3w: No evidence of ubiquitin accumulation / aggregation in SNc	3w: ↓ Spontaneous motor activity (cage activity monitoring)	[107]

All time points are counted from cessation of treatment. 5HT 5HT 5-hydroxytryptamine, ChAT choline acetyl transferase, CV cresyl violet, DA dopamine, DAT dopamine transporter, CFT 2β-carbomethoxy-3β-(4-fluorophenyl)tropane, DARPP-32 α-dopamine and cAMP-regulated phosphoprotein-32, DBH DA β-hydroxylase, DMNV dorsal motor nucleus of the vagus, EPO epoxomicin, FG fluorogold^{TM}, H&E hematoxylin and eosin, i.p. intraperitoneal, LC locus coeruleus, NMB nucleus basalis of Meynert, NR not reported, PET positron emission tomography, p.o. oral, PSI carbobenzoxy-L-isoleucyl-L-gamma-t-butyl-L-glutamyl-L-alanyl-L-leucinal, RP raphe nuclei, s.c. subcutaneous, SN substantia nigra, SNc substantia nigra pars compacta, SNr substantia nigra pars reticulata, Str striatum, TH tyrosine hydroxylase, UPS ubiquitin-proteasome system, VTA ventral tegmental area.

modified form enriched in LBs [86] and development of motor impairment [85].

Stereotaxic infusion in the medial forebrain bundle

Similar as for intranigral delivery, administration of lactacystin to the medial forebrain bundle (mfb) of rats leads to nigral DA neuron loss and PD-like motor deficits [55, 87]. Nigral DA neuron loss was found to be either progressive [55] or non-progressive [87]. Pathologically, nigral cell loss was accompanied by accumulation of α-synuclein in the form of cytoplasmic inclusion bodies [87]. Using MRI to characterize morphological changes occurring after intra-mfb delivery of lactacystin in rats, Vernon and collaborators revealed a distinct pattern of volumetric changes in both nigral and extra-nigral regions [87]. The authors found that lactacystin leads to an early and sustained decrease in cortical volume (evident already at one week post lesion), followed by subsequent decreases in the volume of the midbrain and striatum and a hypertrophy of the lateral ventricles (appearing from three weeks onwards). Interestingly, decreased cortical volume was the best predictor of poor motor function in lactacystin-treated rats. Cortical thinning was not associated with gross neuronal cell loss, but instead might have resulted from a loss of DA innervation from the midbrain [87].

Similar as in rats, intra-mfb administration of lactacystin in mice leads to dose-dependent progressive nigrostriatal DA neurodegeneration and subsequent motor impairment [68, 88]. Apoptotic nigral cell death could be revealed by electron microscopy and immunostaining for cleaved caspase-3. In comparison to nigral DA neurons, VTA DA neurons were less affected, suggesting a pattern resembling neurodegenerative changes occurring in PD [88]. Pathologically, nigral cell loss was accompanied by a marked glial (astrocytic and microglial) reaction and increases in iron levels in the SN [88]. Furthermore, lactacystin administration led to nigral α-synuclein accumulation and formation (in a subset of TH-positive neurons) of α-synuclein-positive intracellular aggregates. Interestingly, the decreases in spontaneous motor behavior in lactacystin-treated mice could be reversed using DA receptor agonist pramipexole [88]. In addition, Xiao and collaborators recently reported that ageing increases the susceptibility of mice to intra-mfb administration of lactacystin [89]. In particular, the authors reported that aged (12 months old) mice suffer from increased loss of nigral DA neurons and striatal DA depletion following intra-mfb lactacystin compared with young (5 month old) mice. Furthermore, a sustained activation of microglial cells could be observed in the aged (but not young) SN following lactacystin [89].

Stereotaxic infusion in the striatum

In addition to the SNc and mfb, the striatum has also been investigated as a possible site of delivery of proteasome inhibitors. In an early study, Fornai and collaborators reported that striatal delivery of proteasome inhibitors, such as lactacystin or epoxomicin, to rats leads to nigral DA neuron loss [50]. Importantly, striatal GABA-ergic neurons were not sensitive to intrastriatal delivery of lactacystin, nor were VTA DA neurons, reflecting the specificity of the lesion. Nigral DA neuron death was apoptotic and accompanied by the formation of LB-like intracellular inclusions in surviving neurons that contained α-synuclein, ubiquitin, parkin and ubiquitin-activating enzyme E1 [50]. In concordance with these findings, Miwa and collaborators reported that intrastriatal administration of lactacystin leads to dose-dependent loss of nigral TH-positive neurons and formation of intracytoplasmic α-synuclein-positive inclusions [90]. Interestingly, they also note that higher concentrations of lactacystin can induce non-specific effects and significantly damage striatal cells. These findings support the notion that lactacystin, once administered intrastriatally, can either be retrogradely transported or initiate retrograde degeneration [91] of nigrostriatal DA neurons, as previously reported with other toxins such as 6-hydroxydopamine (6-OHDA). In contrast with this hypothesis, Lorenc-Koci and collaborators failed to reproduce significant nigrostriatal DA neurodegeneration following intrastriatal administration of lactacystin to rats, even at high doses [77]. The reasons for these discrepancies are currently unclear.

Systemic administration

In 2004, McNaught and collaborators described a model of PD resulting from systemic (subcutaneous) chronic administration of proteasome inhibitor PSI [92]. This model replicated key features of PD, including progressive loss of nigral DA neurons, significant neurodegeneration in extra-nigral regions known to be affected in human PD, and development of a progressive parkinsonian syndrome which was L-

DOPA- and apomorphine- responsive. Importantly, LB-like intracytoplasmic protein inclusions containing α-synuclein, ubiquitin, synphilin-1, γ-tubulin and parkin, and reactive to thioflavin-S (demonstrating their fibrillar nature), developed in affected regions [92]. By reproducing key pathological features as well as replicating the slow and chronic neurodegeneration associated with PD, the systemic proteasome inhibition model has attracted significant attention from the research community. The initial promising findings were, however, difficult to reproduce in subsequent studies. While some investigators confirmed nigrostriatal pathology, including nigral DA neurodegeneration, following systemic administration of PSI in rats [93–97] or mice [53], others failed to find any significant effects of systemic proteasome inhibition on the nigrostriatal pathway [98–103], or reproduced only partial features of the model such as transient motor impairment without DA neuron degeneration [104] or modest loss of striatal DA levels without motor impairment [105] (see Table 4). The reasons for these discrepant findings are unclear. Various factors leading to poor reproducibility between laboratories have been proposed, including batch-to-batch or supplier-to-supplier variation in the properties of PSI (such as purity or solubility), variations in the manner of preparing and/or administering PSI, or fluctuations in the housing conditions of rats/mice receiving PSI which might influence the metabolism of the toxin [106]. In addition, the brain bioavailability of PSI is not well understood, although the compound is lipophilic and believed to cross the blood-brain barrier. However, with the exception of the initial publication of McNaught and collaborators (that confirmed a significant decrease in proteasome function in the midbrain following systemic PSI), most of the subsequent studies did not control for the proper brain bioavailability of PSI after administration (Table 4). In fact, using transgenic mice expressing GFPu (a reporter that acts as substrate for ubiquitin-dependent proteasome degradation), Bove and collaborators failed to identify any increase in GFPu levels in the brain following systemic PSI administration [98]. This indicates that the compound might not have reached inhibitory concentrations in the brain, leading to the observed lack of effect. Furthermore, as Bukhatwa and collaborators point out, both the dose as well as the route of administration, can influence the impact of PSI on the nigrostriatal pathway and motor function [94]. In particular, the authors note that increasing the dose of PSI leads to a surprising loss of effect after subcutaneous administration, while subcutaneous and oral, but not intraperitoneal, administration of the same dose of PSI (8 mg/kg) leads to a significant reduction of nigral DA neurons and ensuing motor deficits [94]. The fact that the route of administration influences the outcome further suggests that proper bioavailability of the toxin is critical for successfully creating the model. The loss of effect with increasing doses of PSI, also reported by McNaught and Olanow [106], is interesting and might indicate that at higher doses PSI becomes less soluble, limiting its absorption from the injection site. In an attempt to achieve a more continuous delivery of the toxin, Shin and collaborators investigated the administration of PSI using osmotic minipumps implanted subcutaneously [107]. Using these conditions, PSI induced a progressive deterioration of locomotor activity, which was however unrelated to nigrostriatal degeneration (as nigral DA neurons were not significantly affected by the toxin) [107]. Given the limited reproducibility of the model amongst different laboratories, and until the reasons for these discrepancies are being elucidated, the use of the systemic proteasome inhibition model of PD remains controversial [108].

Non-human primate models

In the only attempt to date to model proteasome inhibition-induced neurodegeneration in non-human primates, cynomolgus monkeys were treated systemically with PSI, using a similar protocol as proposed in rats. Systemic PSI administration to monkeys failed, however, to produce any significant loss of nigral DA neurons [101]. Similar to the rodent systemic PSI model, various factors might explain this lack of effect, including uncontrolled variables related to the pharmacokinetics and brain penetrance of PSI.

Genetic models

The toxin-based models described above employ proteasome inhibitors to target the 20S catalytic core particle, thereby affecting both 26S and 20S protein degradation pathways. In addition, most of the experiments reported on human post-mortem tissue utilize kinetic assays and fluorogenic peptide substrates that are degraded by the 20S proteasome, thereby specifically assessing the proteolytic activities of the 20S core particle (Table 1). Less is known, however, regarding the particular involvement of 26S proteasomal degradation in PD. Experimental evidence in

this regard was recently provided by Bedford and collaborators, utilizing the Cre/loxP system to generate mice with a targeted deletion of a key subunit of the 19S regulatory particle, Psmc1, in TH neurons ($Psmc1^{fl/fl}$; TH^{Cre}) [109]. Deletion of Psmc1 prevented the correct assembly of the 19S particle with the 20S proteasome, leading to a specific disruption of 26S proteasomal degradation, while 20S degradation remained preserved. Interestingly, in such conditions, the authors observed a striking loss of nigral DA-ergic neurons and striatal DA-ergic fibers, and a substantial depletion of striatal DA content (∼90%) compared to control mice. In addition, surviving neurons developed intracellular eosinophilic inclusions containing α-synuclein, ubiquitin and mitochondria, resembling human pale bodies (precursors to LBs) [109]. This genetic model provides an interesting platform to investigate 26S proteasomal dysfunction in nigral DA neurons and illustrates the possible involvement of this pathway in PD. One of the limitations of the model might be that $Psmc1^{fl/fl}$; TH^{Cre} mice die before postnatal day 28 due to autonomic dysfunction [109].

MECHANISMS UNDERLYING PROTEASOME INHIBITION-INDUCED CELL DEATH

Various studies have been performed in an attempt to decipher the pathways mediating neuronal death induced by proteasome inhibitors. As described below, the determinants of cell death have been suggested to involve diverse cellular pathways related to production of reactive oxygen species, disturbances in mitochondrial function, induction of endoplasmic reticulum (ER) stress, and cytoplasmic accumulation of p53, iron and α-synuclein (Fig. 2, upper panel).

Accumulation of p53

Multiple lines of evidence obtained from *in vitro* and *in vivo* experiments suggest that cell death induced by proteasomal inhibitors is apoptotic in nature. One candidate for regulating apoptotic cell death following proteasome inhibition is transcription factor p53. Accumulation of p53 leads to activation of pro-apoptotic pathways, which can be mediated by transcriptional effects, such as increased expression of pro-apoptotic genes including Puma or Bax, or non-transcriptional effects, for instance by direct actions of p53 on mitochondrial permeability. Of note, p53 is primarily degraded via the proteasome [110]. As a consequence, treatment of PC12 cells or primary neuronal cultures with proteasome inhibitors such as PSI, lactacystin or MG115 leads to accumulation of p53 [52, 53, 111]. Remarkably, inhibition of p53 (either chemical or genetic) is sufficient to prevent proteasome inhibition-induced apoptotic cell death of PC12 cells [52, 53] as well as of primary cortical neurons [111]. These findings suggest that accumulation of p53 following proteasome inhibition might be a key event triggering apoptotic cell death, and some evidence suggests that it does so via non-transcriptional mechanisms [53]. Consistent with such a hypothesis is the finding of increased p53 levels in the SNc of mice treated systemically with PSI at a time point preceding DA neuron loss, as well in the SNc of PD patients [53].

Mitochondrial dysfunction

By treating SH-SY5Y cells with continuous low levels of MG115, Sullivan and collaborators could find that chronic proteasome inhibition leads to a marked reduction in mitochondrial complex I and complex II maximal activities, and an increase in the production of mitochondrial reactive oxygen species [112]. Furthermore, genetic depletion of 26S proteasomes *in vivo* induces the accumulation of mitochondria within intracellular inclusion bodies resembling PD-related pale bodies [109, 113]. These findings suggest that proteasome inhibition influences mitochondrial function and turnover.

Oxidative and nitrosative stress

Treatment of cell lines and primary cell cultures with proteasome inhibitors such as MG132, epoxomicin or lactacystin has been found to lead to glutathione depletion, formation of reactive oxygen species, and increases in levels of protein carbonyls, malondialdehyde, 8-hydroxyguanosine and 8-hydroxy-2'-deoxyguanosine [51, 62, 114–116]. Notably, treatment with antioxidants can prevent proteasome inhibition-induced cell death of PC12 cells [62] as well as of cultured mesencephalic neurons [115], suggesting that this state of oxidative stress can be cytotoxic. Evidence for oxidative stress following proteasome inhibition has also been obtained *in vivo*, as intrastriatal administration of lactacystin in rats leads to an increase in the oxidative stress marker HO-1 in nigral neurons [90], while genetic depletion of neuronal 26S proteasomes in mice leads to gen-

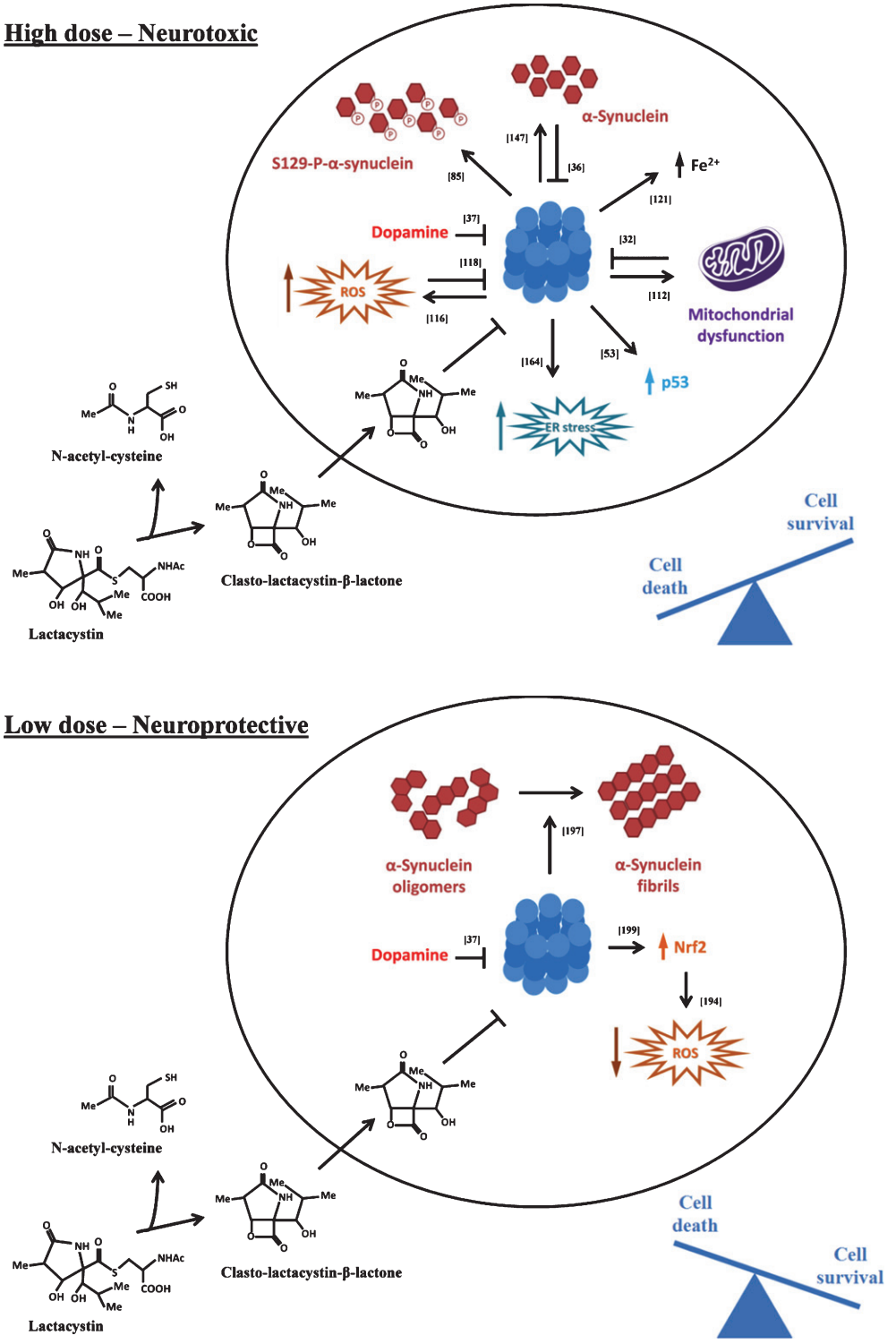


Fig. 2. Molecular pathways recruited following proteasome inhibition. At high, lethal doses (upper panel), proteasome inhibitors activate multiple downstream toxic pathways that together converge in inducing neuronal death. At lower, sub-lethal doses (lower panel), proteasome inhibitors can lead to a neuroprotective response, characterized by activation of anti-oxidant pathways and clearance of α-synuclein oligomers into larger, potentially less toxic fibrils. Lactacystin is exemplified as a typical proteasome inhibitor, although similar effects are observed with structurally distinct inhibitors. See text for details. ER endoplasmic reticulum, ROS reactive oxygen species.

eration of reactive oxygen species and accumulation of malondialdehyde [117]. In addition, proteasome inhibitors epoxomicin and lactacystin have been found to increase the levels of reactive nitrogen species and 3-nitrotyrosine in cell cultures, while nitric oxide synthase inhibitor L-NAME protects against lactacystin-induced toxicity [116]. In turn, reactive oxygen species [118] and oxidative stress end products such as 4-hydroxy-2,3-trans-nonenal [119] can inhibit proteasome function, suggesting a close interaction between the two pathways during neurodegeneration.

Iron dysregulation

Accumulating evidence suggests that iron dysregulation might be a key mechanism underlying proteasome inhibition-induced DA neuron death [120]. Treatment of the MES23.5 DA-ergic cell line with lactacystin has been found to cause a marked increase in labile iron (Fe^{2+}) levels. Accumulation of iron seemed to be cytotoxic, as iron chelator deferoxamine reduced lactacystin-induced neuron death [121]. Similarly, *in vivo*, iron levels have been found to be increased in the SN following intranigral [81] or intra-mfb [88, 122, 123] administration of lactacystin. Evidence suggesting that such an increase might be detrimental for nigral DA neurons has been provided by the findings that both chemical [123] as well as genetic [122] iron chelation are neuroprotective against lactacystin-induced DA neurotoxicity *in vivo*. Iron accumulation induces the formation of reactive oxygen species (such as hydroxyl radicals, following the Fenton reaction) as well as the formation of α-synuclein aggregates which can contribute to the demise of nigral DA neurons [124].

Accumulation of α-synuclein

Accumulation of the presynaptic protein α-synuclein plays a central role in PD pathogenesis. Evidence that increased levels of α-synuclein are toxic to nigral DA neurons comes from the findings that duplication or triplication of *Snca* (the gene encoding α-synuclein) can induce PD in humans with a gene-dosage effect [125], while accumulation and aggregation of α-synuclein in the form of LBs is a pathological hallmark of sporadic PD [3]. Furthermore, overexpression of α-synuclein (for instance via viral vector delivery) leads to nigral DA neurodegeneration [126, 127], while recent *in vivo* findings support the hypothesis that the process of α-synuclein aggregation is toxic to nigral DA neurons [128, 129]. As such, a detailed understanding of the mechanisms regulating α-synuclein degradation is of critical importance in elucidating its aberrant accumulation in synucleinopathies. A great deal of effort has been invested in deciphering the degradation pathways controlling α-synuclein in neurons [130]. The results of these studies seem to indicate that α-synuclein is a substrate for both proteasomal and autophagic degradation, although whether or not α-synuclein is turned over via the proteasome has remained to a certain degree contentious. Cell-free models in which recombinant α-synuclein was incubated with purified 20S [13, 14, 131–133] or 26S [13] proteasomes provide direct evidence for proteasomal degradation of this protein. Similarly, experiments performed in DA-ergic cell lines such as PC12 and SH-SY5Y have indicated that application of proteasome inhibitors lactacystin and epoxomicin leads to an increase in the levels of α-synuclein [14, 134–137]. The notion that α-synuclein is a substrate for the proteasome was, however, challenged by the failure of various laboratories to replicate significant α-synuclein accumulation in PC12 cells, HEK293 cells and primary mesencephalic cultures following application of structurally distinct proteasome inhibitors such as lactacystin, MG132 or PSI [54, 138–142], or found only modest accumulation of α-synuclein in primary mesencephalic cultures treated with epoxomicin [143]. The reasons for these discrepancies are currently unclear, but may relate to the use of different protocols of administration of proteasome inhibitors (e.g. dosing, incubation time, see [14]) or the use of distinct cell models and/or culture conditions which could lead to different baseline expression levels of α-synuclein. In addition, proteasome inhibition can induce a compensatory activation of autophagy as a means to maintain cellular proteostasis [137, 144–146]. As α-synuclein is a substrate for autophagic degradation [130], this might explain the lack of effect seen in certain models.

In order to address this controversy and critically assess the degradation pathways of α-synuclein, Ebrahimi-Fakhari and collaborators applied multi-photon imaging and cranial windows to study the turnover of α-synuclein *in vivo* [147]. In a seminal study, the authors reported that topical treatment of the cortex with clasto-lactacystin-β-lactone induced accumulation of endogenous α-synuclein in non-transgenic mice as well as in human α-synuclein transgenic mice, providing direct evidence that the

proteasome system regulates α-synuclein turnover *in vivo* [147]. In contrast, topical application of BafA1, an inhibitor of the autophagy-lysosomal pathway, led to increased levels of α-synuclein in human α-synuclein transgenic mice but not in nontransgenic mice. The authors propose a model in which the proteasome system degrades α-synuclein under both physiological conditions and under increased α-synuclein burden, whereas the autophagy-lysosomal pathway is recruited to degrade α-synuclein only when intracellular levels become elevated [148].

In view of these findings, and considering the toxicity associated with increased α-synuclein expression and aggregation [149], it might be speculated that α-synuclein plays a relevant role in mediating proteasome inhibition-induced cell death. Surprisingly, such a hypothesis has not been investigated in detail. In a recent study, Bir and collaborators reported that accumulation of α-synuclein following lactacystin administration to SH-SY5Y cells plays an important role in mediating proteasome inhibition induced toxicity, as siRNA-mediated knockdown of α-synuclein was able to completely prevent lactacystin-induced cell death [61]. At the same time, using α-synuclein knock-out mice, Paine and collaborators reported that genetic depletion of 26S proteasomes in nigral DA neurons of mice leads to nigral DA neuron death and formation of intracellular inclusion bodies, irrespective of the presence or absence of α-synuclein [113]. This suggests that in certain scenarios inhibition of the proteasome system might cause cellular toxicity independent of α-synuclein. Further studies would be instrumental to define the role played by α-synuclein in proteasome inhibition-induced cell death.

Besides changes in expression, α-synuclein can also undergo post-translational modifications, such as oxidation, nitration, phosphorylation or truncation, which might play important roles in mediating its aggregation and/or toxicity [150, 151]. Previous studies analyzing the composition of LBs revealed high levels of α-synuclein phosphorylated at Ser129 [86, 152], indicating that this post-translational modification might be associated with fibril formation and/or toxicity. Although its role is not completely understood [153], studies evaluating the impact of authentically phosphorylated α-synuclein at Ser129 have revealed gain-of-function toxic roles in both *Drosophila* [154] and rat [155] α-synuclein-based models. Interestingly, Machiya and collaborators reported a specific increase in Ser129-phosphorylated α-synuclein after treatment of SH-SY5Y cells with proteasome inhibitors lactacystin or MG132 suggesting that Ser129-phopshorylated α-synuclein is a substrate for proteasomal degradation [156]. Similarly, inhibition of the proteasome *in vivo* using lactacystin leads to accumulation of Ser129-phopshorylated α-synuclein in nigral (DA-ergic) neurons [85]. As for α-synuclein, however, it is currently unknown whether the increase in Ser129-phosphorylated α-synuclein following proteasome inhibition contributes to neuronal toxicity or constitutes an epiphenomenon following lesion.

It is interesting to note that while proteasome inhibition can lead to accumulation of α-synuclein, increased levels of α-synuclein can in turn inhibit proteasomal function. Previous findings indicate that α-synuclein, in particular in its oligomeric form [35, 157], can interact with and inhibit the catalytic activity of the 26S proteasome [34, 36]. This, in turn can lead to further α-synuclein accumulation, initiating a pathological feed-back loop [158, 159]. In a compelling study addressing the relation between α-synuclein aggregation and proteasome dysfunction, Chu and collaborators described a decreased expression of the 20S proteasome in SNc neurons of PD patients, but only in cells that contain α-synuclein inclusions [160]. Remarkably, the expression of the 20S proteasome in nigral DA-ergic neurons without α-synuclein pathology in PD was the same as in healthy controls. In a similar manner, the authors describe a decreased expression of the 20S proteasome in nigral DA-ergic neurons with α-synuclein inclusions (but not in those without α-synuclein inclusions) after AAV6 mediated over-expression of α-synuclein in the rat SNc [160]. This would argue for a close relation between α-synuclein aggregation and proteasome dysfunction during the course of PD. Such a view might seem at odds with the finding of intact proteasome activity in brain regions such as the striatum, hippocampus, or cortex [16, 17, 19, 161] that do develop LB pathology in the course of PD. However, these findings should be interpreted with caution, as in the post-mortem studies performed so far evaluating proteasome function in extra-nigral regions, the neuropathology and Braak staging of the patients investigated was not fully described. Notably, extra-nigral regions such as the striatum, hippocampus, and cortex become pathologically involved during later disease stages (Braak stages 4–6) [162], and possible effects on proteasome function in such regions might have been overlooked if using tissue from patients with a short disease duration (e.g. Braak stage 3, in which the SNc is already affected by LB pathology). Case in point might be the

study of Tofaris and collaborators that could reveal a significant decrease in the proteasome activity in the SN, but not frontal, cingulate or occipital cortices from cases with mild pathology and relatively short disease duration (6.3 ± 3 years) [19]. At the same time, in the study of McNaught and collaborators, the proteasome activity in the upper pons at the level of the locus coeruleus (LC) was not affected in patients in which the proteasome activity was found to be decreased in the SNc [17]. This is interesting, as the LC can develop Lewy body pathology at earlier Braak stages (Braak stage 2) than the SNc (Braak stage 3) [162]. This suggests that other factors, besides α-synuclein aggregation, might be required to decrease the proteasome function (e.g. mitochondrial impairment), and the cumulation of such factors occurs in the SNc but not in other regions of the brain. Future investigations are warranted into the integrity of the proteasome system in extra-nigral regions, and ideally controlled for disease staging and LB distribution. Such studies hold the promise to reveal whether the proteasome function in extra-nigral regions succumbs in later stages of disease, and whether this is universally (i.e. outside the SN) linked with the process of LB formation. Alternatively, it may be conceded that proteasome dysfunction and generation of LBs are partly dissociated processes (at least in some brain structures), as evidenced by the lack of proteasome impairment in cingulate and frontal cortices of cases of DLB, regions which stain positive for LBs [19].

Microarray and proteomic studies

In addition to targeted pathway analysis, unbiased screens using microarray or proteomic studies have also revealed insights into mechanisms associated with proteasome inhibition. Microarray studies performed on primary cortical neurons upon exposure to proteasome inhibitor lactacystin revealed a biphasic time-dependent effect, characterized by an initial activation of neuroprotective pathways which was followed, at later stages, by activation of pro-apoptotic pathways [163, 164]. One of the protective responses observed in the early phases was characterized by the upregulation of components of the ubiquitin-proteasome system, such as genes encoding ubiquitins (*Ubb*), proteasome subunits (e.g. *Psma1*, *Psma7*, *Psmc1*, *Psmc3*), E2 ubiquitin-conjugating enzyme (*Ubc*) and ubiquitin protein ligase E3A (*Ube3a*), possibly as part of a regulatory mechanism attempting to counteract the effects of proteasome inhibition. In addition, genes encoding heat shock proteins and molecular chaperones, such as heat shock protein 70 (*Hspa1a*), heat shock protein A (*Hspa9a*), heat shock protein 22 (*Cryac*), heat shock protein 27 (*Hspb8*) and heat shock protein 47 (*Serpinh1*), were upregulated, presumably to prevent accumulation of misfolded/damaged proteins. ER stress-associated genes, such as DNA-damage inducible transcript 3 (*Ddit3*) (also known as CHOP) and CCAAT/enhancer binding protein (*C/EBP*) beta (Cebpb), were also found to be increased in expression at an early time point after proteasome inhibition. As both proteins mediate induction of cell death in conditions of ER stress, this suggests an early pro-apoptotic role of ER stress after lactacystin administration [163]. The later stages of proteasome inhibition were associated with neuronal death and characterized by an upregulation of antioxidant genes (e.g. *Gclm, Gsta4, MgstI, MtI*) and a downregulation of genes involved in cholesterol biosynthesis (e.g. *Hmgcr, Cyp51, Dhcr7, Lss*) [163, 164]. Similar changes could be observed in proteomic screens of PC12 cells incubated with proteasome inhibitors. PSI application to PC12 cells led to upregulation of GRP94 and GRP78, molecular chaperones residing in the ER (and induced in conditions of ER stress), as well as of heat shock protein 27. In addition, PSI led to an upregulation of aldose reductase and a downregulation of galectin-1 [165]. Incubation of PC12 cells with the structurally distinct proteasome inhibitor lactacystin caused, besides others, an increase in the expression of heat shock proteins Hsc70 and GRP78, and aldose reductase, and a decreased expression of TH [166]. The upregulation of aldose reductase, a NADPH-dependent oxidoreductase mediating the reduction of aldehydes and involved in protection against oxidative stress [167], suggests a compensatory reaction of the cells to limit the detrimental effects after PSI or lactacystin administration. Conversely, the downregulation of galectin-1, a β-galactosidase binding lectin induced by brain injury and associated with neuronal regeneration [168], might reflect a decreased capacity to cope with cellular stress in conditions of proteasome inhibition. Aside from the above mentioned studies, performed at acute time points following incubation with proteasome inhibitors (maximum 24–48 h), Ding and collaborators investigated the effects of low-level, chronic proteasome inhibition on gene expression [169]. Incubation of neural SH-SY5Y cells with MG115 during 12 weeks led to a particular gene expression profile, characterized, besides oth-

ers, by an increase in genes related to inflammation (e.g. *STAT1, ISGF3, NPTX1*), apoptosis (e.g. *PIDD, PDCD4*), nuclear homeostasis (e.g. *MAN1*) and lipid metabolism (e.g. *NSMAF*). In addition, the authors note an important downregulation of the synaptic vesicle monoamine transporter, suggesting impaired catecholamine sequestration in synaptic vesicles following chronic proteasome inhibition [169].

CELL TYPE-SPECIFIC VULNERABILITY TO PROTEASOME INHIBITION-INDUCED DEATH

In contrast to other classical PD toxins which achieve specific DA cell death due to their intrinsic mechanism of action (such as the requirement for DAT for uptake of 6-OHDA or MPP^+), the active metabolite of lactacystin, clasto-lactacystin-β-lactone, diffuses freely through the cell membrane, thereby influencing both DA-ergic and non-DA-ergic neurons. It has been argued, however, that DA-ergic neurons might be particularly vulnerable to proteasome-inhibition induced cell death. Petrucelli and collaborators reported that treatment of primary mesencephalic cultures with lactacystin or MG132 leads to a reduction in the number of TH-positive cells, while TH-negative neurons remained unaffected [170]. Similarly, treatment of primary mesencephalic cultures with lactacystin or MG132 induces loss of DA-ergic neurons, with either no [64] or a significantly smaller [65] decrease in viability of GABA-ergic neurons. It has been proposed that the vulnerable nature of DA-ergic neurons could be due to the presence of DA itself. In an elegant study, Fornai and collaborators demonstrated that depleting DA in PC12 cells with reserpine led to a remarkable resistance to lactacystin-induced inclusion body formation and cell death [50]. Conversely, when PC12 cells were exposed to L-DOPA, thereby increasing intracellular DA levels, PC12 cells became more sensitive and demonstrated an increase in inclusion body formation and cell death following lactacystin [50]. Notably, similar results were obtained *in vivo*. Decreasing DA synthesis by systemic administration of TH inhibitor α-methyl-p-tyrosine significantly protected the nigrostriatal DA-ergic pathway against intrastriatal lactacystin administration. Conversely, systemic administration of L-DOPA prior to intrastriatal lactacystin led to an enhancement of the neurodegenerative effects observed at the level of the nigrostriatal pathway [50]. The reasons for which DA neurons are particularly vulnerable to the effects of proteasome inhibitors are not well understood, but might result from the metabolism of DA that leads to generation of reactive oxygen species and oxidation of proteins, providing an additional burden to proteasomal degradation.

At the same time, it should be mentioned that other studies performed in primary mesencephalic cultures failed to provide evidence for the specificity of proteasome inhibitors to induce DA cell death [66, 115, 171]. The reasons for these discrepancies are unclear, but might be due in part to differences in culture conditions or the concentration and duration of exposure to proteasome inhibitors. Of note, Biasini and collaborators reported that application of MG132 and lactacystin to primary mesencephalic cultures at low concentration leads to specific loss of DA content, without changes in GABA, whereas at higher concentrations of inhibitor GABA levels were also decreased [140]. Similar findings have been obtained *in vivo*. Intranigral administration of lactacystin in a dose that causes partial (40%) loss of nigral DA neurons does not damage the GABA-ergic neurons in the surrounding SNr [85]. However, administration of the toxin in a higher dose, causing a severe (90%) loss of nigral DA neurons leads, however, to a partial (27%) loss of non-DA neurons (presumably GABA-ergic) in the SNr and modest (16%) depletion of SN GABA tissue levels [75]. This suggests that, while DA-ergic neurons might be particularly vulnerable to proteasome inhibition, additional effects on non-DA-ergic neurons can also be observed, especially at higher doses of the toxin.

Besides neurons, proteasome inhibitors can diffuse into glial cells. Interestingly, glial cells, especially astrocytes, seem to be particularly resistant to proteasome inhibition-induced toxicity [172]. Cultured astrocytes were found to be resistant to concentrations of proteasome inhibitors that cause neuronal death [173–175]. The mechanisms underlying the resistance of astrocytes to proteasome inhibition-induced toxicity are incompletely understood, but might be related to the high constitutive levels of small heat shock protein HSP25 [176], the intrinsically higher proteasome activity of astrocytes compared to neurons [177] or the increased resistance of astrocytes to proteasome inhibition-induced oxidative stress [114]. However, non-specific effects on glial cells might occur at high doses of proteasome inhibitors and should be carefully considered [79, 175].

Although the indiscriminate mechanism of cell entry of proteasome inhibitors might seem to be a disadvantage, its independence of transport systems, such as DAT (required for the uptake of 6-OHDA and MPP^+), or metabolic enzymes, such as monoamine oxidase B (MAO-B) (required for the metabolization of MPTP) represents a distinct feature compared to classical toxin-based models of PD. In particular, the proteasome inhibition model can be employed to assess neuroprotective compounds that are known to (or might) influence the expression of DAT or MAO-B [178]. In addition, the unrestricted mechanism of action of proteasome inhibitors makes them attractive in modeling extra-nigral pathology in PD. Indeed, neurodegeneration in PD is not restricted to the SNc, but can extend to non-DA-ergic neurons, such as cholinergic neurons in the nucleus basalis of Meynert (NBM), dorsal motor nucleus of the vagus (DMNV) and PPN, noradrenergic neurons in the LC, serotonergic neurons in the raphe nuclei (RN) and hypocretin/orexin neurons in the hypothalamus, which contributes to the development of motor and non-motor symptoms [179–184]. Studies using systemic administration of proteasome inhibitors support the presence of extra-nigral pathology, including noradrenergic degeneration in the LC, cholinergic degeneration in the NBM and DMNV, and serotonergic degeneration in the RN following toxin administration (Table 4), as does the evidence of α-synuclein aggregation in the DMNV following intragastric administration of PSI [185]. Furthermore, intra-cerebral administration models provide further evidence of non-catecholaminergic degeneration following proteasome inhibition, such as the loss of cholinergic neurons in the PPN after intranigral lactacystin administration (Table 3), while injection of lactacystin into the NBM leads to loss of cholinergic neurons within this structure and formation of α-synuclein positive inclusion bodies in surviving cholinergic neurons [186]. The lack of specificity of proteasome inhibitors towards DA neurons might in fact favor the use of this model to study extra-nigral pathology and its impact on motor and non-motor symptoms in PD. In a recent study employing the intranigral lactacystin model to study PPN cholinergic degeneration in PD, Pienaar and collaborators found that selective stimulation of PPN cholinergic neurons using the expression of an excitatory DREADD reversed the lesion-induced motor deficits [187], which might offer additional insights into the clinical benefits observed after deep brain stimulation of this structure in patients with PD [188].

INTERACTIONS BETWEEN PROTEASOME INHIBITION AND PARKINSON'S DISEASE-RELATED GENES

Gene-environment interactions play a key role in the pathogenesis of PD [189]. Various studies have investigated the interactions between proteasomal dysfunction and genetic background, utilizing PD-related mutations. Notably, a synergistic effect could be observed between expression of PD-related α-synuclein mutants A30P and A53T and proteasomal inhibition in cultured cells. Expression of A53T α-synuclein in SH-SY5Y cells has been found to increase the formation of α-synuclein aggregates after lactacystin administration compared to cells expressing wild-type α-synuclein [63]. Furthermore, expression of A30P α-synuclein increased the sensitivity of PC12 cells to lactacystin-induced apoptotic cell death when compared to PC12 cell expressing wild-type α-synuclein [34]. Consistent with these findings, expression of either A53T or A30P α-synuclein in NT-2, SK-N-MC or M17 cells was found to increase susceptibility to proteasome inhibition-induced cell death compared to expression of the wild-type protein [170, 190]. Besides α-synuclein, expression of the G2019S LRRK2 mutation in mice has been found to increase striatal DA loss following intra-mfb administration of lactacystin [89] (Fig. 3). This is particularly interesting as the penetrance of the G2019S LRRK2 in PD patients is incomplete, suggesting that environmental factors might play a role in the pathogenic expression of this mutation.

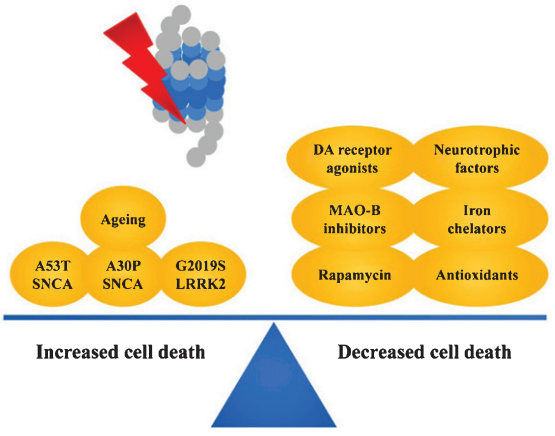


Fig. 3. Factors influencing cell death following application of proteasome inhibitors. See text for details.

INTERACTIONS BETWEEN PROTEASOME INHIBITION AND PARKINSON'S DISEASE-RELATED TOXINS

PD is a multifactorial disorder and the various pathogenic pathways interact in a complex manner to produce death of DA neurons [191]. Although proteasomal dysfunction can lead on its own to DA cell death, a critical question remains on how it interacts with other pathogenic pathways occurring in PD. In order to address this question, studies were performed investigating the reaction of DA cells to simultaneous application of proteasome inhibitors and PD related neurotoxins, such as 6-OHDA, MPP^+ or rotenone.

Hoglinger and collaborators reported that treatment of primary mesencephalic neurons with epoxomicin sensitized DA neurons to death induced by either rotenone, MPP^+ or 6-OHDA [192]. Consistent with these findings, exposure of SH-SY5Y cells to PSI or lactacystin was found to increase cell death induced by 6-OHDA [62] or rotenone [60]. In contrast, the use of sub-toxic levels of proteasome inhibitors has been associated with neuroprotective effects (Fig. 2, lower panel). Using primary cortical cultures, Lee and collaborators reported that low concentrations of proteasome inhibitors MG132 and clasto-lactacystin-β-lactone increased the resistance of cells to oxidative injury associated with paraquat and H_2O_2 treatment [193]. In line with these findings, treatment of PC12 cells [194] or primary mesencephalic neurons [195] with low concentration of lactacystin was protective against 6-OHDA. Comparable findings were observed *in vivo*. Sub-lethal concentrations of lactacystin and MG132, when co-administered intranigrally with 6-OHDA [196] or MPP^+ [197], were found to significantly protect nigral DA neurons against degeneration. Similarly, systemic delivery of PSI at a sub-toxic dose protects against MPTP-induced loss of nigral TH-positive neurons [198]. The mechanisms underlying proteasome inhibition-induced neuroprotection might be related to activation of the Nrf2-ARE pathway and induction of antioxidant defenses [194, 195, 199]. Indeed, Nrf2 is degraded via the proteasome, and its accumulation following proteasome impairment can lead to activation of ARE-dependent transcription of antioxidant genes [199]. In addition, Sawada and collaborators reported that sub-lethal concentrations of lactacystin, PSI and MG132 protect DA neurons in primary mesencephalic cultures against MPP^+-induced neurotoxicity and simultaneously led to the generation of synuclein- and ubiquitin- positive intracellular inclusions which are not observed with either toxic treatment alone [197]. Similarly, Inden and collaborators observed the presence of α-synuclein-positive inclusion bodies after intranigral co-administration of 6-OHDA and lactacystin or MG132, that were not observed with 6-OHDA treatment alone [196]. This suggests that an additional neuroprotective mechanism of proteasome inhibitors might be to stimulate the formation of larger α-synuclein aggregates, which are possibly less toxic than smaller oligomeric species [196].

In conclusion, the level of inhibition of proteasome activity seems to be critical in dictating neuroprotection vs. enhanced cell death following toxic stimuli: while partial inhibition is neuroprotective, a more widespread inhibition enhances the sensitivity to subsequent toxic stimuli.

THE VALIDITY OF THE RODENT PROTEASOME INHIBITION MODEL OF PARKINSON'S DISEASE

The proteasome inhibition model has emerged as a new model of PD that might provide novel clues in our attempts to decipher the complex pathogenesis of PD as well as represent a platform to investigate neuroprotective therapies. Its implementation in pre-clinical research is conditioned, however, by its ability to fulfill the validity criteria for modeling PD. These include requirements of construct validity (i.e. whether the model is induced in a manner that replicates pathogenic pathways relevant in the human disease), face validity (i.e. how closely does the model replicates key biochemical, pathological and behavioral changes as identified in the human disease) and predictive validity (i.e. the fidelity with which the model can positively identify clinically effective therapies) (Table 5).

Construct validity

Post-mortem data obtained from sporadic PD indicate structural and functional defects in the 26/20S proteasome (Table 1). In PD patients, the proteasome seems to be specifically inhibited in the SNc, with sparing of proteasome function in other areas such as the striatum or cortex [17, 19, 161]. As such, animal models based on inhibition of proteasome function fulfill construct validity, and might be especially relevant when administered intranigrally.

Table 5
Validity criteria of the proteasome inhibition model of PD

Construct validity
Catalytic activity of the proteasome decreased in the SN of PD patients. Good construct validity, especially for intra-nigral administration of proteasome inhibitors.
Face validity
Pattern of pathology similar to PD, including vulnerable degeneration of nigral DA-ergic neurons (> VTA DA-ergic neurons), α-synuclein accumulation / aggregation, neuroinflammation and extra-nigral pathology (e.g. cell loss in the PPN). Motor impairment consistently observed.
Predictive validity
Motor dysfunction improved with L-DOPA, apomorphine, and pramipexole.

DA dopamine, SN substantia nigra, PD Parkinson's disease, PPN pedunculopontine nucleus, VTA ventral tegmental area.

At the same time, it should be acknowledged that the causes of proteasome dysfunction in patients with PD are currently unknown. These might include, among others, brain ageing and factors related to underlying pathogenic processes, such as mitochondrial dysfunction, oxidative stress or α-synuclein aggregation. In addition, the decreased expression of structural 20S α-subunits [15, 16] can impede the assembly of the 26S/20S proteasome and, subsequently limit degradation of targeted substrates. Although proteasome inhibitors such as lactacystin are present in the environment as *Streptomyces* natural products and *Streptomyces* spp. (especially when isolated from agricultural soils) can cause loss of DA-ergic neurons in *C. elegans* [200], no epidemiological links exist between exposure to proteasome inhibitors and risk of developing PD. As such, the construct validity of the proteasome inhibition model of PD is limited to reproducing the same functional outcome as in patients with the disorder (i.e. decreased catalytic activity of the 20S proteasome), and not necessarily the same cause of proteasome inhibition as it occurs in PD.

Face validity

Administration of proteasome inhibitors to the nigrostriatal pathway has been found to consistently replicate key pathological hallmarks of PD, including nigral DA neurodegeneration and aberrant α-synuclein accumulation and/or aggregation [50, 79, 81, 82, 85, 87, 88, 90]. The effect on α-synuclein is notable, as accumulation and/or aggregation of endogenous α-synuclein is a feature difficult to reproduce in other toxin-based models of sporadic PD. Similar as in PD, nigral cell loss following proteasome inhibition is accompanied by a persistent neuroinflammatory reaction [82, 88]. Furthermore, the intranigral lactacystin model replicates cholinergic degeneration in the PPN as observed in the human condition [82], while volumetric changes described after intranigral [81] or intra-mfb [87] administration of lactacystin mirror morphological changes (such as reductions in cortical and striatal volume and hypertrophy of the lateral ventricles) occurring in PD. With regards to behavioral changes, proteasome inhibition models induce impairment in spontaneous motor activity, decreased motor coordination, and impaired skilled motor function [78, 85, 87, 88].

In contrast to motor symptoms, the evaluation of PD-relevant non-motor symptoms in proteasome inhibition models has been much more limited. We have recently characterized changes in non-motor behavior in mice intranigrally infused with lactacystin [85]. Our results indicate that nigral proteasome inhibition leads to the manifestation of a complex phenotype, characterized by anxiety-like behavior, somatosensory dysfunction and perseverative motor behavior suggestive of loss of cognitive flexibility. In addition, lactacystin-lesioned mice demonstrate hyperactivity/restlessness and develop spontaneous contralateral circling [85]. Although it is regarded that unilateral damage to the nigrostriatal DA-ergic pathway leads to spontaneous ipsilateral turning [201], intranigral epoxomicin administration in mice was similarly found to induce contralateral turning asymmetry, possibly linked with a hyperactive phenotype of the surviving DA neurons [84]. Furthermore, Konieczny and collaborators recently described an increase in DA release in the ipsilateral striatum after intranigral administration of lactacystin to rats, leading to development of spontaneous contralateral rotations [202]. Interestingly, this spontaneous rotational behavior could be blocked by combined administration of DA D1 and D2 receptor antagonists and represented an early event following toxin administration [202]. The appearance of a hyperkinetic behavior in mice intranigrally lesioned

with lactacystin biased our evaluation of depressive-like behavior using the tail suspension test [85], and might require the use of depression-based paradigms less reliant on motor function and/or investigation of lesioned mice at a later time point post-lesion. In a separate study, Lu and collaborators described excessive somnolence in rats intra-nigrally lesioned with MG132, which might reproduce the nocturnal sleep disturbance and excessive daytime somnolence observed in PD patients [203]. Future studies evaluating non-motor behavior following application of proteasome inhibitors are critical in order to better define the spectrum of PD-related symptoms present in this model.

Predictive validity

With regards to PD-related symptoms, motor impairment in proteasome inhibition induced models of PD has been found to be successfully reversed using clinically effective compounds including L-DOPA [76], apomorphine [79] and pramipexole [88]. In addition, the proteasome inhibition model has been employed as platform to identify (or confirm) promising targets for achieving nigral DA neuroprotection. Genetic or pharmacological studies identified various strategies that can interfere with proteasome-inhibition induced nigral DA cell death, including increasing the expression of glial cell line-derived neurotrophic factor [178, 204–206], genetic [122] or chemical [123] iron chelation, and administration of a wide range of compounds such as MAO-B inhibitors selegiline and rasagiline [207], DA receptor agonists pramipexole [208] and D-264 [209], sodium valproate [210], and rapamycin [211] (Fig. 3). Interestingly, while most of these strategies were found to demonstrate similar effects in classical toxin-based models such as 6-OHDA and MPTP, a recent study failed to identify neuroprotective properties of celastrol (a compound with strong anti-oxidant and anti-inflammatory effects) in the lactacystin model, although being effective in the MPTP model [212]. This suggests that proteasome inhibition models induce DA neurodegeneration by distinct pathways when compared to classical models and might be useful in cross-validation of therapeutic agents.

CONCLUDING REMARKS

Local application of proteasome inhibitors to various regions of the nigrostriatal DA-ergic pathway has been found to consistently lead to nigral DA neuron degeneration and aberrant protein accumulation. This suggests a connection between proteasomal impairment and pathological processes occurring in human PD, including formation of LBs and chronic degeneration of nigral DA neurons.

The proteasome inhibition model is particular by inducing nigral DA neurodegeneration via a distinct, but highly relevant mechanism of action. Notably, the model emulates the accumulation and aggregation of endogenous α-synuclein, a feature which has been difficult to reproduce in non-genetic models of PD. With regards to behavior, rodents treated with proteasome inhibitors show motor impairment which is responsive to DA-ergic drugs. The non-motor phenotype of treated mice is, however, incompletely understood and should be more comprehensively evaluated in future studies. Similar to intracerebral injection of other PD-related toxins, the local proteasome inhibition model is characterized by an acute reaction which, depending on the site of injection, may not be progressive. Systemic proteasome inhibition models might offer a better alternative to model the chronic and progressive nature of neurodegeneration and protein accumulation as it occurs in the human disease. While systemic administration of PSI has been found to lead to inconsistent results, additional studies are warranted to better establish this approach, employing distinct proteasome inhibitors, with favorable pharmacokinetics and blood-brain barrier permeability. Critically, such studies should be controlled for successful engagement of the peripherally delivered proteasome inhibitor with its target in the brain.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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