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PROTEIN DEGRADATION PATHWAYS IN PARKINSON'S DISEASE – CURSE OR BLESSING

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Abstract

Protein misfolding, aggregation and deposition are common disease mechanisms in many neurodegenerative diseases including Parkinson's disease. Accumulation of damaged or abnormally modified proteins may lead to perturbed cellular function and eventually to cell death. Thus neurons rely on elaborated pathways of protein quality control and removal to maintain intracellular protein homeostasis. Molecular chaperones, the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) are critical pathways that mediate the refolding or removal of abnormal proteins.

The successive failure of these protein degradation pathways, as a cause or consequence of early pathological alterations in vulnerable neurons at risk, may present a key step in the pathological cascade that leads to spreading neurodegeneration. A growing number of studies in disease models and patients have implicated dysfunction of the UPS and ALP in the pathogenesis of Parkinson's disease and related disorders. Deciphering the exact mechanism by which the different proteolytic systems contribute to the elimination of pathogenic proteins, like α-synuclein, is therefore of paramount importance. We herein review the role of protein degradation pathways in Parkinson's disease and elaborate on the different contributions of the UPS and the ALP to the clearance of altered proteins. We examine the interplay between different degradation pathways and provide a model for the role of the UPS and ALP in the evolution and progression of α-synuclein pathology.

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With regards to exciting recent studies we also discuss the putative potential of using protein degradation pathways as novel therapeutic targets in Parkinson's disease.

Keywords

Parkinson's disease; neurodegeneration; α-synuclein; autophagy; lysosome; ubiquitin-proteasome system; molecular chaperones

INTRODUCTION

Protein misfolding, aggregation and deposition are common disease mechanisms shared by many neurodegenerative diseases that are collectively referred to as "proteinopathies" or "protein conformation disorders". In Parkinson's disease (PD) the synaptic protein α-synuclein accumulates to form distinctive aggregates termed Lewy bodies and Lewy neurites. Although the mechanisms by which α-synuclein malforms and causes neurodegeneration are not fully elucidated, strong genetic and molecular evidence implicates a crucial role for this protein in PD. Cells and neurons as post-mitotic cells in particular rely on elaborated pathways of protein quality control and removal to maintain intracellular protein homeostasis. Accumulation of damaged or abnormally modified proteins leads to perturbed cellular function and eventually to cell death. Extensive genetic and molecular data in transgenic disease models suggests that many of the proteins responsible for "proteinopathies" cause neuronal dysfunction and neurodegeneration by a toxic gain of function. Thus, it is of great importance to understand the cellular pathways that regulate levels of toxic protein species.

Functional steady-state levels of any given protein are determined by synthesis, post-translational modification, degradation and release. The first line of defense against misfolded proteins prone to aggregation are molecular chaperones that can stabilize or refold such protein species and that, in addition, are also intimately associated with mechanisms of protein degradation [41,63]. Under pathological conditions with massive protein misfolding and cellular stress, however, the subsequent depletion of chaperones and the rising levels of aggregate prone proteins can exceed the refolding capacity of the chaperone system. In this scenario, proteins that no longer can be refolded may be shuttled to proteolytic systems to be eliminated. This is constantly true for the folding of proteins in the endoplasmic reticulum, where a substantial fraction of polypeptides fails to reach their native state and are thus mislocalized to the cytosol where degradation through the proteasome takes place, a process that is referred to as endoplasmic reticulum associated degradation (ERAD) [30,145].

In neurons, the two major proteolytic systems that participate in normal protein turnover and the removal of altered proteins are the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP). While the UPS degrades most short-lived soluble proteins, the ALP is a degradation process that refers to the degradation of intracellular components, proteins and organelles, in lysosomes. The following sections will review the role of intracellular protein degradation pathways in PD and the different contributions of the UPS and ALP to the clearance of altered proteins. With regards to exciting findings from recent studies, a discussion of the potential of protein degradation pathways as a novel therapeutic target in PD will conclude this review.

DYSFUNCTION OF THE UBIQUITIN-PROTEASOME-SYSTEM IN PARKINSON'S DISEASE

The UPS is the major pathway that mediates the degradation of soluble intracellular proteins in the cytoplasm, nucleus and endoplasmic reticulum (ERAD) [reviewed in 30,50,145,169]. The clearance of proteins by the UPS involves an elaborated sequence of events: First, a chain of activated ubiquitin monomers, a small and stable globular protein, is covalently linked to lysine residues of the substrate protein in an ATP-dependent manner. This process is initiated and orchestrated by the sequential action of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). The fact that there is at least one E1 enzyme, but more than ten E2 enzymes and more than a thousand E3 enzymes interacting, underscores the high degree of selectivity of UPS-mediated degradation although it is a vital and constitutively active cell function. The ubiquitin chain serves as a signal for recognition by the proteasome. Proteasomes are small, barrel shapes organelles, that consist of a catalytic core (the 20S proteasome) and regulatory subunits that form two 19S "caps" at either end to regulate the entry and catalytic function of the core. The fully assembled proteasome is referred to as the 26S proteasome. The catalytic core is formed by 28 subunits organized in two outer α-rings and two inner β-rings. The latter comprises the proteolytic chamber that holds three major kinds of protease activity: chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolytic activity. Proteasomal degradation yields short peptide fragments that are then subject to further degradation by peptidases, allowing the recycling of amino acids for the synthesis of novel proteins. The ubiquitin chain is also dissembled by a group of enzymes termed ubiquitin c-terminal hydrolases. It is noteworthy that some proteins do not require ubiquitination to undergo degradation by 26S proteasomes and that other proteins are simply degraded by 20S proteasomes, although the exact relevance of both processes in health and disease remains to be determined.

Following the initial finding that proteins in Lewy bodies are highly ubiquitinated [78,84], numerous components of the UPS, such as proteasomal subunits, ubiquitinating and de-ubiquitinating enzymes and proteasome activators were found to be abundantly present in these aggregates as well [59,79,93,141,183]. Genetic hints for an involvement of the UPS were provided by the discovery of mutations in parkin (PARK2; an E3 ligase that can also modulate 26S proteasome activity) [68,158] and UCH-L1 (PARK5; an ubiquitin hydrolase) [85] that can lead to monogenetic forms of PD.

First evidence for malfunction of the UPS in PD was demonstrated by studies that evaluated proteasome activity in post-mortem brain tissue by using enzymatic assays (Table 1A and Figure 1). Importantly, a significant decrease of the chymotrypsin-like and trypsin-like proteasome activity was noticed in the substantia nigra (SN) of PD patients in comparison to age-matched controls [106,108,154]. However, no deficits or even enhanced proteasome activity was found in other brain regions, arguing that a global perturbation of proteasome function is unlikely a cause or consequence of PD pathology [49,154]. It rather seems plausible that the observed reduction of proteasome activity in the SN is a region specific phenomenon that predisposes vulnerable neurons to subsequent pathological changes or vice versa a reduction in proteasome activity could simply represent a secondary phenomenon to the profound neurodegeneration in this region.

A similar distribution pattern was observed for levels of proteasomal subunits (Table 1A and Figure 1): Genes for several proteasome subunits were significantly downregulated in the SN of PD patients [53] and expression levels of the 20S proteasome core and α-subunits in particular as well as the 19S regulatory caps were significantly reduced in the SN of PD patients [20,29,106,107] and cortex of Dementia with Lewy bodies (DLB) cases [95], but unchanged or even elevated in other extranigral regions [7,49,106,107]. In addition to

studies investigating central proteasome function, a few studies have demonstrated altered proteasome activity in peripheral blood cells of PD patients (Table 1A and Figure 1). Two groups reported reduced 20S proteasome activity in peripheral blood mononuclear cells of PD patients that interestingly seemed to correlate inversely with disease duration and severity suggesting that impaired proteasome function could be an indicator of disease progression [15,157]. Significantly reduced proteasome function was, however, only present in patients treated with both L-dopa and dopamine agonists [15], suggesting an alternative hypothesis by which dopamine levels alter proteasome function, a finding that has been observed under experimental conditions in cultured cells and animal models [66,11,157,177]. A more recent report, however, failed to confirm altered proteasome function in peripheral blood cells of PD patients, arguing that peripheral proteasome function might be a poor disease indicator [19].

A convincing link between proteasome dysfunction and PD has been established by studies in disease models. In α-synuclein-eGFP transfected mouse primary neurons [105], rat ventral mesencephalic primary neurons [109] and cultured PC12 cells [128], application of the proteasome inhibitor lactacystin leads to dose-dependent neurodegeneration and the formation of ubiquitin- and α-synuclein positive inclusions. By systemically applying proteasome inhibitors to rats, McNaught and colleagues seemed to have produced an accurate rat model of PD, both in terms of behavioral or motor deficits and core pathological features [110]. In addition to the progressive and dopamine-responsive motor impairment, treated rats were found to display degeneration of dopaminergic neurons in the SN and decreased levels of dopamine in the striatum. Importantly, surviving neurons were also found to contain α-synuclein and ubiquitin positive inclusion bodies. Hence, this exciting model has received considerable attention, although in the following years several independent laboratories have failed to reproduce most if not all features provided in the initial description [18,75,98,102,140,180]. Only two of five follow-up reports [140,180] replicated dopaminergic cell loss and only one group [180] detected α-synuclein positive aggregates while none could identify a distinct motor phenotype. The extensive variability of findings in this rat model created significant doubt about its utility as an accurate model of PD. Despite this disappointment, related models for proteasome inhibition induced neurodegeneration in rats [45,95,114,161], mice [118,149,172], *Medaka fish* [103] and cultured-cells [126,139] have been validated and used in subsequent studies.

Besides this, the relation between UPS impairment and sporadic PD has been stressed by studies in toxin-based animal models (Table 1A). *In vitro* and *in vivo* studies demonstrated a marked decrease in proteasome activity following exposure to toxins like rotenone or MPTP [12,21,46,27,163,181]. *In vivo*, toxin induced proteasome inhibition was found to cause neuronal cell loss in the SN, dopamine depletion in the striatum and even a disease phenotype [46]. Surprisingly when repeated in mice lacking α-synuclein, UPS impairment was significantly alleviated suggesting that α-synuclein exacerbates the deleterious effects of MPTP on the UPS [46]. Interestingly, a direct inhibitory effect of α-synuclein on proteasome activity has been reported. A number of studies showed that stably increased levels of α-synuclein can lead to impaired proteasome function, potentially paving the way for subsequent pathological changes [25,44,89,122,146,148,150,182]. The reciprocal interaction between α-synuclein and proteasome function suggests a self-perpetuating process in which permanently raised α-synuclein levels impair the UPS, which can in turn lead to further α-synuclein accumulation.

Apart from the reciprocal interaction with α-synuclein metabolism, a recent study interestingly found that overexpression of LRRK2, a protein in which mutations cause autosomal-dominant forms of PD, in cell culture and *zebrafish* impairs proteasomal protein clearance without affecting the catalytic activity or expression of proteasome subunits [88].

Another intriguing study found that in the dopamine depleted striatum of MPTP-lesioned monkeys or 6-OHDA-lesioned mice chronic stimulation of D1 receptors via administration of L-Dopa leads to a significantly impaired chymotrypsin-like activity of the proteasome, arguing for a causal role of proteasome dysfunction in L-Dopa induced dyskinesia, a very compelling link between a focal derangement of protein metabolism, neurotransmission and a behavioral phenotype [11].

Finally, an exciting report by Bedford and colleagues has fundamentally underscored the importance of UPS mediated protein homeostasis for neurodegeneration [9]: A mouse model expressing a conditional deletion of the Rpt2/PSMC1 subunit, an ATPase of the 19S regulatory complex, spatially restricted to the forebrain and a second model engineered to lack the Rpt2/PSMC1 subunit in tyrosine hydroxylase (TH) positive neurons were developed and characterized. As the Rpt2/PSMC1 subunit is vital to the assembly and activity of the 26S proteasome this model represents a model for the specific impairment of the 26S proteasome, while the 20S proteasome remains unaffected. Remarkably, α-synuclein and ubiquitin positive inclusions resembling Lewy bodies developed in the brain regions with impaired 26S proteasome function. In the forebrain restricted knockdown model, progressive neurodegeneration and learning deficits were observed while in the knockdown restricted to TH-positive neurons striking neurodegeneration and inclusion body formation in the SN occurred.

DYSFUNCTION OF AUTOPHAGY IN PARKINSON'S DISEASE

The autophagy-lysosomal pathway (ALP) or autophagy (Greek "to eat oneself") is the general term used to describe pathways that converge into degradation of intracellular proteins or organelles in lysosomes [69,169]. Within the ALP, delivery of targets to the lysosome occurs in three distinct ways that distinguish the respective subtype: macroautophagy, chaperone-mediated-autophagy (CMA) and microautophagy. The final step of all three autophagic processes is the degradation of the substrate protein within lysosomes. Lysosomes are single membrane vesicles that contain a variety of cellular hydrolases including proteases, lipases and glycosidases and that maintain an acidic pH through ATP-dependent proton pumps. Macroautophagy is the "bulk" degradation process that involves the de-novo formation of double membrane bound vesicles that sequester complete regions of the cytosol, including organelles, proteins and lipids. These initial double-membrane vesicles have been termed autophagosomes or autophagic vacuoles. While this compartment lacks proteolytic enzymes, the content can only be degraded by a fusion with lysosomes or late endosomes, which provide the necessary enzymes. The fusion product of autophagosomes and lysosomes is then referred to as an autolysosome.

Interestingly, autophagosomes can be formed anywhere within the cell, independent of the close presence of lysosomes. In neurons for example, autophagosome formation and cargo sequestration can take place in neuronal processes [74]. The long distance from perinuclearly located lysosomes is bridged by cellular transport mechanisms that deliver autophagosomes for the final step of fusion with lysosomes [61]. Without going into much detail, the biogenesis of autophagic vesicles involves 35 autophagy related genes and proteins (Atg) that form two unique ubiquitin-like conjugation systems: The Atg12-Atg5 and the MAP1LC3 (microtubule-associated proteins 1A/1B light chain 3 or LC3 or Atg8)-phosphatidylethanolamine (PE) conjugation [115,117]. Atg12 is linked to Atg5 in a reaction that requires Atg7 and Atg10 (E1- and E2-like enzymes). The Atg12-Atg5 conjugate then associates with Atg16L, which oligomerizes to form a complex. This complex then acts as an E3-like enzyme that determines the sites of LC3 lipidation [48]. Before this step can take place however, LC3 has to be cleaved by Atg4 to generate the cytosolic LC3-I that can subsequently be conjugated to a PE tag by Atg7 and the E2-like enzyme Atg3. The resulting

lipidated form of LC3, LC3-II, is attached to both sides of the autophagosome membrane but is removed from the outer membrane before fusion with lysosomes occurs.

Macroautophagy is an evolutionary highly conserved and tightly regulated pathway. Not only a "bulk" degradation process, increasing evidence suggests the existence of very selective subtypes of macroautophagy, governed by specific cargo recognition molecules (e.g. mitophagy or pexophagy – the selective autophagic degradation of mitochondria or peroxisomes respectively [62,70]). Macroautophagy occurs constitutively in cells and is markedly induced under stress conditions, for instance under starvation [77]. This stress response is mediated by a signaling cascade that involves the serine/threonine kinase mTOR (mammalian target of rapamycin), an important target for pharmacological agents that induce or enhance autophagy [116]. More recent studies also demonstrated that macroautophagy can be enhanced in a mTOR-independent manner [138,165], which may provide additional targets for novel autophagy enhancing compounds.

While macroautophagy is a vital high-turnover cellular pathway under stress conditions, e.g. when essential macromolecules are needed under conditions of nutritional scarcity or when altered intracellular components need to be removed to prevent cell death, CMA's characteristic feature is selectivity. Particular cytosolic proteins that contain a pentapeptide targeting motif (KFERQ) are recognized by molecular chaperones (Hsc70 and co-chaperones) and translocated to the lysosomal surface [5]. Here, chaperones again assist and mediate the binding of the substrate protein to the lysosomal receptor protein LAMP-2a. Assisted by a different set of chaperones located inside the lysosomal compartment, the substrate then crosses the lysosomal membrane in an unfolded state to finally become degraded inside the lysosomal lumen. As it is the case for macroautophagy, CMA is constitutively active in most tissues but becomes recruited under stress conditions. KFERQ-like targeting motifs are estimated to be present in about 30% of all cytosolic proteins, a number that is most likely underrated since post-translational modification can also render proteins prone to recognition by the chaperone-co-chaperone complex.

The third and least well-characterized autophagy subtype, microautophagy, involves the direct sequestration of regions of the cytosol at the lysosomal surface by invagination of the lysosomal membrane. These invaginations then pinch off as vesicles into the lysosomal lumen where rapid degradation takes place [113].

Although each autophagy pathway has individual characteristics, they appear to be interconnected and display compensatory up-regulation upon failure of one of the pathways. Cells respond to a blockage of CMA by activating macroautophagy in a constitutive manner [101]. Although both pathways are not redundant, activation of macroautophagy under basal conditions can preserve protein homeostasis when CMA function is compromised [101]. Vice versa, CMA is enhanced when macroautophagy is dysfunctional [65]. Expanding the crosstalk to non-autophagic pathways, acute UPS inhibition was shown to induce and upregulate autophagic flux in cell culture [38,60,127] and *Drosophila melanogaster* [120] and recently in human α-synuclein transgenic mice [39]. Chronic blockage of the proteasomal pathway leads to constitutively upregulated macroautophagy that however cannot increase upon exposure to cellular stress, as it would be the case under normal conditions [37]. Interestingly, accumulation of p62, an important autophagic adapter protein, in autophagy deficient cells resulted in impaired clearance of proteins through the UPS, although the catalytic activity of the proteasome remained unaffected [76]. The reduced UPS flux could be relieved upon expression of p97/VCP, a chaperone involved in the acquisition of polyubiquitinated proteins for proteasomal degradation, potentially indicating that the UPS and macroautophagy compete for the clearance of ubiquitinated proteins via ubiquitin-binding proteins and p62 [76]. Crosstalk between the different subtypes of autophagy and

coupling to the UPS is of particular interest to neurodegenerative diseases where a primary failure of degradation mechanisms has been proposed [40]. In PD in particular, the accumulation of toxic protein species might be driven by a successive failure of molecular chaperones, the UPS and the ALP [40].

First evidence for an involvement of the ALP in PD was demonstrated by a pathological study that found accumulation of autophagic vacuoles in the SN of PD patients [4]. This accumulation is consistent with either an overproduction (as would be the case following primary UPS failure or as a secondary phenomenon to neurodegenerative changes) or impaired turnover of autophagic vacuoles (as would be the case with primary ALP dysfunction or secondary dysfunction due to neurodegenerative changes). Supportive of both hypotheses, recent studies confirmed the association of autophagic signal molecules and components of CMA [1] and macroautophagy [32,35,151,179] or lysosomes and the ALP in general [29,57,71,86,152] with α-synuclein pathology in both PD and DLB (Table 1B & Figure 2).

Uniformly, all studies found an increase of the autophagosome marker LC3-II [32,35,57,71,151,179] and a decrease of the lysosomal marker LAMP-1 [29,35,71] in both tissue of PD and DLB patients, suggesting the presence of abundant and dysfunctional autophagosomes and lysosomes in synucleinopathies. Whether the observed increase in LC3 expression directly translates into decreased or increased autophagic processing is currently unknown, however the increase in p62, a selective autophagy substrate that is continuously degraded by macroautophagy [14,73], in DLB as observed by Tanji and colleagues does suggest that autophagic flux is impaired [151]. Furthermore LC3 immunostaining widely co-localized with different types of Lewy pathology arguing for an interaction between autophagy and α-synuclein pathology [1,32,35,57,71,151]. Discrepant findings exist regarding levels of the CMA adapter molecule LAMP-2a and the lysosomal protease cathepsin D: LAMP-2a was significantly reduced in the SN pars compacta and amygdala of PD patients [1], while in the lysosomal enriched fraction of temporal cortex tissue of DLB patients LAMP-2a was significantly increased compared to controls [71]. Likewise, cathepsin D immunoreactivity was significantly reduced in substantia nigra neurons of PD patients with an even greater decrease in α-synuclein inclusion bearing cells [29]. In contrast cortical neurons of DLB patients displaying α-synuclein accumulation were found to show abundant enlarged cathepsin D positive lysosomes [32]. It is currently difficult to interpret these apparent differences. Nevertheless the co-localization of LAMP-2a and cathepsin D with Lewy body pathology implies a pivotal role of these lysosomal proteins in α-synuclein aggregation and/or removal.

In peripheral blood mononuclear cells, two studies identified increased LC3-II levels, suggestive of autophagy activation (Table 1B) [123,171].

Important genetic evidence in support of altered autophagy in PD was provided by studies that linked loss-of function mutation in the lysosomal enzyme ATP13A2, a lysosomal ATPase, to Kufor-Rakeb syndrome, a familial, juvenile onset form of Parkinsonism [125] and by the strong association between Gaucher's disease, caused by mutations in the lysosomal enzyme glucocerebrosidase, and the incidence of PD [144]. The latter in particular, has recently received considerable attention and exciting new molecular links to α-synuclein pathology are being explored [34,104,134]. Parkin and PINK1, both linked to autosomal-recessive forms of PD, have also been found to be part of the signaling pathway that controls mitophagy, a cargo-specific autophagic process that eliminates damaged mitochondria [178]. The interplay between α-synuclein and CMA or macroautophagy has been explored in recent years. Mutant A53T and A30P α-synuclein was shown to strongly bind the CMA adapter molecule LAMP-2 on the surface of lysosomes, but are unable to

become internalized and thus act as inhibitors of CMA, hindering their own processing and the degradation of other CMA substrate proteins [33,173]. Relevant to sporadic PD, dopamine induced conformational changes in wild type α-synuclein revealed the same inhibitory effects [100]. The resulting increase in cytosolic α-synuclein levels could favor pathological misfolding and aggregation. Further supporting the crucial involvement of CMA in PD, the turnover of the neuronal transcription factor MEF2D was shown to dependent on CMA and lysosomal degradation, a process that is disrupted by wild-type and mutant α-synuclein, leading to impaired MEF2D function and thus to neuronal degeneration [176].

In the MPTP mouse model of PD, autophagosome accumulation and dopaminergic cell death were found to be preceded by a disruption of lysosomal integrity and clearance caused by abnormal permeablization of lysosomal membranes through mitochondrial induced oxidative stress [35]. The ectopic release of lysosomal hydrolases into the cytosol may therefore contribute to the dopaminergic cell death observed with these toxins.

On the other hand, mice deficient in cathepsin D, a major lysosomal protease, were found to display α-synuclein accumulation that interestingly formed primarily outside of autophagic vacuoles or lysosomes and that was accompanied by significant proteasome impairment [124]. Conversely, cathepsin D overexpression was protective against α-synuclein overexpression induced cell death in cultured cells and *C. elegans* [124]. Similarly ALP impairment by pharmacologic inhibition using Bafilomycin A1 was shown to exacerbate α-synuclein induced toxicity but to reduce aggregate formation in both cell culture and transgenic mice, suggesting the production of toxic oligomeric intermediates when autophagic clearance is dysfunctional [71]. Lysosomal dysfunction was also shown to influence the secretion of α-synuclein via exosomes [2], a recently discovered mechanism that might have important implications for the seeding, regional spreading and progression of α-synuclein pathology and neuronal degeneration [43,54].

Another recent study, using cell culture and α-synuclein transgenic mice, demonstrated that excess levels of α-synuclein can inhibit macroautophagy in the very early stages of autophagosome formation via inhibition of the small GTPase Rab1A [167]. Compelling *in vivo* evidence for this finding was provided by a second study that showed enhanced autophagy in α-synuclein depleted (Snca $^{-/-}$) mice [31].

Mutations in LRRK2, the most prevalent cause of autosomal-dominant PD, cause alterations in the autophagic pathway as well. LRRK2 not only co-localizes with markers of the endosomal-lysosomal pathway in cases of DLB [58], its knock-out [155,156] or overexpression [51] also significantly alters autophagosome formation.

Integral to the concept that constitutive macroautophagy is essential to neuronal survival, studies in knockout models of essential components of this pathway (Atg5 and Atg7) revealed that genetic inactivation of macroautophagy function leads to profound neurodegeneration and the formation of ubiquitinated inclusion bodies [55,72]. A very recent study also demonstrated that cell specific deletion of Atg7 in midbrain dopamine neurons of knockout mice leads to the formation of ubiquitin und p62 positive inclusions, dendritic and axonal pathology as well as delayed and moderate loss of dopaminergic neurons, accompanied by late-onset locomotor deficits [47]. Intriguingly, whole-brain depletion of Atg7 was found to cause presynaptic accumulation of α-synuclein and LRRK2. While the former was caused by reduced α-synuclein turnover, the latter was shown to be attributable to increased transcription [47].

It should be noted that studies that assessed the effects of genetic depletion of CMA function in mice, did not report a distinct neurological phenotype or neurodegeneration [101],

potentially because deleterious effects of CMA dysfunction are masked through a compensatory upregulation of macroautophagy.

Apart from pure dysfunction, aberrant autophagy following exposure to overexpressed A53T mutant α-synuclein [28] neurotoxins [86,112,168] or iron overload [26] importantly demonstrates that prolonged excess levels of autophagy promote cell death. The recent study by Choubey and colleagues in particular demonstrates an interesting example for the concept of "lethal" autophagy in neurodegeneration because it links the overexpression of mutant A53T α-synuclein in primary cortical neurons to excessive autophagy and consequently to mitochondrial loss via mitophagy, bioenergetics deficits and neurodegeneration [28]. Importantly, these findings were recapitulated with the use of rapamycin (with toxicity occurring only at a prolonged exposure of 5 days), a finding that conceivably has broad implication for therapeutic strategies that aim at globally enhancing autophagy (Table 1B).

DEGRADATION OF α-SYNUCLEIN – A CRUCIAL QUESTION FOR PARKINSON'S DISEASE

The simple question how α-synuclein is degraded by neurons has caused much controversy and a body of conflicting results (summarized in Table 2). Deciphering the pathway responsible for degradation of α-synuclein has significant impact for the pathogenesis of α-synuclein aggregation and derived therapeutic approaches in synucleinopathies: α-synuclein may accumulate because of dysfunctional degradation and on the other hand degradation pathways could be targeted pharmacologically to facilitate clearance and to counteract α-synuclein pathology.

As a general concept, excess levels of abnormal proteins cause misfolding, aggregation and proteotoxic stress. Clearance of pathological proteins is a vital cell function and essential for cellular integrity. Mechanisms of protein clearance are even more important to neurons, as post mitotic cells with a long life span that renders them vulnerable to protein accumulation over time and proteotoxic stress.

Current literature, nearly exclusively based on *in vitro* studies and different cell-culture models, provides evidence for the UPS [10,90,91,121,153,164,175] and the ALP [8,32,33,94,100,121,142,147,162,164,177] as the primary mechanism by which unmodified human wild type α-synuclein is degraded, but conflicting findings exist. No role for the UPS was observed in a different set of studies [3,13,82,94,121,129,162,177] and the role of the ALP was challenged by a number of experiments [82,135].

Two groups convincingly demonstrated that unfolded, non-ubiquitinated α-synuclein can undergo degradation via both the 26S and 20S proteasome *in vitro* [90,91,153]. Degradation through the latter was found to yield truncated α-synuclein species that are known to promote the formation of inclusions [91].

Mutant A53T and A30P α-synuclein is degraded by both the UPS [10,164,175,177] and macroautophagy [130,135,164,177,179], although this was questioned in other studies [3,82]. Importantly, as explained in the previous section, Cuervo and colleagues demonstrated that mutant α-synuclein cannot be degraded by CMA [33] and modified α-synuclein, such as dopamine or nitration induced species, were shown to behave similarly in this respect [100]. A53T mutant α-synuclein was further shown to increase upon inhibition of a specific cytosolic peptidase, the Puromycin-sensitive aminopeptidase, that promotes autophagic clearance by enhancing autophagosome formation [111].

Serine129 phosphorylated α-synuclein was found to be degraded by the UPS [94] and macroautophagy [94,147] but not by CMA [100]. Recent studies also convincingly demonstrated that α-synuclein oligomers are targeted for macroautophagic [179] and lysosomal [82] degradation but are not a substrate for CMA [100], as opposed to the dimeric form of α-synuclein which was found to be translocated and degraded within the lumen of isolated lysosomes [100]. Toxin-induced aggregates and aggregate prone species of α-synuclein were shown to be subject to proteasomal and lysosomal degradation [82,143] although not unequivocally [82]. Alpha-synuclein containing aggresomes were found to be amenable to autophagic clearance induced by starvation or rapamycin treatment in cultured cells [170]. Rat α-synuclein, containing the A53T substitution just like familial cases of PD [80], seems to be degraded by the ALP, macroautophagy [162] and CMA [33,162], although ambiguous findings concerning the latter exist [33]. A definite role for the UPS, however, was negated [33,94,128,162]. Mouse α-synuclein, again sharing the A53T mutation, was shown to be degraded by CMA [96,162] what again contradicts findings for the human A53T mutant protein.

Two recently published *in vivo* reports using genetic and pharmacologic strategies to upregulate macroautophagy function in human wild type α-synuclein transgenic mice demonstrated enhanced clearance of α-synuclein via this pathway [32,147]. A third *in vivo* study argued for an increased association of mouse α-synuclein with CMA in a paraquat-based toxin model of α-synuclein overexpression [96].

Our own work addressing the role of UPS and the ALP in PD through a novel *in vivo* paradigm that employs multiphoton imaging and cranial windows to study α-synuclein turnover in the living mouse brain [159,160], demonstrates clear but distinct roles for both pathways [39,40]. Our results show that α-synuclein turnover is mediated by the UPS both in non-transgenic mice with only endogenous levels of α-synuclein and in transgenic mice with pathologically raised α-synuclein burden. Consistent with previous reports, we found that the UPS becomes increasingly impaired as mice age and importantly that stably raised α-synuclein levels impact proteasome activity in a negative fashion. Turning to autophagy, we found that macroautophagy is not crucially involved in the regular turnover of endogenous levels of α-synuclein but becomes recruited to eliminate excess levels of α-synuclein when the pre-existing burden is raised as it is the case in α-synuclein transgenic mice.

Shedding light on the role of ubiquitination in the degradation process of α-synuclein, another recent study found a pivotal role for the deubiqitinase USP9X in controlling α-synuclein's fate [131]. Levels of USP9X were found to be significantly reduced in the SN of PD cases and cortex of DLB patients, potentially through sequestration into Lewy bodies, leading to impaired deubiquitinase activity in recovered tissue extracts. Monoubiquitination of α-synuclein by the E3-ubiquitin ligase SIAH [87] and deubiquitination by USP9X might be critical for Lewy body formation as a number of studies have demonstrated that monoubiquitination promotes α-synuclein aggregation and inclusion formation that are toxic to cells [83,87,132]. Important to the degradation of α-synuclein, Rott and colleagues demonstrated that, in the absence of proteolytic impairment, monoubiquitination promotes degradation through the proteasome while deubiquitinated α-synuclein is cleared through autophagy in cultured cells [131]. Another recent study by Tofaris and colleagues showed that Nedd4, a protein of the endosomal-lysosomal pathway, acts as an E3 ligase that mediates α-synuclein ubiquitination at lysine residue 63 and promotes degradation through lysosomes [152].

Taken together, early studies addressing the degradation mechanism of α-synuclein in cell-culture models have produced conflicting results and no clear finding was established for

any of the α-synuclein species examined. Variability in the findings might result from the use of different in vitro or cell culture models, the use of non-neuronal cultures, different transfection paradigms, different culture conditions, the use of different pharmacological inhibitors at a different dose and incubation time, different expression levels of α-synuclein in the individual experimental settings, differences in the degradation of different α-synuclein species, e.g. rodent versus human α-synuclein or monomeric, soluble α-synuclein versus aggregated, insoluble α-synuclein species. Recent *in vivo* studies, including our own work, however, have provided new evidence for distinct roles of the ubiquitination machinery, the UPS and ALP in α-synuclein turnover and pathology (Figure 3).

ENHANCED DEGRADATION OF α-SYNUCLEIN AS A THERAPEUTIC STRATEGY

Advanced understanding of the implications of protein degradation pathways for age-related neurodegenerative diseases has led to an intensive search for small-molecules that can modulate the activity of chaperones, the UPS or ALP. Development of these compounds for clinical use might provide a novel disease modifying strategy for PD and related protein conformation disorders. In the case of molecular chaperones numerous proof-of principle studies in disease models have provided encouraging results [36,41].

As for mechanisms of protein degradation, the UPS has successfully been explored as a drug target for treatment of several forms of cancer, most importantly hematologic malignancies such as multiple myeloma [42]. While proteasome inhibitors like bortezomib are approved and established therapeutics for these diseases, the challenge for neurodegenerative diseases is to develop targeted strategies to facilitate clearance of unwanted proteins. Such “enhancers” of proteasomal degradation could in theory correct or ameliorate a primary UPS dysfunction in PD (Figure 3). Enhanced degradation of α-synuclein and other potentially pathogenic proteins through the proteasome could possibly slow down or even prevent protein misfolding and accumulation. A recent and exciting study for example explored the proteasome-associated deubiquitinating enzyme USP14 as a target for proteasome enhancement [81]. USP14 is known to inhibit proteasomal degradation by trimming of ubiquitin chains on target proteins that would otherwise determine their clearance. By blocking USP14, proteasomal degradation of substrates, including the proteins tau and TDP-43, both linked to neurodegenerative diseases, can be facilitated [81]. Studies of proteasome enhancement in models of PD or α-synucleinopathies respectively are awaited eagerly.

Autophagy enhancement has emerged as a very promising route to clear unwanted protein species and to prevent neurodegeneration [56,99,133]. Autophagy is constitutively active at a low basal level but is induced in response to various forms of cell stress. It therefore represents an essential mechanism by which neurons can adapt to acute changes in intracellular protein homeostasis (Figure 3). Small molecule enhancers of autophagosome formation are being explored for neurodegenerative diseases and can be classified into two groups: mTOR-dependent and mTOR-independent enhancers of autophagy.

Rapamycin (Sirolimus) inhibits the activity of mTOR, a protein-kinase that has a key role in controlling autophagy [184], and thus rapamycin stimulates autophagosome formation and autophagic protein turnover. As a therapeutic, rapamycin is an FDA- and EMA-approved immunosuppressant used to prevent graft rejection after transplantation of organs or coronary stents and has shown promising results in studies for various forms of cancer [17]. Important to neurodegenerative diseases associated with aging, rapamycin strongly ameliorates the disease phenotype in models of Alzheimer’s disease, Huntington’s disease, amyotrophic lateral sclerosis and PD [17] and has been shown to exhibit strong anti-aging

effects in several species [184]. In toxin based mouse models of PD, the beneficial effects of rapamycin [92,118,119] has been explained by two distinct mechanisms: The first study by Malagelada et al. reported that mTOR inhibition using rapamycin effectively blocks translation of the pro-cell death signaling molecule RTP180 and thus prevents neurodegeneration [97]. In a second study by Dehay et al. the neuroprotective effect was attributed to an induction of autophagy and a restoration of lysosomal deficiency by a boost in lysosomal biogenesis [35]. As discussed above rapamycin and related small molecule enhancers of rapamycin attenuate α-synuclein accumulation in neuronal cell bodies and synaptic terminals through autophagy enhancement [32,130,138,147,164,179].

While mTOR inhibitors clearly show potential as future therapeutics their use might be limited because of their significant impact on other pathways that are regulated in a mTOR-dependent manner. mTOR-independent inducers of autophagy comprise a heterogeneous group of compounds of which trehalose is probably studied best. Trehalose is a stable disaccharide with unique physicochemical properties: It directly acts as a chemical chaperone that can stabilize misfolded proteins but it also facilitates the clearance of aggregate-prone proteins including α-synuclein via the autophagic pathway [22,135]. Interestingly, simultaneous treatment with trehalose and rapamycin had an additive effect on the clearance of mutant A30P α-synuclein [135]. L-type calcium channel blockers such as verapamil were identified as autophagy enhancers in a small-molecule screen and were found to effectively promote degradation of A53T mutant α-synuclein [165]. Lithium, a mood-stabilizing drug used in bipolar disorder, is an autophagy inducer through its inhibitory effect on inositol monophosphatases therefore depleting the intracellular signaling molecule IP3 [136]. Although lithium enhances autophagic flux independent of mTOR signaling, it has the opposite effect through its inhibition of GSK3β, which leads to mTOR activation and therefore alleviates autophagy [137]. Nevertheless enhanced clearance of mutant α-synuclein [136] and neuroprotective effects [67,174] through lithium were demonstrated in cell culture models and mice, underscoring the potential of lithium and related molecules for further evaluation.

Two important caveats to the putative potential of enhanced protein degradation have to be kept in mind: 1) Enhanced protein degradation and enhanced autophagy in particular is a double-edged sword, since both impaired and unregulated, excessive protein degradation can be detrimental to neuronal integrity. Hence, for example simply upregulating autophagic flux by applying autophagy-enhancing drugs may not be a feasible strategy and should be assessed with great caution. Determining the primary molecular mechanism responsible for dysregulation of protein degradation pathways in PD will allow the development of tailored therapeutic interventions that restore deficits. 2) The exact role of mature protein aggregates or Lewy bodies respectively for neurodegeneration remains unclear, therefore the consequences of preventing, dissolving or removing mature aggregates are uncertain. Notably the presence of soluble oligomers as opposed to mature aggregates correlates best with neurotoxicity [52,64,166]. In fact neurons may employ aggregate formation as a protective mechanism to prolong survival [6,23]. Supporting this theory, promotion of aggregate formation, which may act as scavengers for toxic intermediate oligomeric species, might be a promising neuroprotective approach [16].

CONCLUDING REMARKS AND FUTURE CHALLENGES

Protein degradation pathways play an integral role in the development of neurodegenerative diseases. Their successive failure, as a cause or consequence of early pathological alterations in vulnerable neurons at risk, might provide a key step in the pathological cascade that leads to spreading neurodegeneration (Figure 3). Exploring the crosstalk between the UPS and the

different subtypes of autophagy will reveal crucial insights into progression of dysfunctional protein degradation and/or the prevention thereof.

Keeping the balance in protein homeostasis is a primary goal for interventions that aim at mechanisms for protein removal. The targeted development of small molecules that specifically induce the ubiquitination and proteasomal or lysosomal degradation of aberrant and potentially toxic proteins will provide a novel generation of therapeutics for PD and related diseases. Deciphering the exact mechanism by which the different proteolytic systems contribute to the elimination of pathogenic proteins, like α-synuclein, is therefore of paramount importance. The short review of the existing studies on α-synuclein degradation highlights the difficulties faced in the attempt to answer this question. The few *in vivo* studies addressing α-synuclein's degradation in a more relevant and accurate experimental paradigm have provided new and much needed insights into the roles of the UPS and ALP in PD. It is now on future research to translate mechanistic insights into therapeutic strategies that are amenable to further testing and evaluation in disease models and patients. Therapeutic use of degradation pathways in PD to overcome a dysfunction in protein homeostasis may turn a "curse" into a "blessing".

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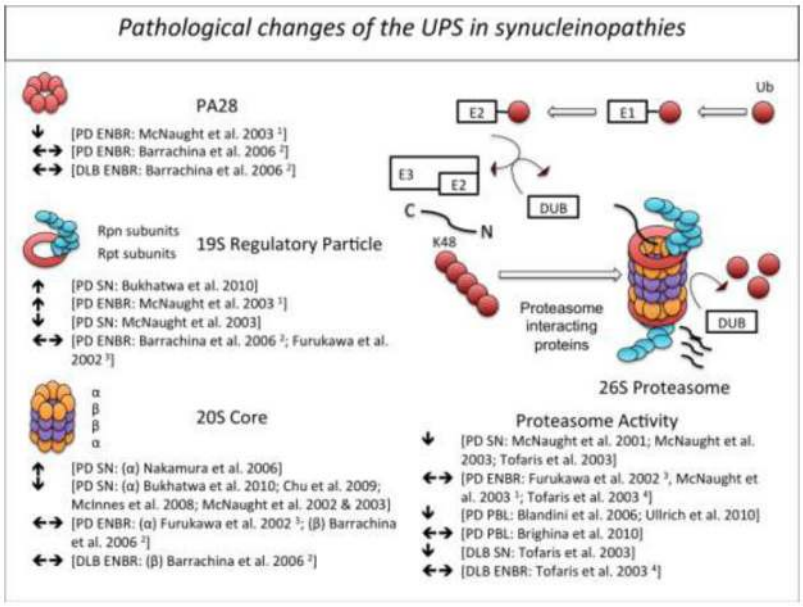

Fig. 1.

Pathological alterations of the ubiquitin-proteasome system in patients with Parkinson's disease (PD) or Dementia with Lewy bodies (DLB). [1] cerebral cortex, striatum, hippocampus, pons, cerebellum; [2] frontal cortex; [3] putamen, caudate nucleus, frontal cortex, occipital cortex and cerebellar cortex; [4] frontal cortex, cingulate cortex, occipital cortex. DUB (deubiquitinating enzyme); EBNR (extranigral brain region); PBL (peripheral blood lymphocyte); Ub (ubiquitin); SN (substantia nigra)

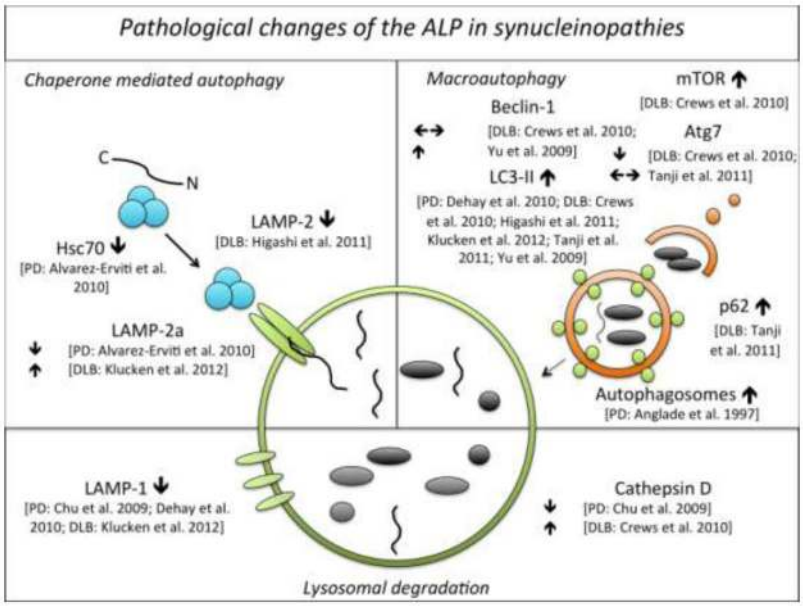

Fig. 2.

Pathological alterations of the autophagy-lysosomal pathway in patients with Parkinson's disease (PD) or Dementia with Lewy bodies (DLB)

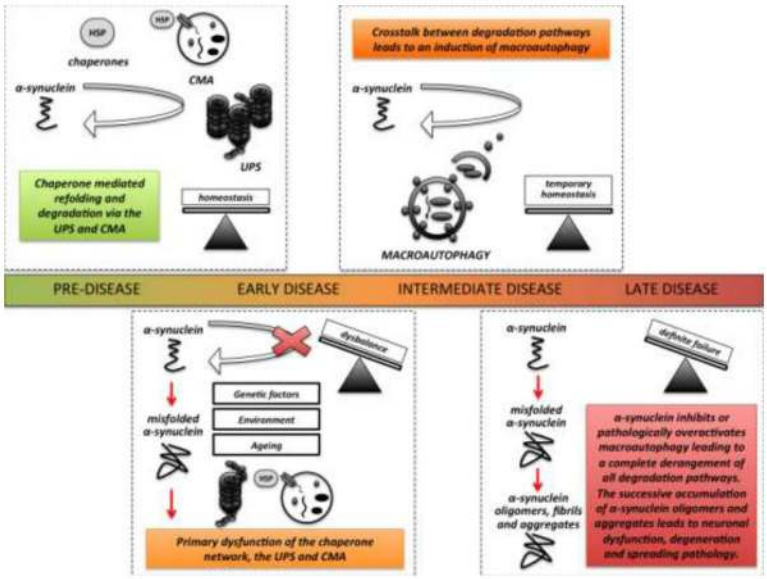

Fig. 3.

The successive failure of protein degradation pathways might provide a key step in the pathological cascade that leads to α-synuclein pathology, neuronal dysfunction and spreading neurodegeneration. The regular turnover of unmodified α-synuclein is largely mediated via both the ubiquitin-proteasome system (UPS) and chaperone-mediated autophagy (CMA). In the early disease stage, primary dysfunction of protein degradation, caused and influenced by genetic, environmental and age-related factors, may lead to excess levels of α-synuclein and the formation of pathologically modified species that overwhelm the capacity of chaperones, the UPS and CMA. Accumulating α-synuclein leads to further and continuous impairment of these pathways therefore eventually creating a vicious cycle. In the next disease stage, crosstalk between degradation pathways drives the induction of macroautophagy and temporary protein homeostasis is achieved before this balance finally tilts. Entering the late disease stage, α-synuclein inhibits or disproportionally overactivates dysfunctional macroautophagy and lysosomal degradation, leading to a complete derangement of all protein degradation pathways. Uncontrolled accumulation of α-synuclein produces oligomeric species that cause neuronal dysfunction and degeneration. At the same time, excess levels of α-synuclein and impaired degradation trigger α-synuclein secretion, thus promoting seeding or protein templating and therefore potentially contributing to a spread of the pathology. CMA (chaperone mediated autophagy); HSP (heat shock proteins); UPS (ubiquitin-proteasome-system)

Table 1

UPS and ALP dysfunction in synucleinopathies

Table 1A. Dysfunction of the UPS in PD & DLB	**Reference**
Reduced proteasome activity in the SN of PD patients	[106,108,154]
Reduced expression of proteasomal subunits in the SN of PD patients or cortex of DLB patients	[20,29,95,106,107]
Reduced proteasome activity and expression of UPS related proteins in peripheral blood mononuclear cells	[15,157]
Altered proteasome function in toxin-based models of PD	[12,21,46,27,163,181]
Altered proteasome function in α-synuclein overexpressing rodent models	[24,44,39]

Table 1B. Dysfunction of the ALP in PD & DLB	Reference
Altered levels of autophagy-related proteins in PD or DLB patients	[1,29,32,35,57,71,86,151,152,179]
Altered autophagy function in peripheral blood cells	[123,171]
Altered autophagy function in toxin-based models of PD	[86,112,168]
Altered autophagy function in α-synuclein overexpressing rodent models	[29,32,179]

Table 2

Published studies on α-synuclein degradation

reference	α-synuclein	UPS	ALP	model system
Ancolio et al. 2000	WT & A53T	**NO** (Lactacystin, PSI)	n.a.	Transiently & stably transfected HEK293 cells or TSM1 neurons
Batelli et al. 2011	WT	**NO** (MG132)	**Yes** (3-MA)	Inducible TetOn(α-syn) PC12 cells
	A30P	**Yes** (MG132)	**Yes** (3-MA)	
Bennett et al. 19910	WT & A53T His-tagged	**YES** (β-lactone)	n.a.	Transiently transfected SH-SY5Y cells
Crews et al. 2010	WT	n.a	**YES** (Rapamycin, Atg7 upregulation)	***Human WT α-synuclein tg mice***, B103 cells
Cuervo et al. 2004	Rat	**NO** (Epoxomicin)	**YES** (NH_4Cl) **NO** (3-MA)	Rat ventral midbrain cultures
	WT	n.a.	**YES** (NH_4Cl, serum removal)	Stably transfected PC12 cells & isolated lysosomes
	A53T, A30P	n.a.	**NO** (CMA)	Isolated lysosomes
Ebrahimi-Fakhari et al. 2011	Mouse and human α-synuclein in α-synuclein transgenic mice; α-synuclein-eGFP in a multiphoton imaging based i*n vivo* paradigm	**YES** (CLBL, MG132)	**YES** (BafA1, HCQ)	***Human α-synuclein transgenic mice, α-synuclein-eGFP transgenic mice and non-transgenic mice***
Lee et al. 2004	WT, A53T, A30P Myc- and His-tagged	**YES** for aggregates (β-lactone, epoxomicin)	**YES** for aggregates (BafA1, E64, cathepsin I) **NO** for aggregates (3-MA, rapamycin)	AV-mediated infection of COS-7 cells & SH-SY5Y cells Rotenone induced aggregate formation paradigm
		n.a.	**YES** for oligomeric aggregates (BafA1)	
		NO for soluble monomer (β-lactone, epoxomicin)	**NO** for soluble monomer (BafA1, E64)	
	Rat	n.a.	**YES** for oligomeric aggregates (BafA1)	Rat primary cortical neurons
Liu et al. 2003; 2005	WT	**YES** (MG132)	n.a.	Isolated 20S and 26S proteasomes
Machiya et al. 2010	S129P	**YES** (Lactacystin, MG132)	**YES** (3-MA, CQ)	Stably transfected SH-SY5Y cells
	WT	**NO** (Lactacystin, MG132)	**YES** (CQ)	Stably transfected SH-SY5Y cells
	S129P	**YES** (Lactacystin, MG132)	n.a.	Rat primary cortical neurons
	Rat	**NO** (Lactacystin, MG132)	n.a.	Rat primary cortical neurons
Mak et al. 2010	Mouse α-synuclein in a paraquat based overexpression model	n.a.	**YES**	***Paraquat treated C57BL/6 mice***

reference	α-synuclein	UPS	ALP	model system
Martinez-Vicente et al. 2008	S129P; S129E; Oligomeric forms induced by nitrating agents; nitrated & dopamine modified forms WT monomers and dimers	n.a. n.a.	**NO** (CMA) **YES** (CMA)	Isolated lysosomes
Paxinou et al. 2001	WT	**YES** (MG132) **NO** (Lactacystin)	**YES** (NH_4Cl)	Stably transfected HEK293 cells
Rideout et al. 2001	Rat	**NO** (Lactacystin, PSI)	n.a.	PC12 cells
Riedel et al. 2010	A53T	n.a.	**YES** (17-AAG, NH_4Cl, rapamycin)	OLN-93 cells
Rott et al. 2011	Monoubiquitinated α-synuclein	**YES** (Lactacystin, MG132)	**YES,** but only small effect (3-MA, CQ, NH_4Cl, Wortmannin)	Transfected SH-SY5Y cells
	Deubiquitinated α-synuclein	**YES**, but only small effect (Lactacystin)	**YES** (3-MA)	
Sarkar et al. 2007	WT, A53T, A30P	n.a.	**YES** for A53T & A30P (Trehalose) **NO** for WT (Trehalose)	Stably transfected PC12 cells
Shin et al. 2005	SynT (WTSynEGFPΔ 155)	**YES** (ALLN)	**YES** (NH_4Cl)	Stably and transiently transfected H4 cells
Spencer et al. 2009	WT, Ser129P	n.a.	**YES** (Beclin-1 upregulation, 3-MA, BafA1, rapamycin)	B103 cells
	WT	n.a.	**YES** (Beclin-1 upregulation)	***Human WT α-synuclein tg mice***
Sevlever et al. 2008	WT	n.a.	**Yes** (shRNA to cathepsin D)	3D5 cells
Tofaris et al. 2001	WT	**YES** (Lactacystin)	n.a.	Stably transfected SH-SY5Y cells & isolated 20S proteasomes
Tofaris et al. 2011	Ubiquitinated α-synuclein	**YES,** but not in Nedd4 overexpressing cells (Bortezomib)	**YES** (CQ, 3-MA)	SH-SY5Y and HEK293 cells
Vogiatzi et al. 2008	Rat & overexpressed WT	**NO** (Epoxomicin)	**YES** (3-MA, Lamp downregulation, DQWT mutant)	PC12 cells
	WT	n.a.	**YES** (DQWT mutant)	SH-SY5Y cells
	Rat	**NO** (Epoxomicin)	**YES** (3-MA, Lamp2a downregulation)	Rat primary cortical neurons
	Mouse α-synuclein	n.a.	**YES** (Lamp2a downregulation)	Mouse primary cortical neurons
	Rat synuclein	n.a.	**YES** (Lamp2a downregulation, 3- MA)	Primary ventral mid- brain cultures
Webb et al. 2003	WT, A53T, A30P HA tagged	**YES** (Lactacystin, epoxomicin)	**YES** (BafA1, 3-MA, rapamycin)	Inducible TetOn(α-syn) PC12 cells

reference	α-synuclein	UPS	ALP	model system
Wong et al. 2008	WT α-synuclein in lactacystin induced aggresomes	n.a.	**YES** (Starvation, rapamycin, 3-MA)	SH-SY5Y cells
Yamada et al. 2006	WT, A53T, A30P Myc-His tagged	**YES**	n.a.	Archaeal 20S proteasomes in HEK293 & Neuro2a cells
Yu et al. 2009	WT, A53T	n.a	**YES** for oligomers (Beclin-1 downregulation, Atg5 downregulation; 3MA, rapamycin)	Transfected M17 cells

Abbreviations: 3-MA (3-Methyladenine); AV (adenovirus); BafA1 (Bafilomycin A1); CLBL (Clasto-lactacystin β-lactone); CMA (chaperone mediated autophagy); CQ (Chloroquine); HCQ (Hydroxychloroquine); PSI (Z-lle-Glu(OtBu)-Ala-Leu-al); WT (wild type)