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Chapter 17

Role of Autophagy in Parkinson's Disease

Grace G.Y. Lim, Chengwu Zhang and Kah-Leong Lim

Additional information is available at the end of the chapter

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1. Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative movement disorder whose occurrence crosses geographic, racial and social boundaries affecting 1-2% of the population above the age of 65 (Dorsey et al., 2007). Clinically, the disease is attended by a constellation of motoric deficits that progressively worsen with age, which ultimately leads to near total immobility. Although pathological changes are distributed in the PD brain (Braak et al., 2003), the principal lesion that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) of the midbrain. This neuronal loss results in a severe depletion of striatal dopamine (DA) and thereby an impaired nigrostriatal system that otherwise allows an individual to execute proper, coordinated movements. Accordingly, pharmacological replacement of brain DA via L-DOPA administration represents an effective symptomatic recourse for the patient (especially during the initial stages of the disease) and remains a clinical gold standard treatment for PD. However, neither L-DOPA nor any currently available therapies could slow or stop the insidious degenerative process in the PD brain. Thus, PD remains an incurable disease. Invariably, the debilitating nature and morbidity of the disease present significant healthcare, social, emotional and economic problems. As the world population rapidly ages, these problems undoubtedly would also increase. According to a recent report, more than 4 million individuals in Europe's five most and the world's ten most populous countries are currently afflicted with PD (Dorsey et al., 2007). In less than 20 years' time, the number of PD sufferers is projected to increase to close to 10 million (i.e. in 2030). This is definitely a worrying trend, and one that aptly emphasizes the urgency to develop more effective treatment modalities for the PD patient. Towards this endeavour, a better understanding of the molecular mechanism(s) that underlies the pathogenesis of PD would definitely be helpful, as the illumination of which

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would allow the identification and therapeutic exploitation of key molecules/events involved in the pathogenic process.

Although a subject of intense research, the etiology of PD unfortunately remains incompletely understood. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic and post-mortem analysis, as well as *in vitro* and *in vivo* modelling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including *α-synuclein*, *parkin*, *DJ-1*, *PINK1* and *LRRK2*, whose mutations are causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration (Lim and Ng, 2009; Martin et al., 2011). Collectively, these studies implicate aberrant protein and mitochondrial homeostasis as key contributors to the development of PD, with oxidative stress likely acting an important nexus between the two pathogenic events.

2. Aberrant protein homeostasis & PD

Perhaps the most glaring evidence suggesting that protein homeostasis has gone awry in the PD brain is the presence of intra-neuronal inclusions, known as Lewy Bodies (LBs), in affected regions of the diseased brain in numbers that far exceed their occasional presence in the normal brain (Lewy, 1912). These signature inclusions of PD comprise of a plethora of protein constituents that include several PD-linked gene products such as α-synuclein, parkin, DJ-1, PINK1 and LRRK2. In a recent report, Wakabayashi and colleagues have documented more than 90 components of the LB and have grouped them into 13 functional groups (Table 1) (Wakabayashi et al., 2012). Among these, α-synuclein is recognized as the major component of LB and thought to be the key initiator of LB biogenesis.

However, whether LB biogenesis represents a cytoprotective or pathogenic mechanism in PD remains debatable. Notwithstanding this, how proteins aggregate to form LB is intriguing in the first place, as the cell is endowed with several complex surveillance machineries to detect and repair faulty proteins, and also destroy those are beyond repair rapidly (Fig. 1). In this surveillance system, the chaperones (comprising of members of the heat-shock proteins) represent the first line of defense in ensuring the correct folding and refolding of proteins (Liberek et al., 2008). When a native folding state could not be attained, the chaperones will direct the misfolded protein for proteolyic removal typically by the proteasome. Proteins that are destined for proteasome-mediated degradation are usually added a chain of ubiquitin via a reaction cascade that involves the ubiquitin-activating (E1), -conjugating (E2) and -ligating (E3) enzymes, whereby successive iso-peptide linkages are formed between the terminal residue (G76) of one ubiquitin molecule and a lysine (K) residue (most commonly K48) within another. The (G76-K48) polyubiquitinated substrate is then recognized by the 26S proteasome as a target for degradation (Pickart and Cohen, 2004). It is noteworthy to mention that although the G76-K48 chain linkage is the most common form of polyubiquitin, ubiquitin self-assembly can occur at any lysine residues within the molecule (at positions 6, 11, 27, 29, 33, 48 and 63)

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(Pickart, 2000; Peng et al., 2003). In addition, proteins can also be monoubiquitinated. Notably, both K63-linked polyubiquitination and monoubiquitination of proteins are not typically associated with proteasome-mediated degradation (Pickart, 2000; Peng et al., 2003).

Group	Components	Remarks
1	α-synuclein; Neurofilaments	Structural Elements
2	Agrin; 14-3-3; Synphilin-1; Tau	α-synuclein-binding proteins
3	Dorfin; GSK-3β; NUB1; Parkin; Pin1; SIAH-1	Synphilin-1-binding proteins
4	Ubiquitin; E1; UbcH7; TRAF6; TRIM9; Proteasome subunits; PA700; PA28; β-TrCP; Cullin-1; HDAC4; NEDD8; p38; p62 (Sequestosome 1); ROC1; UCHL1	UPS-related proteins
5	LC3; GABARAP; GATE-16; Glucocerebrosidase; NBR-1	Autophagosome-lysosome system
6	γ-tubulin; HDAC6; Peri-centrin	Aggresome-related proteins
7	DJ-1; CHIP; Clusterin/apolipoprotein J; DnaJB6; Heat Shock Proteins; Torsin A, SOD1 & 2; FOXO3a	Stress response-related proteins
8	CaMKII; Casein Kinase II; CDK5, G-Protein Coupled Receptor Kinase 5; LRRK2; PINK1; IκBα; NFκB; p35; phospho-lipase C-δ; Tissue Transglutaminase	Signal transduction-related proteins
9	MAP1B; MAP2; Sept4/H5	Cytoskeletal proteins
10	Cox IV; Cytochrome C; Omi/HtrA2	Mitochondria-related proteins
11	Cyclin B; Retinoblastoma Protein	Cell cycle proteins
12	Amyloid Precursor Protein; Calbindin; Choline Acetyltransferase; Chromogranin A; Synaptophysin; Synaptotagmin; Tyrosine Hydroxylase; VMAT2	Cytosolic Proteins
13	Complement Proteins; Immunoglobulin	Immune-related proteins

Table 1. Components of Lewy Body (Wakabayashi et al., 2012)

Whilst the coupling of chaperone and ubiquitin protein system (UPS) provides an efficient way for the cell to deal with protein misfolding, there are times when the capacity of these systems may be exceeded by the production of misfolded proteins (e.g. under conditions of cellular stress). In such cases, aggregation-prone proteins that failed to be degraded may be transported along microtubules in a retrograde fashion to the microtubule organizing center to form an "aggresome", a term originally coined by Johnston and Kopito more than a decade ago (Johnston et al., 1998). According to the model, aggresome formation represents a cellular response towards proteasome impairments and their localization to the juxta-nuclear region is to facilitate their capture by lysosomes and thereby their clearance by macroautophagy (hereafter referred to as autophagy). Consistent with this, aggregation-prone proteins often generate aggresome-like structures when ectopically expressed in cultured cells in the presence of proteasome inhibition (Wong et al., 2008). Moreover, several groups including ours have demonstrated that autophagy induction promotes the clearance of aggresomes whereas the reverse is true when the bulk degradation system is inhibited (Fortun et al., 2003; Iwata et al., 2005b; Opazo et al., 2008; Wong et al., 2008).

Together, the chaperone, ubiquitin-proteasome and autophagy systems thus function in synergism to effectively counterbalance the threat of protein misfolding and aggregation. Accordingly, aberrations in one or more of these systems would be expected to promote protein aggregation and inclusion body formation, as in the case of affected neurons in the PD brain where LBs occur.

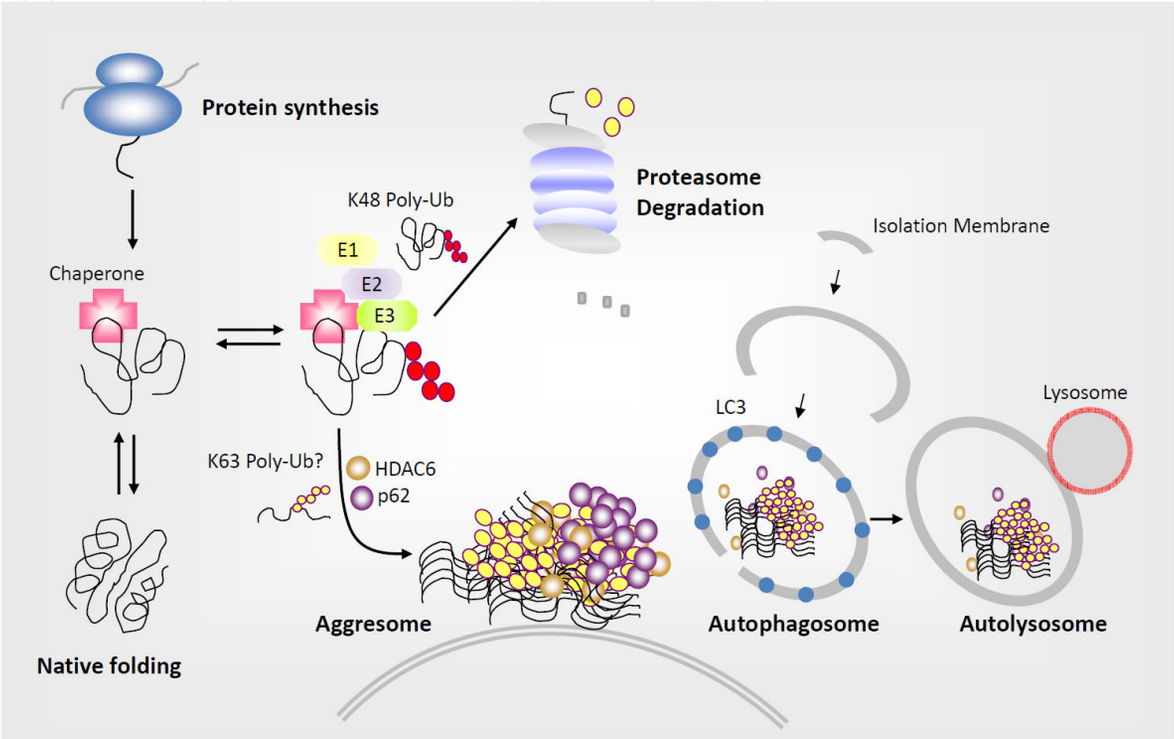


Figure 1. Schematic depiction of the collaboration among the chaperone, ubiquitin-proteasome and autophagy systems in the maintenance of intracellular protein homeostasis.

3. Biogenesis of Lewy bodies – An aggresome-related process reflecting failed autophagy?

As mentioned earlier, α-synuclein is a major component of LBs, suggesting that aberrant α-synuclein homeostasis contributes to the biogenesis of these inclusion bodies in the PD brain. The presynaptic terminal-enriched α-synuclein protein is an interesting molecule in that it is typically unfolded (or intrinsically disordered) in its native state, although the protein is extremely sensitive to its environment and can be moulded into an assortment of structurally unrelated conformations including a fibrillization-prone partially folded structure as well as various α-helical and β-sheet species occurring in both monomeric and oligomeric states (Uversky, 2007). Along with this conformation flexibility, α-synuclein also tends to misfold and becomes aggregated in the process. PD-associated mutations, including missense substitutions (A53T, A30P and E46K), duplication or triplication are

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known to enhance α-synuclein accumulation and aggregation (Giasson et al., 1999; Narhi et al., 1999; Conway et al., 2000; Uversky, 2007). Further, several groups have demonstrated in different experimental models that various exogenous neurotoxicants linked to PD, including pesticides, herbicides and metal ions, significantly accelerate the aggregation of α-synuclein (Manning-Bog et al., 2002; Uversky et al., 2002; Sherer et al., 2003). Not surprisingly, α-synuclein accumulation and aggregation can lead to impairments of the chaperone and UPS systems [For a recent review, see (Tan et al., 2009)]. Under such conditions, the isolation of α-synuclein aggregates into an aggresome would represent an alternative way by which the protein could be cleared, i.e. via autophagy. Indeed, emerging evidence suggest that LB biogenesis may be an aggresome-related process (Olanow et al., 2004). Because the protofibrillar, oligomeric forms of α-synuclein are thought to be more toxic than fibrillar, aggregated α-synuclein species, aggresome formation may also be regarded as a "protective" response that serves as a trap to immobilize soluble toxic forms of α-synuclein. However, this process has to be coupled to the active removal of the aggresomes by autophagy, as the unregulated growth of an inclusion body could conceivably affect cellular functions, physically or otherwise.

The relevance of aggresome formation to LB biogenesis in PD is exemplified by their striking similarities to each other in terms of structural organization, protein composition and intracellular localization (Olanow et al., 2004). For example, aggresome-related proteins such as γ-tubulin and HDAC6 can be found in LB (Table 1). HDAC6 plays an important role during aggresome formation by facilitating the retrograde transport of ubiquitinated misfolded proteins along the microtubule network to the γ-tubulin-positive MTOC by the dynein motor complex (Kawaguchi et al., 2003). Moreover, LBs are also immunopositive for p62 and NBR1, which are autophagy adapter proteins capable of binding to ubiquitinated substrates and the autophagosome protein LC3 (Bjorkoy et al., 2005; Pankiv et al., 2007; Kirkin et al., 2009). By virtue of this binding property, p62 and NBR1 may provide a link between aggresome-related proteins and their clearance by the autophagy machinery. Interestingly, all the three ubiquitin-binding autophagy receptors, i.e. p62, HDAC6 and NBR1, show preference for K63-linked polyubiquitin chains (Olzmann et al., 2007; Tan et al., 2008a; Kirkin et al., 2009), suggesting that this form of ubiquitin modification may underlie the formation as well as autophagic degradation of protein aggregates. Consistent with this, we found that K63-linked ubiquitination promotes the formation of inclusion bodies associated with PD and other neurodegenerative diseases and importantly, acts as a cargo selection signal for their subsequent removal by autophagy (Tan et al., 2008a; Tan et al., 2008b). As per our original proposal (Lim et al., 2006), it is tempting to think that the cell may switch to an alternative, proteasome-independent form of ubiquitination under conditions of proteasome-related stress that could help divert cargo proteins away from an otherwise overloaded proteasome. All these would culminate to the ultimate clearance of these proteins by autophagy (Fig. 1).

What remains curious about LB biogenesis is that it apparently takes place in the presence of constitutive autophagy, which is a characteristic of post-mitotic neurons (Wong and Cuervo, 2010). Moreover, α-synuclein is itself a substrate for autophagy (Webb et al., 2003).

Although α-synuclein can also be degraded by the proteasome, the aggregates of which appear to be preferentially cleared by the autophagy system (Petroi et al., 2012). Consistent with this, autophagy is recruited as the primary removal system in transgenic mice over-expressing oligomeric species of α-synuclein (Ebrahimi-Fakhari et al., 2011). Further, the protein can also be removed via chaperone-mediated autophagy (CMA), a specialized form of lysosomal degradation by which proteins containing a particular pentapeptide motif related to KFERQ are transported across the lysosomal membrane via the action of the integral membrane protein LAMP-2A and both cytosolic and lumenal hsc70 (Klionsky et al., 2011). Notably, the intralysosomal level of α-synuclein is significantly increased along with LAMP-2A and hsc70 in mice treated with the herbicide paraquat (which induces parkinsonism) or expressing α-synuclein as a transgene (Mak et al., 2010). Thus in theory, the level of α-synuclein, whether present as soluble or aggregated species, should be effectively managed in neurons under normal conditions or even when they are undergoing stress. Indeed, even in the PD brain, LB takes a significant length of time to develop. Given this, and that the autophagy system arguably represents the final line of cellular defense against the buildup of protein aggregates, the simplest explanation that could account for the presence of LB in PD is that the autophagy system has either become suboptimal in its function or is otherwise impaired altogether during the disease pathogenesis process.

4. Autophagy and PD

Morphological evidence of autophagic vacuole (AV) accumulation is certainly evident in PD as well as in several other neurodegenerative disorders (Anglade et al., 1997). However, whether the phenomenon represents attempts by the neuron to clean up its cobwebs of aggregated proteins, or a prelude to cell death, or simply a failure in AV consumption remains poorly understood. Notwithstanding this, two elegant studies conducted in 2006 aptly illustrated the importance of competent autophagy function to neuronal homeostasis (Hara et al., 2006; Komatsu et al., 2006). By means of targeted genetic disruption of essential components of the autophagy process (Atg5 or Atg7), these studies demonstrated that ablation of autophagy function in neural cells of mice results in extensive neurodegeneraion that is accompanied by widespread inclusion pathology, suggesting that autophagy failure can precipitate protein aggregation and subsequent cell death in affected neurons.

Supporting a role for failed autophagy in PD in the face of α-synuclein accumulation, α-synuclein was recently demonstrated to inhibit autophagy when over-expressed, both *in vitro* and *in vivo* (Winslow et al., 2010). The inhibition apparently occurs at a very early stage of autophagosome formation, which is likely a result of disrupted localization and mobilization of Atg9, a multi-spanning membrane protein whose associated vesicles are important sources of membranes for the synthesis of early autophagosomes (Yamamoto et al., 2012). Interesting, the reverse, i.e. autophagy enhancement, was observed when α-synuclein is depleted via RNAi-mediated knockdown (Winslow et al., 2010), suggesting that the protein might play a regulatory role in the synthesis of autophagosome. More-

over, targeted disruption of autophagy (via *Atg7* deletion) in midbrain dopaminergic neurons results in abnormal presynaptic accumulation of α-synuclein that is accompanied by dendritic and axonal dystrophy, reduced striatal DA content, and the formation of somatic and dendritic ubiquitinated inclusions (Friedman et al., 2012). Significant age-dependent loss of nigral dopaminergic neurons were also recorded in these *Atg7* conditionally knockout mice (*Atg7*-cKOTH), with 9 month old *Atg7*-cKOTH mice exhibiting about 40% reduction in the number of SN neurons that is accompanied by markedly decreased spontaneous motor activity and coordination relative to controls (Friedman et al., 2012). Together, these results suggest that failure in autophagy function precipitates inclusions formation in dopaminergic neurons that leads to their demise.

Besides macroautophagy, α-synuclein can also affect the function of CMA. For example, disease-associated α-synuclein mutants bind to the CMA lysosomal receptor with high affinity but are poorly translocated, resulting in the blockage of uptake and degradation of CMA substrates (Cuervo et al., 2004). The increase in cytosolic α-synuclein levels that ensued could favour its aggregation and concomitantly, amplify the burden of misfolded protein load for the cell. Interestingly, DA modification of α-synuclein also impairs CMA-mediated degradation by a similar mechanism (Martinez-Vicente et al., 2008). In this case, membrane-bound DA-α-synuclein monomers appear to seed the formation of oligomeric complexes, which consequently placed the translocation complex under siege. Consistent with this, CMA inhibition following L-DOPA treatment is more pronounced in ventral midbrain cultures containing dopaminergic neurons than in non-DA producing cortical neurons. Importantly, α-synuclein appears to be the principal mediator of DA-induced blockage of CMA, as ventral midbrain cultures derived from α-synuclein null mice are relatively spared from the inhibitory effects of DA on CMA (Martinez-Vicente et al., 2008). More recently, Malkus and Ischiropoulos demonstrated that CMA activity in the adult brain of A53T α-synuclein-expressing transgenic mice varies across different regions, with brain regions vulnerable to α-synuclein aggregation displaying marked deficiencies in CMA (Malkus and Ischiropoulos, 2012). Their results support an integral role for the lysosome in maintaining α-synuclein homeostasis and at the same time, provides an explanation to why certain brain regions are vulnerable to inclusion formation and cellular dysfunction while others are spared.

Perhaps the most direct evidence linking lysosomal dysfunction to PD is the demonstration that loss-of-function mutations in a gene encoding for the lysosomal P-type ATPase named ATP13A2 cause a juvenile and early-onset form of parkinsonism that is also characterized by pyramidal degeneration and dementia (Ramirez et al., 2006). In patient-derived fibroblasts as well as in ATP13A2-silenced primary mouse neurons, deficient ATPase function results in impaired lysosomal degradation capacity that concomitantly enhanced the accumulation and toxicity of α-synuclein (Usenovic et al., 2012). Importantly, silencing of endogenous α-synuclein ameliorated the toxicity in neurons depleted of ATP13A2, suggesting that ATP13A2-induced parkinsonism may be contributed by α-synuclein accumulation amid functional impairments of the lysosome. Supporting this, overexpression of wild type ATP13A2 suppresses α-synuclein-mediated toxicity in *C. elegans* while knockdown of ATP13A2 expression

promotes the accumulation of misfolded α-synuclein in the animal (Rappley et al., 2009). Together, these studies demonstrate a functional link between ATP13A2-related lysosomal dysfunction and α-synuclein in promoting neurodegeneration.

Besides *α-synuclein* and *ATP13A2*, several other PD-linked genes have also been associated directly or indirectly with the autophagic process. For example, emerging evidence suggest that mutations in LRRK2 promote dysregulation in autophagy, although the role of LRRK2 in controlling autophagy-lysosome pathway is likely to be complex (discussed further in section 6). In the case of parkin, which has the ability to promote K63-linked ubiquitination, we and others have shown that the ubiquitin ligase is involved in aggresome formation and thereby their removal via autophagy (at least indirectly) (Lim et al., 2005; Olzmann et al., 2007). Consistent with its role as an "aggresome-promoter", parkin-related cases are frequently (although not exclusively) devoid of classic LBs, as revealed by a number of autopsy studies (Takahashi et al., 1994; Mori et al., 1998; Hayashi et al., 2000). In recent years, the attention to parkin-autophagy axis has however shifted towards its ability to remove damaged mitochondria via a specialized form of autophagy known as "mitophagy", a term originally coined by Lemasters (Lemasters, 2005). Accordingly, impairment in mitochondrial quality control due to failed mitophagy in parkin-deficient neurons is now thought to be a key mechanism that predisposes them to degeneration.

5. Mitophagy and PD

A role for mitochondria dysfunction in the pathogenesis of PD has long been appreciated. Through post-mortem analysis performed as early as 1989, several groups have recorded a significant reduction in the activity of mitochondrial complex I as well as ubiquinone (co-enzyme Q10) in the SN of PD brains (Schapira et al., 1989; Shults et al., 1997; Keeney et al., 2006). Moreover, mitochondrial poisoning recapitulates PD features in humans and represents a popular strategy to model the disease in animals (Dauer and Przedborski, 2003). Similarly, impairment of mitochondrial homeostasis via genetic ablation of TFAM, a mitochondrial transcription factor, in dopaminergic neurons of mice (named MitoPark mouse) results in energy crisis and neurodegeneration (Sterky et al., 2011).

Rather than being solitary and static structures as depicted in many textbooks, mitochondria are now recognized to be dynamic and mobile organelles that constantly undergo membrane remodeling through repeated cycles of fusion and fission as well as regulated turnover via mitophagy. These processes help to maintain a steady pool of healthy mitochondrial essential for energy production and beyond (e.g. calcium homeostasis). Following the seminal discovery by Youle group that identified parkin as a key mammalian regulator of mitophagy (Narendra et al., 2008), intensive research is now focused on elucidating the precise mechanism underlying parkin-mediated mitophagy and whether impaired clearance of damaged mitochondria may trigger the demise of dopaminergic neurons in the PD brain.

Mechanistically, the picture regarding parkin-mediated mitophagy that has emerged thus far is depicted in Figure 2.

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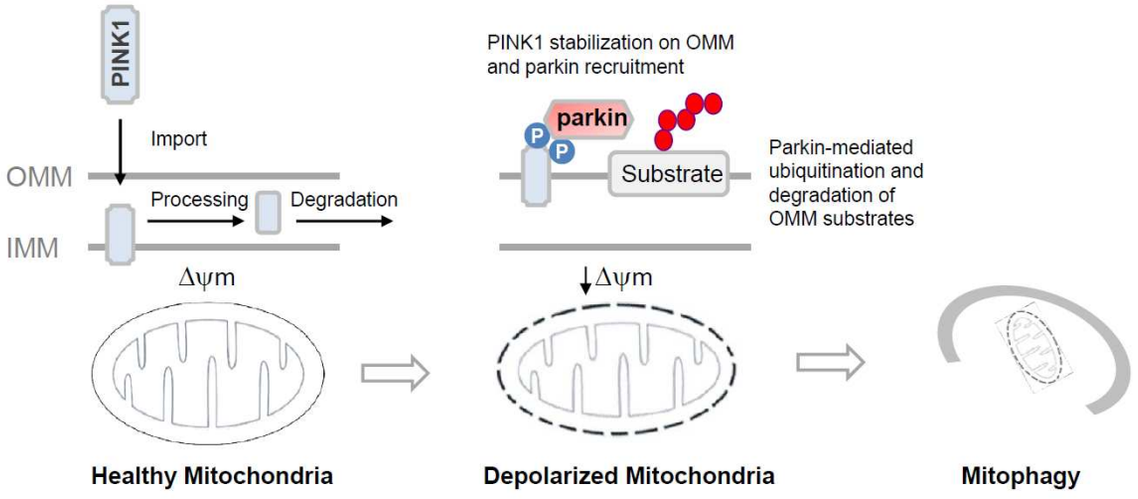


Figure 2. Model of Parkin/PINK1-mediated mitophagy

In this model, another PD-linked gene known as PINK1, which is a mitochondrial serine/threonine kinase, collaborate closely with parkin to bring about the mitophagy process. Briefly, a key initial event that occurs upon mitochondrial depolarization is the selective accumulation of PINK1 in the outer membrane of the damaged organelle. Normally, PINK1 accumulation in healthy mitochondria is prevented by the sequential proteolytic actions of mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like protease (PARL) that rapidly cleaves the protein to generate an unstable 53 kDa PINK1 species that is usually degraded by the proteasome or by an unknown "proteasome-like" protease (Becker et al., 2012; Greene et al., 2012). In depolarized mitochondria, PINK1 stabilization on the outer membrane enables the protein to recruit parkin to the organelle, a process that is apparently dependent on PINK1 autophosphorylation at Ser228 and Ser402 (Okatsu et al., 2012). Once recruited onto the mitochondria, parkin becomes activated and promotes the ubiquitination and subsequent degradation of many outer membrane proteins (Chan et al., 2011; Yoshii et al., 2011) including the pro-fusion mitofusin proteins (Poole et al., 2010; Ziviani et al., 2010), the elimination of which is thought to prevent unintended fusion events involving damaged mitochondria and thereby their re-entry into undamaged mitochondrial network from occurring. Mitophagy induction then occurs, which likely involves parkin-mediated K63 ubiquitination that will help recruit the autophagy adaptors HDAC6 and p62 that subsequently lead to mitochondrial clustering around the peri-nucleus region. By virtue of their association with the autophagy process, the concerted actions of p62 and HDAC6 will presumably facilitate the final removal of damaged mitochondria by the lysosome (Ding et al., 2010; Geisler et al., 2010; Lee et al., 2010). However, a recent study from Mizushima's lab revealed that the initial cargo recognition step of mitophagy does not involves the interaction between LC3 and the adaptor molecules. Rather, parkin recruitment on the mitochondria induces the formation of ULK1 (Atg1) puncta and Atg9 structures (Itakura et al., 2012). Because ULK1 complex functions as an essential upstream nucleation step of the hierachical autophagy cascade, their results suggest that mitophagosome is generated in a de novo fashion on

damaged mitochondria. Autophagosomal LC3 is however important for the efficient incorporation of damaged mitochondria into the autophagosome at a later stage. Notwithstanding this, how parkin participates in the de novo synthesis of isolation membrane awaits further clarifications. Interestingly, the whole mitophagy process bears striking resemblance to the formation and autophagic clearance of aggresomes. Indeed, we have termed the mitochondrial clustering phenomenon as (formation of) "mito-aggresomes" (Lee et al., 2010). Importantly, several groups, including ours, have demonstrated that PD-associated parkin mutants are defective in supporting mitophagy due to distinct problem at recognition, transportation or ubiquitination of impaired mitochondria (Lee et al., 2010; Matsuda et al., 2010), thereby implicating dysfunctional mitophagy in the development of parkin-related parkinsonism.

Given the pivotal role of parkin/PINK1 pathway in mitochondrial quality control, it is perhaps not surprising to note that deficiency in parkin or PINK1 function results in the accumulation of abnormal mitochondria in several parkin/PINK1-related PD models. This defect is perhaps most prominently observed in *Drosophila* parkin or PINK1 mutants, especially in their flight musculature, which is plagued by pronounced mitochondrial lesions and muscle degeneration (Greene et al., 2003; Clark et al., 2006; Park et al., 2006; Wang et al., 2007). Importantly, parkin over expression in pink1-/- flies significantly ameliorates all the mutant phenotypes, although the reverse, does not happen, i.e. pink1 over expression in parkin null flies does not compensate for the loss of parkin function. These results suggest that parkin acts in the same pathway but downstream of pink1 (Clark et al., 2006; Park et al., 2006). The hierachy is consistent with the proposed model of parkin/PINK1 pathway in the regulation of mitochondrial quality control, although parkin in this case can apparently do the job in the complete absence of pink1. Notably, several other studies also suggested that mitophagy can take place in a PINK1-deficient background (Dagda et al., 2009; Cui et al., 2010; Dagda et al., 2011). Conversely, Seibler and colleagues found PINK1 to be essential for parkin-mediated mitophagy. They demonstrated that parkin recruitment to depolarized mitochondria is impaired in human dopaminergic neurons derived via the induced pluripotent stem cells route from PINK1-related PD patients, a defect that can be rescued by the re-introduction of wild-type PINK1 into PINK1-deficient neurons (Seibler et al., 2011).

As with the case with virtually all the biological models initially proposed, the parkin/PINK1 mitophagy model is currently less than perfect and clearly needs be continually updated with each new piece of significant data. The relevance of mitophagy to sporadic PD is also debatable, although we and others have previously shown that parkin dysfunction (presumably triggering mitophagy deficiency) may also underlie the pathogenesis of sporadic PD (Pawlyk et al., 2003; LaVoie et al., 2005; Wang et al., 2005a). Perhaps one of most challenging tasks at hand is to demonstrate unequivocally that mitophagy impairment, instead of a generalized impairment in the autophagy process, contributes directly to neurodegeneration *in vivo*. This would require the genetic differentiation of targeted components that are exclusively involved in mitophagy. Currently, key components of mitophagy and autophagy tend to overlap. Even parkin appear to subserve both types of autophagy processes (and more). Thus, although mitochondrial quality control is invariably important for neuronal survival, whether failure in the removal of damaged mitochondria is in itself a driver of disease pathogenesis or is a

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consequence of a progressive and general decline in autophagy function in the PD brain remains to be clarified.

6. Autophagy induction as therapeutic strategy for PD?

If failure in autophagy function were to underlie PD pathogenesis, it follows intuitively that stimulation of autophagy in the PD brain might be beneficial for the patient. Indeed, work from Rubinsztein lab and others have demonstrated that autophagy enhancement promotes beneficial outcomes in several experimental models of PD, supporting that such an approach could represent a viable therapeutic strategy (Rubinsztein et al., 2012).

Notably, most neurodegenerative disease-associated proteins, including α-synuclein, that are prone to aggregation are substrates of autophagy. Accordingly, pharmacological or genetic enhancement of autophagy can in theory help remove these aggregation-prone proteins and concomitantly reduce their associated toxicity. Rapamycin, an inducer of mTOR (mammalian Target of Rapamycin), is widely established to be a potent autophagy inducer. Expectedly, rapamycin treatment of cellular or animal models of α-synucleinopathies reduces the levels of both soluble and aggregated species of α-synuclein in an autophagy-dependent manner (Crews et al., 2010). Similarly, trehalose also accelerates the clearance of α-synuclein by means of its ability to induce autophagy, albeit in an mTOR-independent manner (Sarkar et al., 2007). Further, trehalose-treated cells are protected against subsequent pro-apoptotic insults. Together, trehalose and rapamycin exert an additive effect in the clearance of aggregate-prone proteins (Sarkar et al., 2007). Perhaps unsurprisingly, rapamycin can also rescue failed mitophagy in parkin deficient cells and result in improved mitochondrial function (Siddiqui et al., 2012), suggesting that generalized autophagy activation can help clean up all the cellular "cobwebs" be it protein aggregates or damaged organelles. More recently, Steele and colleagues showed that latrepirdine, a neuroactive compound associated with enhanced cognition and neuroprotection, also stimulates the degradation of α-synuclein and concomitantly protects against α-synuclein-induced toxicity in 3 model systems: yeast, differentiated SH-SY5Y cells and wild type mouse (Steele et al., 2012). The beneficial effects of latrepirdine again appear to be related to autophagy induction, as evident by the elevation of several autophagy markers in mouse brain following chronic administration of the compound. Using a genetic approach, Spencer and colleagues demonstrated via lentivirus-mediated gene transfer of beclin 1, a key promoter of autophagy, that genetic enhancement of autophagy in α-synuclein overexpressing mice ameliorates the synaptic and dendritic pathology in these transgenic animals and reduces the accumulation of the protein *in vivo* (Spencer et al., 2009). Taken together, these studies support the therapeutic applications of autophagy induction in PD, particularly in preventing the accumulation of α-synuclein.

Notwithstanding the above promising findings regarding the protective effects of autophagy induction, it is important to recognize that autophagy induction is a "double-edge sword" that can cut both ways, i.e. being protective or pro-death under different conditions. One therefore have to consider this caveat in considering autophagy induction as a

therapeutic strategy for PD. Notably, the parkinsonian neurotoxin MPP+ that induces selective loss of dopaminergic neurons has been demonstrated by several groups to activate autophagy (Zhu et al., 2007; Xilouri et al., 2009; Wong et al., 2011), a process that appears to act through the dephosphorylation of LC3 (which enhances its recruitment into autophagosomes) (Cherra et al., 2010) and/or CDK5-mediated phosphorylation of endophilin B1 (which promotes its dimerization and recruitment of the UVRAG/Beclin 1 complex to induce autophagy) (Wong et al., 2011). In this case, autophagy induction is apparently harmful to dopaminergic neurons. Moreover, stimulation of autophagy also contributes to neuronal death induced by overexpression of α-synuclein (Xilouri et al., 2009). Conversely, inhibition of autophagy pharmacologically with 3-methylalanine (3-MA) or genetically via Atg5 or Atg12 gene silencing significantly attenuates neuronal loss associated with MPP + treatment or mutant α-synuclein expression, as is the case with knockdown of CDK5 or endophilin B1 (Wong et al., 2011). Along these lines, we found that mutant α-synuclein-associated toxicity is aggravated by the accumulation of iron, which act together to trigger autophagic cell death. The toxicity that α-synuclein-iron elicits can be ameliorated by pharmacological inhibition of autophagy (Chew et al., 2011). Interestingly, autophagy activation elicited by mutant α-synuclein overexpression can also result in excessive mitophagy and thereby unintended loss of mitochondria, which in turn promotes bioenergetics deficit and neuronal degeneration (Choubey et al., 2011). Further supporting a "pathological" role for autophagy, loss of DJ-1 function associated with recessive parkinsonism has been found to increase (instead of decrease) autophagic flux, although it is currently unclear how this relates to neuronal death in the context of DJ-1 deficiency (Irrcher et al., 2010).

Finally, mutations in LRRK2, which currently represent the most prevalent genetic contributor to PD, are also implicated in aberrant autophagy induction. For example, transgenic mice expressing disease-associated LRRK2 mutants (R1441C and G2019S) frequently exhibit increased incidence of autophagic vacuoles in their brain (Ramonet et al., 2011). Similarly, cells expressing G2019S LRRK2 mutant show increase autophagosome content and autophagy-dependent shortening of neurites (Plowey et al., 2008). Conversely, ablation of LRRK2 in mice promotes impairment of the autophagy pathway as evident by the accumulation of p62, lipofuscin granules, ubiquitinated proteins and α-synuclein-positive inclusions in their kidneys (Tong et al., 2010). The relationship between LRRK2 and autophagy is however complicated. For example, Gomez-Suaga and colleagues have recently demonstrated that LRRK2-induced accumulation of autophagosome is related to the ability of the kinase to activate a calcium-dependent protein kinase kinase-beta (CaMKK-beta)/adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway via modulation of NAADP-dependent Ca2+ channel on lysosomal membrane (Gomez-Suaga et al., 2012). However, they also detected at the same time a reduction in the acidification of lysosomes that can compromise autophagosome turnover and thereby autophagy (Gomez-Suaga et al., 2012), suggesting that autophagy is actually impaired rather than activated in LRRK2-expressing cells. Consistent with this, another study revealed that the expression of LRRK2 R1441C mutant leads to impaired autophagic balance that is characterized by AV accumulation containing incompletely degraded materials and increased levels of p62 (Alegre-Abarrategui et al., 2009).

Accordingly, siRNA-mediated knockdown of LRRK2 expression results in increased autophagic activity and prevented cell death caused by inhibition of autophagy in starvation conditions. Thus, the precise role of autophagy in LRRK2-related parkinsonism is anybody's guess at this moment, begging again caution in the proposed use of autophagy inducers as a therapeutic recourse.

In a related development, we recently found that disease-associated LRRK2 G2019S mutant can trigger marked mitochondrial abnormalities when overexpressed in *Drosophila*, a phenotype that can be rescued by parkin co-expression (Ng et al., 2012). Given the role of parkin in promoting mitophagy, it is tempting to speculate that the LRRK2 mutant may retard the clearance of damaged mitochondria via mitophagy in the absence of parkin overexpression. Indeed, the mitochondrial phenotype LRRK2 G2019S mutant induces in the flight muscle is reminiscent of that brought about by the loss of parkin function. Alternatively, this could also be a result of LRRK2-induced impairment in autophagy in general. Importantly, we further found that pharmacological or genetic activation of AMPK can effectively compensate for parkin deficiency to bring about a significant suppression of dopaminergic and mitochondrial dysfunction in mutant LRRK2 flies (Ng et al., 2012). Our results suggest a neuroprotective role for AMPK that might be related to mitophagy/autophagy modulation. AMPK is an evolutionarily conserved cellular energy sensor that is activated by ATP depletion or glucose starvation (Hardie, 2011). When activated, AMPK switches the cell from an anabolic to a catabolic mode and in so doing, helps to regulate diverse cellular processes that impact on cellular energy demands. Interestingly, like parkin, AMPK can also regulate mitophagy and also autophagy through its ability to phosphorylate the autophagy initiator ATG1 (Egan et al., 2011; Kim et al., 2011). Lending relevance to our findings, a recent report demonstrated that AMPK is activated in mice treated with MPTP and that inhibition of AMPK function by compound C enhances MPP(+)-induced cell death (Choi et al., 2010). More recently, a PD cohort-based study revealed that Metformin-inclusive sulfonylurea therapy reduces the risk for the disease occurring with Type 2 diabetes in a Taiwanese population (Wahlqvist et al., 2012). Metformin is a direct activator of AMPK. Together, these findings suggest that AMPK activation may protect against the development of PD, presumably via its ability to maintain energy balance via the modulation of autophagy as well as a range of other cellular processes. Given that caveats associated with direct autophagy induction, and that excessive autophagy can result in energy crisis especially in the aged brain, perhaps AMPK activation, through its ability to maintain both protein and energy homeostasis, would represent a better approach than direct autophagy induction as a therapeutic strategy for PD.

7. Conclusion

In essence, the case for autophagy dysfunction as a contributor to the pathogenesis of PD is rather compelling. As evident from the above discussion, virtually all the major PD-associated gene products have some direct or indirect relationship with the autophagy-lysosome axis. What is less clear is whether autophagy induction is neuroprotective or is a key driver of neurodegeneration. One can envisage that the activation of autophagy may be beneficial in

the short term (particularly when the induction is transient and timely), but deleterious when it is becomes chronic or excessive. Finding the tipping autophagy threshold point between neuroprotection and neurodegeneration would therefore be an important endeavour, the clarification of which has important implications for the future development of autophagy-related therapeutics for the PD patient.

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Author details

Grace G.Y. Lim[1], Chengwu Zhang[2] and Kah-Leong Lim[1,2,3]

1 Department of Physiology, National University of Singapore, Singapore

2 National Neuroscience Institute, Singapore

3 Duke-NUS Graduate Medical School, Singapore

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