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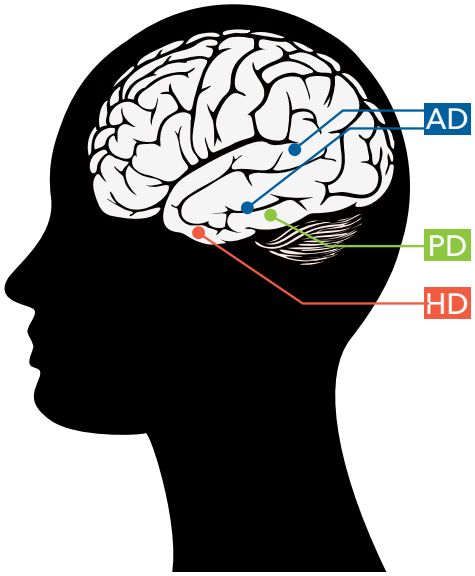
WHITE PAPER

AUTOPHAGY IN NEURODEGENERATIVE DISEASES:

AT THE INTERSECTION OF CELLULAR HOMEOSTASIS AND DISEASE



Neurodegenerative diseases affect neuronal function in various brain regions. Alzheimer's affects several brain regions, but the hippocampus is the first region impacted, resulting in short-term memory loss. In Huntington's disease, the basal ganglia, involved in organizing motor movements, is the brain region first affected. Lastly, Parkinson's disease affects primarily dopaminergic neurons in the substantia nigra leading to loss of controlled-body movements.

ABSTRACT

Neurodegenerative diseases (NDDs) differ substantially in their effects on various neuronal populations and brain regions, however, the accumulation of intracellular, pathogenic protein aggregates and the progressive loss of cellular function due to neuronal synaptic impairment seem to be common to all NDDs. Although the molecular nature of the specific pathogenic proteins differs among NDDs, each condition seems to be associated with the chronic accumulation of vesicular organelles within affected neurons. It has therefore been hypothesized that dysfunctions in autophagy are involved in the accumulation of proteins and damaged organelles in NDDs such as Alzheimer's, Huntington's and Parkinson's Diseases. In this Review, we focus on dysfunctional autophagy in NDDs and the key molecular factors that link autophagy to neurodegeneration.

INTRODUCTION

Neurodegenerative diseases (NDDs) encompass a broad spectrum of conditions classified as proteinopathies, which are characterized by the presence of aggregated proteins in specific neurons and glia within the central nervous system (CNS).[1,2] These diseases differ in the specific cells and regions of the brain that they affect as well as in the key molecular factors or proteins involved in their pathogenesis. Commonly, aggregates contain abnormally processed proteins with altered biophysical properties, which result in improper neuronal function and eventual cell death. In most NDDs, protein aggregates are localized to specific subcellular compartments, however, extracellular aggregates may also be present, as exemplified by the deposition of amyloid-β (Aβ) plaques in Alzheimer's Disease (AD).

Although abnormal protein processing is the archetypal dysfunction associated with NDDs, other factors contributing to the overall pathology include mitochondrial malfunction, oxidative stress and other environmental stressors.[2,3] A multifactorial etiology is a common trait of sporadic NDDs as opposed to early onset or familial NDDs, which may be more clearly associated with specific genetic mutations.[4] Therefore, to better understand the mechanisms underscoring the development of sporadic NDDs, recent studies have increasingly focused on the pathogenic role of dysfunction in specific pathways controlling cellular death and survival decisions as well as organelle and protein homeostasis.[4]

1. Apoptosis
2. Autophagy
3. Mitochondrial quality control
4. Ubiquitin-proteasome system

Among these pathways, autophagy represents the principal cellular mechanism involved in the removal of aggregated proteins and thus is expected to play a significant role in NDDs.

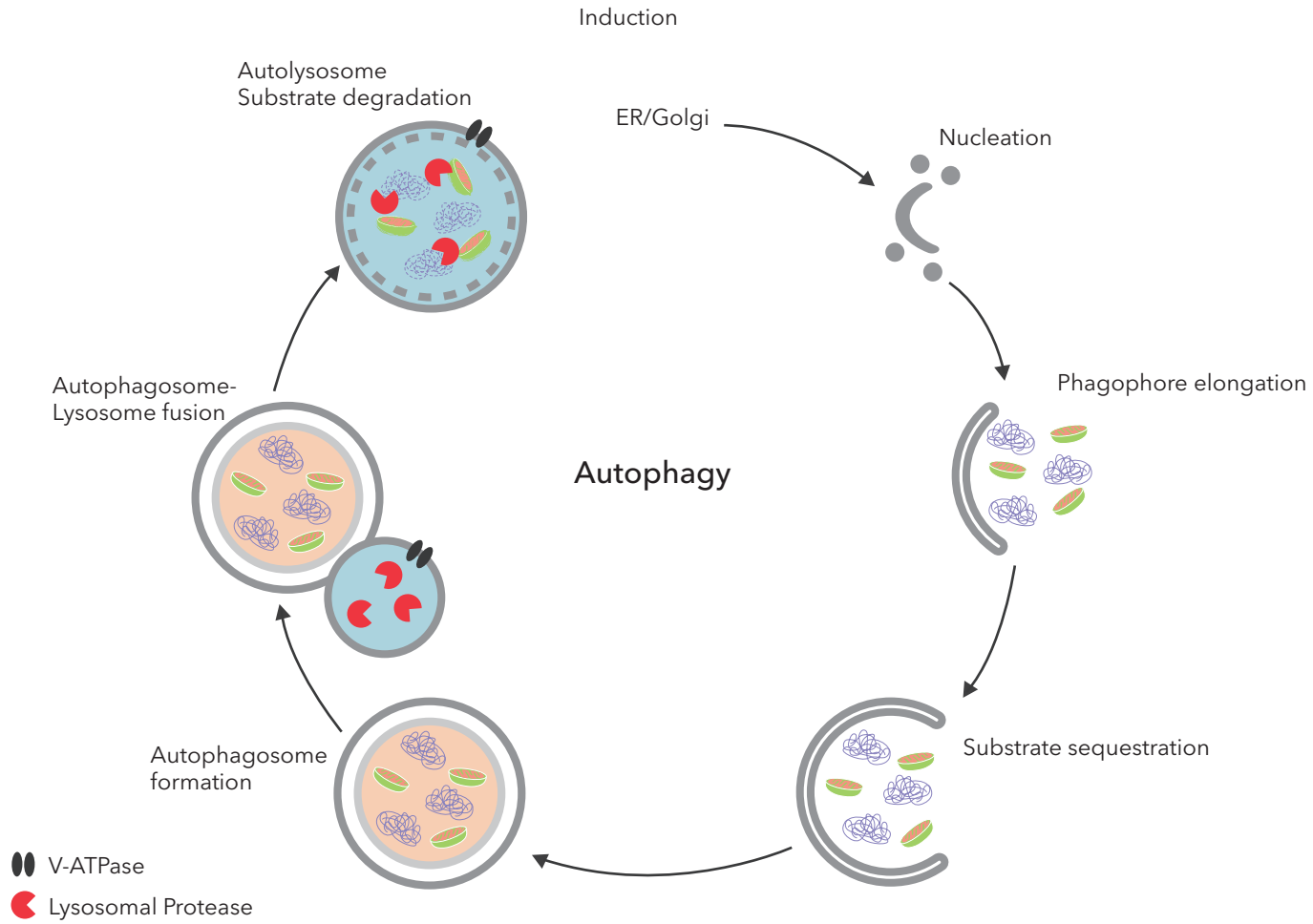


FIGURE 1. Autophagy depends on the formation of the autophagosome for the sequestration of cytosolic content including damaged or unwanted proteins and organelles. **Induction** and **Nucleation** are mediated by the ATG/ULK1 and the Class III PI3-K complex, respectively. Addition of membrane during the process of **Phagophore elongation** depends on two ubiquitin like conjugation systems including (1) ATG7/ATG10 and (2) ATG7/ATG3. ATG4 and ATG7/ATG3 are critical for the lipidation of LC3-I to LC3-II. Association of LC3-II to the forming autophagosome allows sequestration of cytoplasmic content. **Fusion** of the mature autophagosome with lysosomes, a process mediated by SNARE proteins, forms the autophagolysosome where cytosolic cargo is hydrolyzed.

Autophagy is the process by which cells eliminate damaged organelles and proteins via the formation of a double-membraned vesicular engulfing organelle termed the autophagosome (**FIGURE 1**). For a comprehensive overview of the signaling molecules involved in autophagy and mechanisms regulating this process refer to: www.novusbio.com/support/autophagy-handbook. Briefly, following the sequestration of excess, long-lived or damaged cytoplasmic contents by the autophagosome, fusion with the lysosome results in the formation of the autophagolysosome, a proteolytic and hydrolytic compartment in which the cytoplasmic content is degraded. Degradation within the autophagolysosome allows the recycling of basic cellular components back into the cytoplasm to serve as an energy source or for the synthesis of new proteins and organelles.

The process of autophagy may occur in bulk, however, selective autophagy pathways, which use autophagy receptors to bind and sequester specific cargo proteins, have also been identified and are thought to play a significant role in protein homeostasis. As a protein quality control mechanism, autophagy is thought to protect neurons from the accumulation of toxic protein aggregates that otherwise would lead to neuronal degeneration and eventual cell death.

AUTOPHAGY IN NEURONS

Autophagy plays a critical role in cellular homeostasis and is constitutively active at a basal level in every cell. For neurons, efficient basal autophagic activity is a requisite due to their unique physiological and morphological attributes.[5,6,7]

1. **Neurons are terminally-differentiated (non-dividing) cells: cytoplasmic remodeling through cell division does not occur in these cells and thus neurons are solely dependent on autophagy to eliminate large protein aggregates and defective organelles.[8]**

2. **Neurons have a complex morphology: they have several compartments including soma, axon and dendrites, and require precise regulation of specific protein pathways to maintain axonal protein trafficking as well as the highly energetic process of synaptic transmission.[5]**

Early studies demonstrated that the regulation of autophagy differs in neuronal and non-neuronal cells. For example, scarce autophagosome numbers were found in healthy brains, suggesting low basal autophagy or that autophagosomes are quickly turned over in neurons.[7,9,10] Moreover, the typical

increase in autophagosomes observed in non-neuronal cells following starvation was absent in neurons, suggesting a lack of responsiveness to nutrient deprivation.[9,11] More recently, it was demonstrated that rapamycin and everolimus–inhibitors of the cell growth and metabolic regulatory kinase, mTOR, induce autophagy in non-neuronal cells as evidenced by an increase in autophagosomes, but not in primary neurons.[12] Moreover, lithium chloride, a known inducer of autophagy in non-neuronal cells, failed to elicit an increase in autophagosomes in primary neurons.[12] Together these findings indicate that neurons differ from non-neuronal cells in both mTOR-dependent and -independent autophagy inducing pathways.[12] However, autophagy is inducible in neurons as treatment with niguldipine, trifluoroperazine and loperamide effectively increased the number of autophagosomes.[12]

Studies with primary cortical neurons revealed that neuronal basal autophagy is both efficient and relatively robust.[7] Under normal conditions, cortical neurons were found to have a negligible number of autophagosomes, in agreement with earlier studies.[9,11] Following inhibition of lysosomal degradation, accumulation of autophagosomes occurred in primary cortical neurons, supporting the presence of relatively high basal autophagic activity in these neurons.[7] However, it has been proposed that the level of basal autophagy is not inheritably high in all neurons, instead basal autophagy levels vary throughout the CNS and high autophagy levels appear to be required by neurons exposed to high levels of stress.[13]

EARLY CONNECTION BETWEEN AUTOPHAGY DEFICITS AND NEURODEGENERATION

One of the initial findings linking autophagy with NDDs came from electron microscopy analysis of neocortex tissue, from the biopsies of AD patients and non-AD control brains.[10] The accumulation of autophagosomes in conjunction with the presence of dystrophic neurites led to the proposal that autophagy may be dysfunctional in the brains of AD patients.[10] Whether the accumulation of autophagic organelles occurred as the result of aberrant autophagy induction or due to the inhibition of autophagy via impaired lysosomal degradation remains inconclusive.

Studies with primary cortical neurons revealed that interruption of autophagic flux results in changes that closely resemble the accumulation of autophagic organelles and the neurite dystrophy associated with AD.[7] These findings agreed with previous observations from *in vivo* mouse models where the expression of *Atg5* or *Atg7*, required for autophagosome formation, was selectively ablated from Purkinje neurons.[11,14] In these studies the absence of either ATG5 or ATG7 proteins led to the accumulation of abnormal membranous structures, triggered axonal morphology changes characterized by swelling and dystrophy, and ultimately resulted in the degeneration of Purkinje cells.[11,14] Overall, these studies demonstrated that basal autophagy plays a critical role for axon homeostasis in neurons.

This stereotypical accumulation of autophagosomes is associated with various NDDs including Huntington's (HD), Parkinson's (PD) and AD.[5,7,10] A standing question in the field is whether the role of the elevated number of autophagosomes in NDDs aims at resolving the pathogenesis or rather contributes to neurodegeneration.[5,8,11,13]

SELECTIVE AUTOPHAGY AND NEURODEGENERATION

Selective autophagy pathways target specific cytosolic components for degradation by the autophagolysosome. Specificity for cytosolic cargo (e.g., mitochondria, endoplasmic reticulum and aggregated proteins) partly depends on selective autophagy receptors that bind directly or via ubiquitin to the cytosolic components.[15] These receptors also bind microtubule-associated light chain 3 (LC3), a protein that incorporates into the autophagosome membrane, thereby sequestering the cytosolic cargo into the lumen of the autophagosome. Additionally, selective autophagy receptors can interact with adaptor proteins which modulate the receptor-cargo interactions as well as the binding of receptors to lipidated LC3 within autophagosomes.[16] Post-translational modifications of receptors and LC3 further modulate the overall selectivity for cytosolic cargo and influence degradation efficiency.[16]

Autophagy receptors can be found within aggregates together with other proteins implicated in NDDs. Recently, various mutations in selective autophagy receptors have been associated with NDDs.[16] Here we review findings that have helped to establish a connection between various neurodegenerative conditions including AD, HD and PD with dysfunctions in critical steps of autophagy.

ALZHEIMER'S DISEASE (AD)

Late onset AD represents the most common NDD in older adults that leads to dementia due to significant neuronal loss and synaptic impairment.[16,17] As a proteinopathy, AD's pathology is underscored by the accumulation of both extra- and intra-cellular protein products. Amyloid plaques form extracellularly, near affected neurons, from the deposition of amyloid-β (Aβ) fragments produced by the cleavage of the amyloid precursor protein (APP).[1,16,17] Intracellularly, neurofibrillary tangles accumulate consisting of hyperphosphorylated Tau protein aggregates.[1,16,18]

Findings in animal models and patients support the involvement of dysfunctional protein processing in the pathogenesis of AD.[10] Various proteins involved in the pathology of AD have functions that intersect with the autophagy pathway. For example, presenilins (PS) are part of the γ-secretase complex and are involved in the cleavage of APP, however they are also needed for the acidification and proper function of lysosomes, thus impacting autophagy (**FIGURE 2**).[1] Presenilin mutations, particularly mutations in *PS1*, have been linked to early onset AD.[1,19] Mutations in both genes, *PS1* and *PS2*, are linked to familial autosomal dominant AD.[1,20]

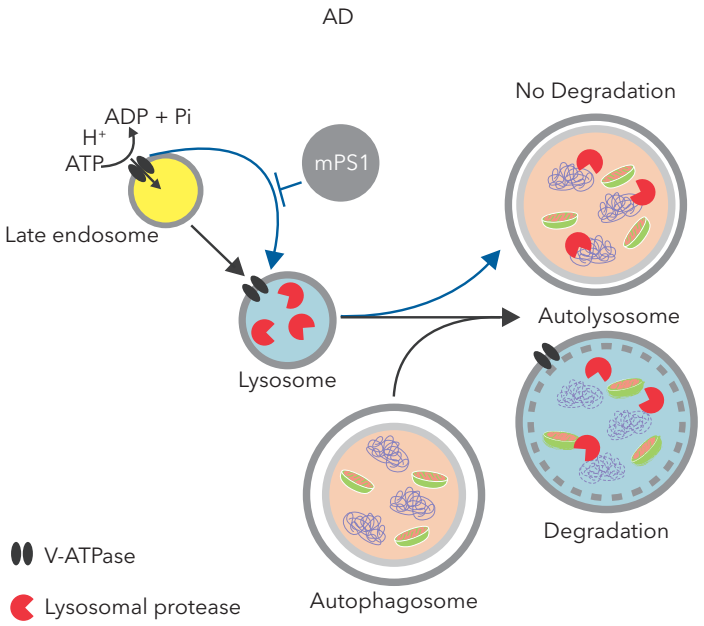


FIGURE 2. Mutations in Presenilins 1 and 2 (PS1 and PS2) have been implicated in the pathogenesis of AD. PS1 is involved in the glycosylation and trafficking of the lysosomal H+-ATPase (v-ATPase). Mutant PS1 (mPS1) affects these functions leading to impaired lysosomal acidification and reduced autophagic degradation.

Recently, genome-wide association studies (GWAS) linked phosphatidylinositol-binding clathrin assembly protein (PICALM), involved in endocytosis, with increased AD risk.[21] PICALM was shown to play a role in autophagy and the clearance of Tau, a known autophagy target.[21] In AD brains, PICALM was found to be truncated or at decreased levels, suggesting that loss of function of PICALM may be associated with increased AD risk.[22] In agreement with these findings, *in vitro* and *in vivo* studies demonstrated that PICALM plays various roles in autophagy including phagophore elongation, autophagosome formation and autophagosome-lysosome fusion.[21]

The mammalian target of rapamaycin (mTOR) regulates autophagy through its role as a nutrient sensor.[23] Inhibition of mTOR activity, by rapamycin or starvation, increases autophagic activity.[23] Altered mTOR activity has been previously reported in AD where increased mTOR activity *in vitro* and in an *in vivo* animal model of AD correlated with the accumulation of Aβ.[24] Previous studies in human AD brains, established a similar correlation between mTOR hyperactivity and Tau hyperphosphorylation.[25,26,27] Additionally, pharmacological inhibition of mTOR activity attenuates the cognitive decline associated with Aβ accumulation in an animal model of AD.[24,25]

Lastly, the autophagy receptor p62/SQSTM1 targets multiple cytosolic components for degradation via autophagy including mitochondria, peroxisomes and protein aggregates.[15] In the AD brain, phosphorylated p62/SQSTM1 is found at abnormal levels and associated with neurofibrillary tangles.[19,28] p62/SQSTM1 is thought to play a role in the clearance of hyperphosphorylated Tau and Aβ.

HUNTINGTON'S DISEASE (HD)

HD is a neurodegenerative polyglutamine disorder associated with mutant forms of the protein huntingtin (Htt). Neurodegeneration in polyglutamine disorders is characterized by the presence of proteins with abnormally expanded polyglutamine tracts (polyQ) and their associated intracellular aggregates. Although Htt is broadly expressed, the presence of the mutant form of Htt (mHtt), with over 35 glutamine residues (>Q35), primarily affects neuronal populations in the striatum and cortex.[16,29] HD pathology is associated with the accumulation of toxic amino terminal fragments containing polyQ extensions, which are produced through the cleavage of mHtt by caspases and calpains.[30-33]

Dysfunctional autophagy is implicated in HD pathogenesis, as evidenced by the accumulation of autophagic vesicles in neurons of postmortem brain specimens.[5,29,34] Htt is a substrate of autophagy and functions as a scaffold for autophagy signaling through direct interaction with p62/SQSTM1 and the autophagy-initiating kinase, ULK1.[35,36] Aggregates of mHtt are degraded by autophagy and are known to sequester Beclin1, a key autophagy initiator, and mTOR (**FIGURE 3**).[29,37,38] In some HD models, accumulation of mHtt has been linked with defects in autophagosome-lysosome fusion.[39,41] However, treatment with rapamycin reduces mHtt aggregates and neuronal death in HD animal models[5,37,40], which demonstrates that autophagy can be induced and suggests that downstream dysfunction in autophagolysosome formation and maturation do not underlie HD pathology.[29] Additionally, in HD animal models, autophagosomes form properly, but are devoid of cargo, suggesting that inefficient targeting of cytosolic components to the autophagosome may cause impaired clearance of protein aggregates.[29,39]

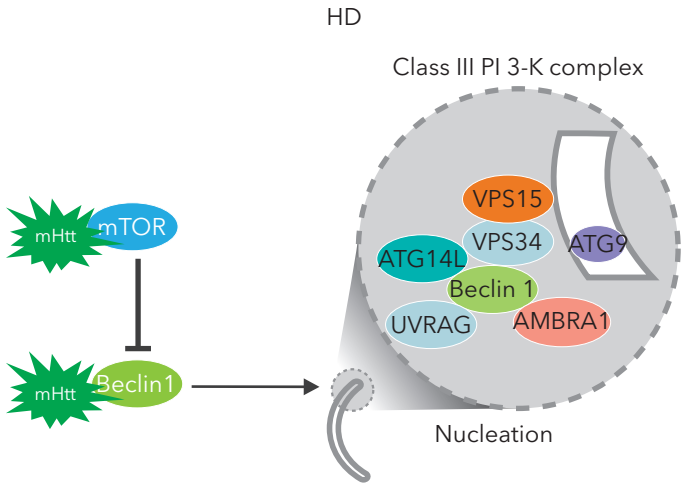


FIGURE 3. Upon induction of autophagy, nucleation occurs following the release of Beclin1 from Bcl-2 inhibition and the formation of the class III phosphatidylinositol 3-kinase (Class III PI3-K) complex. This complex is involved in the production of phosphatidylinositol 3-kinase and the targeting of ATG proteins to the forming membrane to give rise to the phagophore. The mutant form of Htt (mHtt) with expanded polyQ repeats forms toxic intraneuronal aggregates that sequester and inactivate mTOR, a regulator of autophagy, resulting in the induction of autophagy. In contrast, mHtt aggregates sequester Beclin1, leading to nucleation impairment.

The selective autophagy receptor p62/SQSTM1 and the adaptor, autophagy-linked FYVE protein (ALFY), play important roles in the degradation of mHtt aggregates.[39,42] The expression of *ALFY* mRNA is reduced in the brains of HD patients.[43] Additionally, the expression of mHtt increases the interaction between p62/SQSTM1 and ULK1 and results in increased phosphorylation of p62/SQSTM1 within its ubiquitin binding domain (UBD) at a serine residue (S409).[44,45] Phosphorylation of p62/SQSTM1 at S409 enhances the binding affinity of p62/SQSTM1 for ubiquitin and improves the degradation of protein aggregates including those with expanded polyQ repeats, which shows that autophagy is induced in response to mHtt expression.[16,44]

Recently, polyQ containing proteins including Htt and ataxin3 were shown to interact with Beclin1, a major regulator of the autophagy-promoting class III phosphatidylinositol 3-kinase complex.[46] The polyQ extension in Htt is required for interaction with Beclin1 and longer polyQ extensions have increased affinity for Beclin1.[46] Expression of mHtt (Q111) in striatal cells causes a reduction in Beclin1 expression and, moreover, transgenic mice expressing mHtt show decreased Beclin1 expression and impairments in starvation-induced autophagy.[46] These defects are present in young mice even before substantial accumulation of mHtt aggregates, which implicates dysfunctional autophagy as an underlying mechanism of HD pathology.

PARKINSON'S DISEASE (PD)

PD is characterized by the loss of midbrain dopaminergic neurons, specifically in the substantia nigra.[5,16,29] Like other NDDs, substantial accumulation of intracytoplasmic protein aggregates occurs in affected neurons. In PD, the protein α-synuclein accumulates within dopaminergic neurons forming inclusions or Lewy bodies.[16,29] Another pathological feature of PD is the accumulation of damaged mitochondria, which is thought to trigger neuronal death.[5] Neuronal death in PD occurs via apoptosis and necrosis mechanisms.[29,47]

The main protein implicated in PD pathogenesis, α-synuclein, is targeted for degradation by chaperone-mediated autophagy (CMA) and macroautophagy.[5,48-50] CMA represents the main mechanism for removal of α-synuclein, however mutant forms of α-synuclein (e.g., A53T and A30P) impair CMA function by outcompeting wild type α-synuclein for binding to chaperone molecules and thus leading to the formation of Lewy bodies.[5,17,49,51] Blockade of CMA is thought to engage macroautophagy for the removal of mutant α-synuclein leading to the accumulation of autophagic organelles within dopaminergic neurons.[5,17,52]

Some cases of familial PD have been associated with α-synuclein gene duplication and increased expression of α-synuclein both *in vitro* and *in vivo* leads to decreased macroautophagy.[5,53] The mechanism underscoring this effect pertains to the mislocalization of ATG9, a transmembrane protein involved in the formation of the initial membrane precursor to the phagophore.[5]

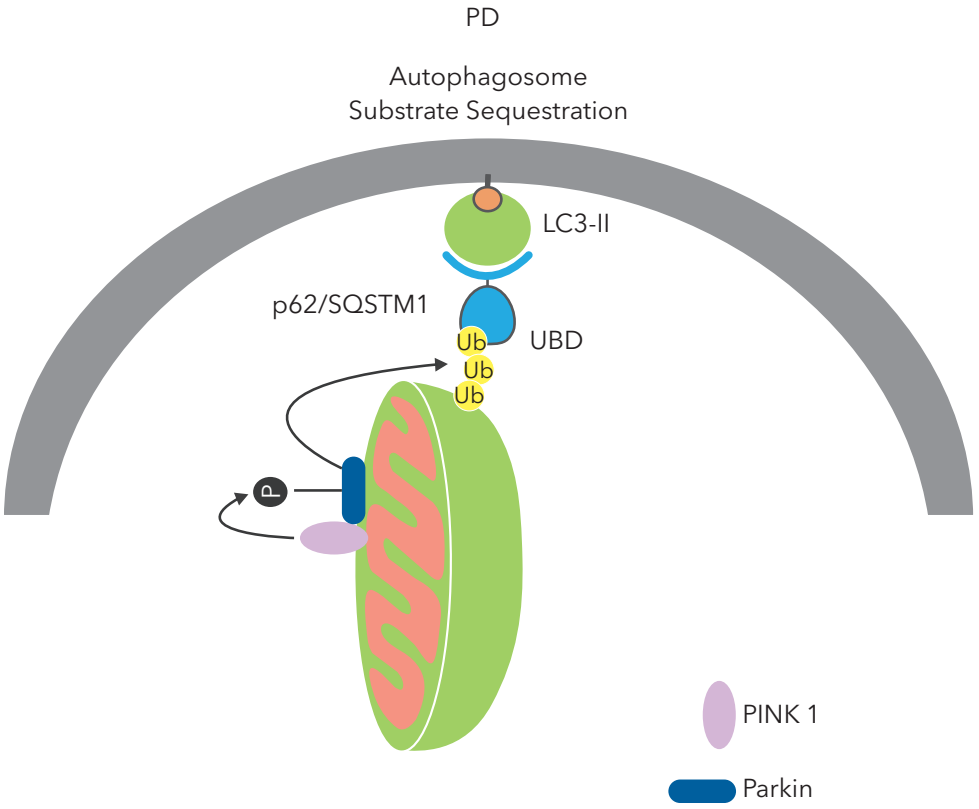


FIGURE 4 PINK1 and Parkin participate in the process of mitophagy, a type of selective autophagy that targets damaged mitochondria for elimination. Following stressors that result in the depolarization of the mitochondrial membrane, PINK1 accumulates on the outer mitochondrial membrane where it recruits and phosphorylates Parkin. Ubiquitination of mitochondrial proteins by Parkin facilitates the targeting of damaged mitochondria for degradation. PINK1 and Parkin mutations lead to dysregulated mitophagy and have been implicated in PD.

Two other PD-associated proteins, PINK1 and Parkin play central roles in mitochondrial homeostasis by their participation in the process of mitophagy.[15] In response to mitochondrial damage and loss of membrane potential, the serine/threonine protein kinase, PINK1, associates with the outer mitochondrial membrane and phosphorylates the E3 ligase, Parkin, along with mitochondrial ubiquitin.[15,54-57] Phosphorylated Parkin, localized to the outer mitochondrial membrane, is activated, and initiates the ubiquitination of multiple mitochondrial proteins.[15,58,59] Selective autophagy receptors (e.g., p62/SQSTM1) recognize these ubiquitinated proteins and degrade the damaged mitochondria through autophagy.[15] Since PINK1 and Parkin mutations are predominant in autosomal recessive cases of PD, defective mitophagy has been implicated in the pathogenesis of PD (FIGURE 4).[5,29,60]

TABLE 1. MOLECULAR MECHANISMS CONNECTING NEURODEGENERATIVE DISEASES WITH AUTOPHAGY DYSFUNCTION.

NEURODEGENERATIVE CONDITION	TARGETS AT INTERSECTION OF NDDS AND AUTOPHAGY	MECHANISM FROM HUMAN BRAIN, IN VIVO ANIMAL MODELS OR IN VITRO STUDIES	IMPLICATIONS FOR AUTOPHAGY ACTIVITY
Alzheimer's Disease	PS1 and PS2 Mutated	Impaired glycosylation and targeting of the proton pump H^+ ATPase (v-ATPase) to lysosomes Impaired lysosomal acidification[19]	Decreased degradation of protein aggregates Accumulation of Autophagic Vesicles
	PICALM Truncated Decreased expression	Impaired regulation of endocytosis Impaired endocytosis of SNARE proteins: VAMP2, 3 and 8[21]	Impaired phagophore elongation, autophagosome formation and autophagosome-lysosome fusion Decreased degradation of protein aggregates
	Amyloid Precursor Protein (APP) Mutated High levels of Aβ	Increased PI3K/AKT signaling and phosphorylation of the proline-rich AKT substrate 40 (PRAS40) Increased mTOR signaling[24]	Inhibition of autophagy Accumulation of Aβ and Tau
	p62/SQSTM1 Abnormal levels Altered phosphorylation	Associated with neurofibrillary tangles[28]	Decreased autophagy of specific cargo
Huntington's Disease	Htt	Htt aggregates sequester mTOR[37] and Beclin1[38]	Induction and inhibition of autophagy, respectively
	Beclin1	Decreased Beclin1 levels[46]	Impaired starvation-induced autophagy
	ALFY	Decreased expression[43]	Decreased protein aggregate clearance
	p62/SQSTM1 Phosphorylated at UBD	Increased affinity for ubiquitin[44,45]	Increased protein aggregate clearance
Parkinson's Disease	α-synuclein overexpressed	Mislocalized ATG9[5]	Inhibition of autophagy induction
	α-synuclein mutated (A53T and A30P)	Inhibition of Chaperone-mediated autophagy[49,51,52]	Triggers compensatory macroautophagy
	ATPase type13A2 mutated	Defective lysosomal acidification[61,62]	Decreased autophagy
	PINK1/Parkin mutated	Decreased ubiquitin labeling of mitochondrial proteins[5,29,60]	Decreased mitophagy

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CONCLUSIONS

Recent studies have identified several protein targets with functions that intersect key steps in autophagy with neurodegeneration (TABLE1). While the molecular characteristics of these targets differ among NDDs, the mechanisms by which they influence autophagy often coincide. Many questions remain to be addressed regarding the role of autophagy as a homeostatic process in NDDs to effectively harness the power of this pathway to restore neuronal health.

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