



# Quality Control in Neurons: Mitophagy and Other Selective Autophagy Mechanisms

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## Abstract

The cargo-specific removal of organelles *via* selective autophagy is important to maintain neuronal homeostasis. Genetic studies indicate that deficits in these pathways are implicated in neurodegenerative diseases, including Parkinson's and amyotrophic lateral sclerosis. Here, we review our current understanding of the pathways that regulate mitochondrial quality control, and compare these mechanisms to those regulating turnover of the endoplasmic reticulum and the clearance of protein aggregates. Research suggests that there are multiple mechanisms regulating the degradation of specific cargos, such as dysfunctional organelles and protein aggregates. These mechanisms are critical for neuronal health, as neurons are uniquely vulnerable to impairment in organelle quality control pathways due to their morphology, size, polarity, and postmitotic nature. We highlight the consequences of dysregulation of selective autophagy in neurons and discuss current challenges in correlating noncongruent findings from *in vitro* and *in vivo* systems.

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## Introduction: Bulk *versus* Selective Autophagy in Neurons

Neurons are highly polarized, terminally differentiated cells that transmit information through the propagation of electrical signals. Due to these unique properties, neurons rely heavily on the targeted delivery and regulated removal of proteins and organelles along the axon. Defects in the removal of aggregated proteins and damaged or aged organelles can lead to cellular stress and eventual neurodegeneration or death. Furthermore, there is strong genetic evidence linking mutations in intracellular trafficking proteins to a range of neurodegenerative diseases [1].

In the neuron, multiple maintenance mechanisms are in place to degrade cellular debris. Macroautophagy (hereafter referred to as autophagy) is the major clearance pathway to nonselectively remove dysfunctional proteins and organelles from the cell [2]. This evolutionarily conserved pathway involves the engulfment of cytoplasmic components into a

double-membraned vesicle or autophagosome, which fuses with late endosomes or lysosomes to form autolysosomes. Engulfed cargos are degraded by lysosomal enzymes, which allow for recycling of cellular components [3–5]. The protein machinery driving this pathway, autophagy-related proteins (ATGs), has been well investigated in yeast and mammals (for reviews, see Refs. [6–8]).

In neurons, autophagy is critical for maintaining cellular homeostasis, as neuron-specific knock-outs (KOs) of key ATG proteins are sufficient to induce cellular degeneration [3,4]. A constitutively active pathway has been described that functions under basal conditions [9,10]. *In vitro* and *in vivo*, autophagosomes are formed in the distal axon and mature through fusion with lysosomes as the organelle is retrogradely trafficked to the soma [11–16]. Observations to date suggest that this constitutive pathway for autophagy takes up cargo nonselectively, engulfing both protein aggregates and mitochondrial fragments at the axon terminal [13,17].

In contrast to the nonselective bulk removal of cytoplasmic components, selective autophagy involves the degradation of specific cargos through the autophagic pathway [18,19]. Autophagosome formation is mediated by cargo-specific autophagy receptors. These receptors are cargo recognition proteins that contain unique domains that allow them to simultaneously bind, directly or indirectly, to the cargo targeted for degradation (usually through ubiquitination) and to the phagophore or growing autophagosome *via* interaction with membrane-associated mATG8 proteins. The sequestration of each organelle or aggregate that is targeted and cleared by this pathway may require specific cues, although all pathways converge downstream in their use of the core components of the nonselective pathway to generate autophagosomes that engulf the cargo [18]. Specific types of selective autophagy may use multiple autophagy receptors or single receptors may mediate the removal of several types of organelles, suggesting some redundancy in the system [19]. Since it is crucial to remove damaged or aged proteins and organelles from the neuron, the promiscuity of the receptors may act as a failsafe mechanism.

Several types of selective autophagy have been reported to date, including the removal of mitochondria by mitophagy, endoplasmic reticulum (ER) by ER-phagy, aggregated proteins by aggrephagy, ribosomes *via* ribophagy, peroxisomes by pexophagy, and so on. Much of this work was performed in non-neuronal systems. However, given the key role for autophagy in the maintenance of neuronal health and the accumulating genetic data implicating autophagy deficits in neurodegeneration, it is essential to develop a mechanistic understanding of the neuron-specific aspects of autophagy. Here, we summarize progress examining selective autophagy in neurons, highlighting the three best understood mechanisms: mitophagy, ER-phagy, and aggrephagy. We discuss how damaged organelles signal for degradation and the molecular players required for cargo recognition and sequestration. Neurons are highly polarized cells with distinct somal, dendritic and axonal compartments; as both dendritic and axon terminals may be located far from the soma, these cells face unique challenges. We emphasize the diverse mechanisms that mediate quality control in neurons and focus on how dynamics, long-distance trafficking, and compartmentalization contribute to the overall effectiveness of the pathway. Finally, we discuss the pathophysiological roles of these pathways and implications to disease states.

## Selective Autophagy

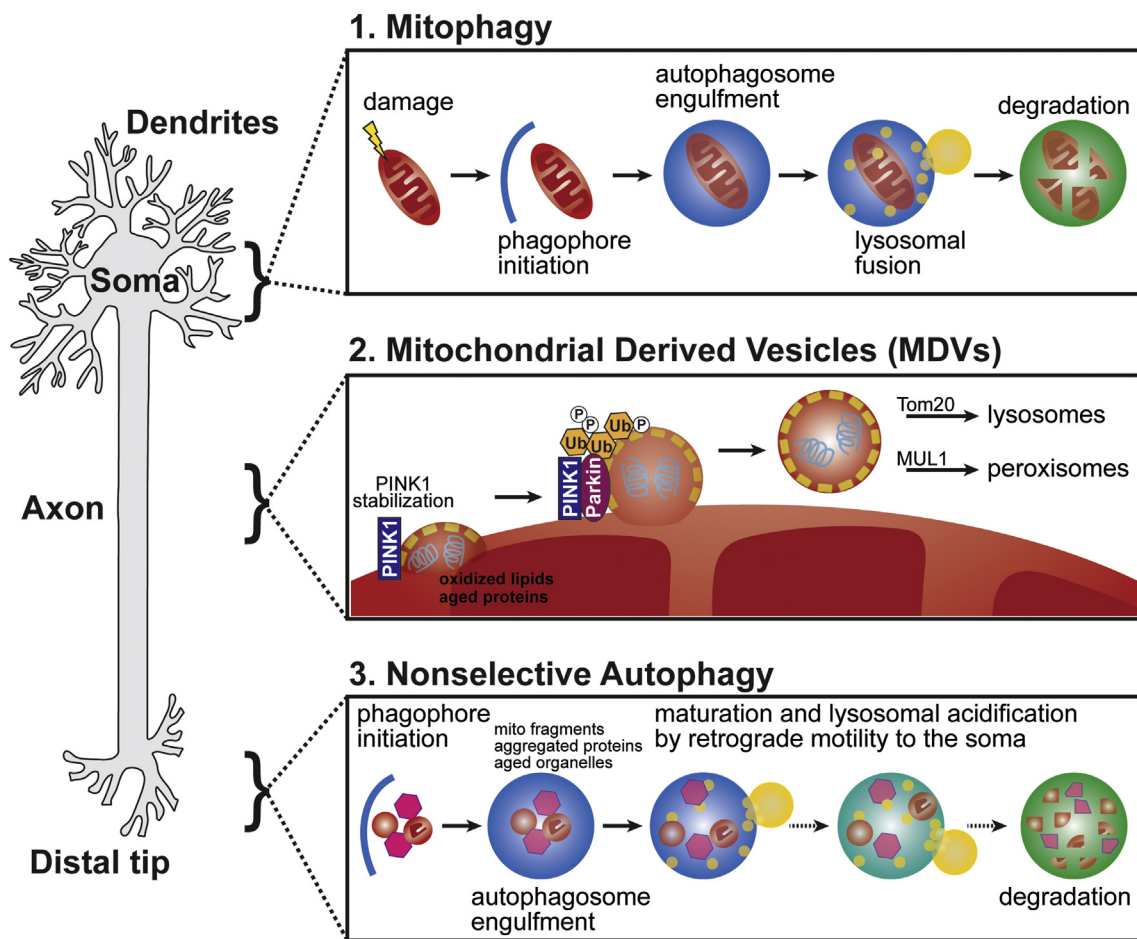
Many of the proteins identified as autophagy receptors have a microtubule-associated protein

1A/1B-light chain 3 (LC3)-interacting region (LIR) that mediates binding to the growing autophagosome, and a ubiquitin binding domain (UBAN, UBA, or UBZ) which allows for binding to the damaged protein or organelle [19]. It should be noted that ubiquitin-independent selective autophagy has also been reported [20]. In addition, most autophagy receptors are cytosolic proteins that translocate to the cargo upon activation of the pathway. One exception is Nip-like protein X (NIX), a mitophagy receptor with a transmembrane domain that is found on the outer mitochondrial membrane (OMM) [21,22]. Selectivity can be further enhanced by post-translational modifications of autophagy receptors or by receptor oligomerization. For example, oligomerization of the autophagy receptor p62 enhances and stabilizes LC3 binding [23]. Similarly, phosphorylation or ubiquitination of receptors can stabilize cargo binding [18,24,25].

Although the types of selective autophagy differ by the organelles that are sequestered, commonalities among the parallel pathways do exist [18]. For instance, all of the pathways are thought to initiate through some type of stress, perturbation, or age-dependent turnover of the organelle or protein aggregate. The damaged organelle or aggregate cues receptor-mediated specificity for degradation, and all use the components of the autophagic pathway for elimination. Selective autophagy pathways appear to be important for neuronal homeostasis, as they are implicated in neurodegenerative diseases. Mutations in the mitophagy components OPTN and TBK1 cause familial forms of amyotrophic lateral sclerosis (ALS; [26,27]) and mutations in PINK1 and Parkin cause familial forms of Parkinson's disease (PD; [28–30]). Similarly, mutations in the autophagy receptor p62 cause ALS, frontotemporal dementia, and Paget's disease of bone [31], and mutations in the ER-phagy receptor family with sequence similarity 134 member B (FAM134B) cause sensory and autonomic neuropathy [32].

## Mitophagy

Mitophagy is the selective sequestration and elimination of damaged mitochondria [33–35]. Neurons, in particular, rely heavily on the mitochondrial network to support local energy demands at synaptic sites. Thus, the prompt removal of dysfunctional mitochondria is critical for neuronal health and maintenance. *In vivo*, damage to mitochondria occurs through aging, localized reactive oxygen species (ROS) production, exposure to toxins, hypoxia, or developmental programming. Experimentally, mitochondrial damage and mitophagy can be initiated *via* multiple triggers, including oxidative stress through antioxidant removal, chemical uncouplers (i.e., CCCP, carbonyl cyanide m-chlorophenyl



**Fig. 1.** The mechanisms of neuronal mitochondrial quality control. (1) Damaged mitochondria are removed from the cell via a process termed mitophagy. Organelles are marked for degradation, sequestered by an autophagosome, and degraded by lysosomal fusion. Additional mechanisms exist to remove organelle components, including MDVs and the nonselective removal of mitochondrial fragments by autophagy. (2) MDVs are budded vesicles thought to contain oxidized lipids and damaged/aged proteins. These small vesicles utilize PINK1/Parkin phospho-ubiquitination signaling and undergo cargo-based targeting to peroxisomes and lysosomes. Additional budded vesicles have been reported, such as SNPH-positive vesicles that are Parkin-independent. (3) In the distal axon, mitochondrial fragments, protein aggregates, and other cytosolic components are nonselectively engulfed by the constitutive formation autophagosomes. Cargos are degraded through lysosomal fusion and acidification as autophagosomes mature through retrograde trafficking. Each of these molecularly distinct pathways may contribute to a different aspect of mitochondrial regulation.

hydrazine; Antimycin A; Oligomycin; rotenone), hypoxia, iron chelation, and the local generation of ROS [36–38], although not all of these approaches may effectively model physiological changes in mitochondrial health.

Initial investigation of the mitophagy pathway started with the discovery of two genes that were linked to familial PD, *park6* encoding PINK1 (PTEN-induced kinase 1) and *park2* encoding Parkin [28–30]. In immortalized cell lines, these proteins were found to be critical for the clearance of damaged mitochondria. Since these early discoveries, multiple mitophagy pathways have been reported (Fig. 1; [21,39,40]). Some of these pathways do

not involve either PINK1 or Parkin, sparking questions as to which pathways are most relevant *in vivo* and under which circumstances. In general, it is thought that the multiple mitophagy mechanisms, identified to date, work in parallel to maintain mitochondrial health. However, whether there is a predominant pathway whose dysregulation leads to the progressive functional and structural degeneration of the neuron is highly debated.

#### PINK1/Parkin-dependent mitophagy

PINK1/Parkin-dependent mitophagy is the best-described pathway for mitochondrial quality control

and initially was characterized in immortalized cell lines. This is a highly regulated process that involves the translocation of several proteins to the damaged organelle. Under basal conditions, the serine–threonine kinase PINK1 is proteolytically processed at the OMM by a mitochondrial intermembrane protease presenilin-associated rhomboid-like protein (PARL), which keeps the relative PINK1 concentration low [41,42]. Under cellular or environmental stress, mitochondrial depolarization leads to a failure in PINK1 processing and its subsequent accumulation on the OMM [43,44]. Autophosphorylation of the kinase enhances activity, which leads to the recruitment, phosphorylation, and activation of Parkin, an E3 ubiquitin ligase [43,45,46]. In addition, PINK1 phosphorylates ubiquitin [47,48]. Together, these proteins create a feedforward amplification mechanism whereby ubiquitin binding and PINK1-phosphorylation of Parkin induce full activation of the protein and thus conjugation of ubiquitin chains to OMM substrates [i.e., voltage-dependent anion channel and mitofusin (Mfn) 2]; in turn, this leads to new sites for PINK1 phosphorylation and the resulting amplification of the initial response [47,49–51]. As a result, phospho-ubiquitination of OMM proteins by PINK1 and Parkin generates a specific signal and a platform for the recruitment of autophagy receptors for organelle degradation.

#### *PINK1/Parkin-dependent mitophagy in nonneuronal cell lines*

In non-neuronal cell lines overexpressing Parkin, multiple autophagy receptors translocate to mitochondria after mitophagy induction via CCCP or local ROS generation, including optineurin (OPTN), nuclear dot protein 52 kDa or calcium-binding and coiled-coil domain-containing protein 2 (NDP52/CALCOCO2), tax1 binding protein 1 (TAX1BP1), neighbor of BRCA1 gene 1 protein (NBR1), and p62/sequestosome 1 (SQSTM1) [24,38,52–54]. Translocation and mitochondrial interactions are mediated by the ubiquitin domain found in all of these proteins. Initial studies suggested that p62 might mediate PINK1/Parkin-dependent mitophagy [53], but additional work showed that p62 can cluster damaged mitochondria but does not facilitate mitophagy [38,52,54]. To investigate the functional contribution of each receptor in mitophagy, one study generated a cell line lacking all five receptors (OPTN, NDP52, TAX1BP1, NBR1, and p62). In this penta-KO background, cells failed to remove damaged mitochondria, but overexpression of OPTN, NDP52, or, to a lesser extent, TAX1BP1 rescued mitophagy [52]; similar results were reported using transient knock-down in a wild-type background [24,38]. While defects were seen in the penta-KO cells, individual depletion of either OPTN or NDP52 was not reported to cause a

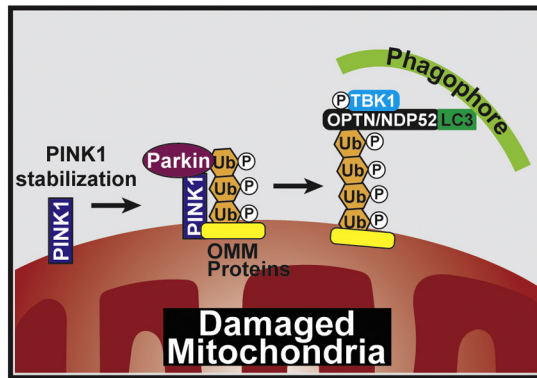
long-term defect in mitochondrial clearance [52]. In contrast, transient depletion of OPTN was shown to cause a significant delay in mitophagy, indicating a kinetic impairment upon OPTN loss [24,38].

Autophagy receptor specificity is enhanced by posttranslational modifications, such as the phosphorylation of autophagy receptors by TANK-binding kinase 1 (TBK1). TBK1 has been shown to phosphorylate multiple receptors at sites that are relevant to selective autophagy, for example OPTN, NDP52, TAX1BP1, and p62 [25,55–57]. However, interactions with OPTN appear to be the most functionally relevant. TBK1 binds and phosphorylates OPTN at sites in or near both the UBAN and LIR motifs, which activates the protein [24,25,52,56]. For instance, TBK1-dependent phosphorylation of OPTN S177 is necessary for efficient LC3 recruitment [24,52,56]. TBK1 is co-translocated with OPTN to the surface of damaged organelles, where phosphorylation helps to stabilize OPTN on the OMM [24,25]. In cells in which TBK1 was either depleted or pharmacologically inhibited, autophagosome engulfment of damaged mitochondria was impaired [24]. In addition, expression of disease-associated mutants in either OPTN or TBK1 disrupted the pathway leading to accumulation of damaged mitochondria [24,25,38,52]. More recently, TBK1 was shown to cooperate with NDP52 to recruit induction complex components of the autophagic machinery, UNC-51-like autophagy acting kinase 1 (ULK1) through interactions with FAK family kinase-interacting protein of 200 kDa (FIP200) [58]. Surprisingly, NDP52 and TBK1 could target ULK1 to cargo and mediate autophagosome biogenesis in the absence of mATG8s, indicating that selective autophagy receptors might participate more upstream in the signaling pathway than was initially thought.

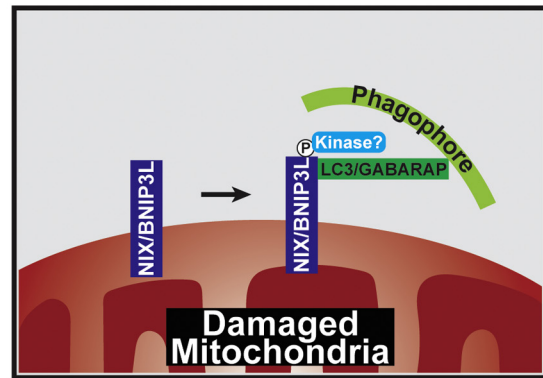
A novel family of mitochondrial matrix proteins has been implicated in mitophagy signaling, illustrating further redundancy in the PINK1/Parkin-dependent mitophagy pathway. In nonneuronal cell lines, 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (NIPSNAP1) and NIPSNAP2 accumulated on the OMM of depolarized mitochondria [59]. NIPSNAP1 is highly expressed in dopaminergic neurons of mice [60], and the homologs appear to be redundant [59]. NIPSNAP1 immunoprecipitated with Parkin and interacted with LC3B and autophagy receptors, p62 and NDP52, to target the sequestration of damaged organelles. When NIPSNAP1 and NIPSNAP2 were depleted from cells, Parkin was efficiently recruited to depolarized mitochondria, but autophagy receptor recruitment was impaired, suggesting that NIPSNAPs are required for autophagy receptor recruitment and mitophagy. Consistent with a role of mitophagy in PD, zebrafish models show loss of NIPSNAP1 leads to increased mitochondrial dysfunction, fewer



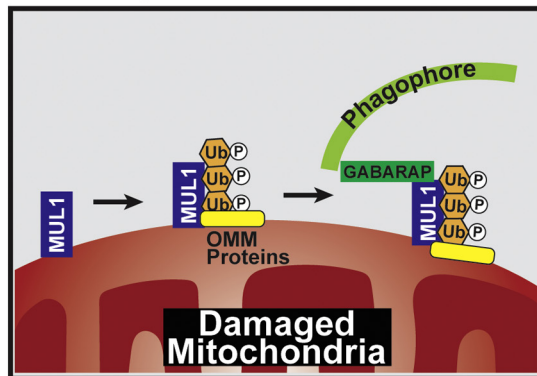
## 1. PINK1/Parkin-mediated



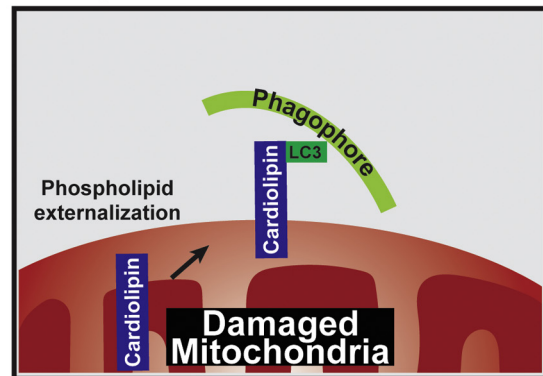
## 2. Receptor-mediated



## 3. Ubiquitin-mediated



## 4. Lipid-mediated



**Fig. 2.** The diverse pathways to induce mitophagy. Within the neuron, multiple mechanisms exist to signal cargo engulfment, including PINK1/Parkin, OMM receptors, ubiquitin cascades, and lipids. (1) PINK1/Parkin-mediated mitophagy initiates with PINK1 stabilization on the OMM, which recruits Parkin. Phospho-ubiquitination of OMM substrates (voltage-dependent anion channel, Mfn2, etc.) by PINK1/Parkin recruits autophagy receptors, OPTN and NDP52. Phosphorylation of OPTN by TBK1 helps to stabilize it on the mitochondrial surface. These autophagy receptors link the damaged organelle to the autophagosome through interactions with ubiquitin and LC3 on the mitochondrial membrane and growing phagophore, respectively. (2) Several autophagy receptors are anchored within the OMM, including BNIP3 and NIX/BNIP3L. Highlighted is the role of NIX/BNIP3L, which is phosphorylated by an unknown kinase, leading to activation and binding of NIX/BNIP3L to LC3 or GABARAP on the phagophore. (3) Additional E3 ligases have been reported to regulate mitophagy independent of Parkin. MUL1 has similar substrates to Parkin and can directly bind to GABARAP, suggesting that it can function independently to mediate engulfment. (4) Externalization of cardiolipin, normally found on the IMM, to the OMM is a unique mechanism for lipid-mediated mitophagy. Cardiolipin can bind directly to LC3 to induce mitophagy.

dopaminergic neurons, and impaired motor neuron function [59].

#### *PINK1/Parkin-dependent mitophagy in primary neurons*

Work in nonneuronal cell lines has established a critical role of PINK1 and Parkin in mitophagy, but studies translating these findings to mitophagy in primary neurons or *in vivo* have yielded some surprising results. Furthermore, the unique characteristics of neurons have raised additional questions, such as whether all neuronal mitochondria are

subject to the same quality control mechanisms and whether mitophagy is spatially regulated, a point we return to below.

Thus far, work in neurons has confirmed the molecular players involved, suggesting that the PINK1–Parkin mitophagy pathway is conserved across cell types (Fig. 2). In cultured neurons, Parkin is selectively recruited to damaged mitochondria after induction of mitophagy with multiple damaging agents, including CCCP, Antimycin A, and rotenone [37,48,61,62]. Interestingly, Parkin translocation was shown to be differentially regulated depending on the damaging agent and by the presence or absence

of antioxidants in the culture media [62]. Furthermore, Parkin translocation has been reported to occur over a long time scale, ~18 h after damage [61], much slower than the temporal translocation dynamics observed in HeLa cells where LC3 engulfment can be observed within an hour of insult [24,38]. In contrast to the observations of Cai *et al.* [61], local damage to axonal mitochondria using the mitochondrially targeted photosensitizer mitoKillerRed was reported to occur within an hour [37].

A “double”-hit strategy of antioxidant removal coupled to treatment with a mitochondrial damaging agent has been used to induce mitophagy in neurons but is not needed in nonneuronal cells [37,48,62]. Moreover, only a subset of mitochondria appears to enter the mitophagic pathway after global treatment of neurons. In contrast, the integrity of the mitochondrial network in nonneuronal cells was lost and the majority of organelles were sequestered following similar treatments. It may be that post-mitotic neurons have additional quality control mechanisms in place to preserve mitochondrial health in order to prevent the turnover of the entire network; these mechanisms may be key in terminally differentiated and post-mitotic cells.

#### *PINK1/Parkin-dependent mitophagy in vivo*

PD is a neurodegenerative disease that leads to the progressive degeneration of dopaminergic neurons in the substantia nigra. Since loss-of-function mutations in *park2* and *park6* genes are linked to familial PD [28–30], transgenic models were generated to recapitulate aspects of the disease *in vivo*. Thus far, most of the work has utilized mouse and fly model systems. Interestingly, loss of PINK1 or Parkin in mouse models does not cause a robust PD phenotype, such as dopaminergic neuron loss. However, these phenotypes are observed in *Drosophila* mutant flies. These thought-provoking findings have pushed the field to generate new mitochondrial probes to better understand mitophagy *in vivo* and question our understanding of this mechanism.

**Rodent models.** KO of neither PINK1 nor Parkin in mice was reported to be lethal. Initial Parkin KO mice unexpectedly displayed only minor phenotypes, with no loss of the dopaminergic neurons, a hallmark of PD in humans [63,64]. As a result, additional Parkin KO mice were generated through the deletion of different exons compared to the first two lines generated. Still, these mice failed to exhibit any substantial motor or behavioral phenotype, or degeneration of dopaminergic neurons [65,66]. In two different lines of PINK1 KO mice, dopaminergic loss was not observed [67,68]. In aged mice, dopamine

levels were lower than in control mice but dopamine turnover was upregulated, without significant changes in the number of dopaminergic neurons [67]. However, striatal slices exhibited decreased evoked dopaminergic release [68].

In addition, triple KO mice lacking PINK1, Parkin, and DJ1 (a protein encoded by *park7*, another gene linked to familial PD; [69]) showed no loss of dopaminergic neurons after 24 months [70]. Interestingly, KO rat models of PINK1 and DJ1 showed significant dopaminergic neuron loss at 8 months, with motor defects starting at 4 months; similar to mice, Parkin KO rats had no phenotype [71]. Collectively, these data point to a non-essential role of PINK1 and Parkin in neuronal mitophagy, suggesting that other mitochondrial quality control pathways play a more significant role than was initially proposed. It is possible that PINK1 and Parkin are crucial in humans but the shorter lifespan of mice fails to allow for full manifestation of the disease, as the average onset of PD in humans is in the early 1960s.

***Drosophila* models.** In sharp contrast to the mouse models, which only displayed small changes in dopaminergic levels, null and mutant flies of either PINK1 or Parkin showed clear PD phenotypes. Multiple studies reported that a subset of dopaminergic neurons had morphology defects or abnormal shrinkage [72,73]. In addition, locomotive defects, reduced lifespan, and sterility were all observed [74,75]. Consistent with the Parkin phenotype in flies, PINK1 null or mutant flies exhibited dopaminergic neuron degeneration along with defects in with flight muscle and locomotion [76–78]. These results seem contradictory to the mouse models' findings that suggest a minimal role for PINK1 and Parkin in neuronal mitophagy. Furthermore, work in nonneuronal cell lines and primary neuronal cultures corroborates the *Drosophila* results, suggesting that PINK1 and Parkin are important in facilitating mitophagy. However, overexpression of mitophagy-associated proteins in culture may upregulate PINK1/Parkin-dependent mitophagy, making it appear to be more significant than it truly is *in vivo*. It is possible that other mitophagy pathways play a more significant role than were initially proposed, such as partially redundant mechanisms that utilize alternative E3 ligases or PINK1/Parkin-independent mitophagy pathways.

**Mitochondrial form and function.** In addition to investigating the onset of PD, the PINK1 and Parkin KO model systems have been used to study the role of these proteins *in vivo*. No morphological changes were observed in striatal mitochondria from mice depleted of Parkin [79]. However, one study using PINK1-deficient mice reported no gross changes to mitochondrial ultrastructure, but an increase in large

swollen mitochondria in the striatum of old mice compared to young mice [80]. Consistent with the later finding, transmission electron microscopy of flight muscles in Parkin or PINK1 null or mutant flies illustrated muscle degeneration with enlarged swollen mitochondria and disrupted cristae [74,75,77]. Mitochondrial fragmentation was also observed in primary mouse embryonic fibroblasts (MEFs) from PINK1 KO mice [81]. In *Drosophila* motor neurons, somal mitochondria appeared abnormal but axonal mitochondria were unaffected [82,83], suggesting tissue- and compartment-specific phenotypes. Thus, loss of PINK1 and Parkin in *Drosophila* and mice, to a lesser extent, alters mitochondrial morphology.

The muscle degeneration and morphological phenotypes of PINK1 mutant flies can be rescued by overexpression of human PINK1, illustrating functional conservation between species [77,78]. These phenotypes could also be rescued by overexpression of Parkin, but not DJ1 [76–78]. It is important to note that PINK1 expression could not rescue Parkin phenotypes and double mutants phenocopied individual PINK1 and Parkin mutants [76,78]. These findings provide strong *in vivo* evidence that (1) PINK1 and Parkin function in a common pathway and (2) Parkin is downstream from PINK1 [76–78].

Functionally, loss of PINK1 in mice and flies altered mitochondrial membrane potential, increased ROS production, decreased mitochondrial DNA, impaired ATP synthesis, and decreased ATP levels [76,81,82]. Mitochondria isolated from PINK1 KO mice had significantly lower  $\text{Ca}^{2+}$  load capacity [67], and striatum ATP levels were reduced *in vivo* [81]. In motor axons of PINK1 null *Drosophila*, mitochondrial flux was altered with no change in organelle density or length [82]; however, Parkin nulls had a decreased number of axonal mitochondria [83]. Purified dopaminergic neurons from Parkin *Drosophila* mutants accumulate enlarged depolarized mitochondria [84]. Despite significant gross morphological changes, null PINK1 and Parkin mice exhibit decreased expression of proteins involved in oxidative stress, resulting in impaired mitochondrial respiration and increased sensitivity to ROS [79]. These findings are consistent with reports from Parkin loss-of-function mutants in *Drosophila* [75,85]. In *Drosophila*, PINK1 and Parkin have a role in the maintenance of mitochondrial integrity *in vivo* and protein depletion leads to PD phenotypes. However, in mice, there is sparse evidence implicating PINK1 and Parkin in mitochondrial maintenance and no reports of PD phenotypes in KO neurons. It is possible that mice have developed compensatory mechanisms or tolerate a higher threshold for mitochondrial damage than flies or humans. Mitochondrial dysregulation and impairment are early cellular manifestations that ultimately lead to tissue degeneration, and this is apparent in

*Drosophila* models. However, more work is needed to fully understand the disparate findings in these model systems.

**Mitochondrially targeted fluorescent probes.** Due to the difficulty of studying mitophagy *in vivo*, transgenic mice with mitochondrially targeted fluorescent probes were generated, including mito-Timer, mt-Keima, and mito-QC. Mito-Timer is a variant of DsRed fluorescent protein that localizes to the inner mitochondrial membrane *via* a targeting sequence and transitions from green to red over time (~48 h) allowing for temporal monitoring of mitochondria [86]. The mito-Timer construct is routinely used in neuronal cultures, but the reporter mice have primarily been used to study cardiac mitochondria [87,88]. There is potential for the reporter mice to be used in other tissues, such as the brain, as the slow oxidation rate of mito-Timer makes it an ideal probe to study relative age-related mitochondrial dynamics.

Generation of mt-Keima reporter mice took advantage of the Keima protein, a coral-derived pH-dependent fluorescent probe that is resistant to lysosomal degradation; the Keima moiety is targeted to the mitochondrial matrix through the mitochondrial targeting sequence of cytochrome *c* oxidase subunit-VIII (COX-VIII) [89–91]. Mitophagic flux can be monitored through ratiometric analysis. At physiological pH, the shorter wavelength excitation dominates, but fluorescence shifts to longer wavelength excitation in acidic environments. *In vivo*, basal mitophagy levels were lower in the cortex, substantia nigra, and striatum compared to the dentate gyrus and lateral ventricle, illustrating tissue-specific differences in mice [89]. In addition, mitophagy was reduced in the dentate gyrus of aged mice compared to young mice [89]. In contrast, basal mitophagy was shown to increase with age in dopaminergic neurons of genetically engineered flies expressing the mt-Keima reporter [92]. This increase in frequency was suppressed by depletion of Parkin, demonstrating a critical role of the PINK1/Parkin mitophagy pathway in age-dependent basal mitochondrial turnover. Aging has been correlated with mitochondrial dysfunction, accumulation of mitochondrial DNA mutations, and mitochondrial morphological defects [93–97]. It is possible that mitochondrial impairment is due to a decrease in mitophagy with aging. On the other hand, mitophagy could be upregulated with age as a result of increased mitochondrial dysfunction in order to maintain organelle homeostasis. As with autophagy, mitophagy modulation impacts neuronal longevity and lifespan [74,97,98], but more work is needed to clarify the role of mitophagy in aging.

The mito-QC reporter mouse line has a constitutive knock-in of mCherry-GFP-FIS1 [99,100].



Mitophagy is monitored by the loss of the pH-sensitive GFP signal, while the fluorescence of the fused mCherry moiety is retained in the acidic environment produced by fusion of the autophagosome with lysosomes. As a result, the dually labeled mitochondrial network appears yellow and damaged organelles undergoing mitophagy are red. Tissue-wide analysis illustrated that constitutive mitophagy occurs in multiple organs, providing further evidence that mitophagy is a homeostatic pathway. Immunolabeling of brain sections showed that Purkinje neurons undergo pronounced mitophagy, and suggested high turnover in the soma but minimal turnover in the axonal projections and dendritic arbors [99]. To further investigate the role of PINK1 in mitophagy *in vivo*, a cross between PINK1 KO and *mito-QC* reporter mice was generated [101]. Surprisingly, in tyrosine hydroxylase-positive dopaminergic neurons of the midbrain, basal mitophagy was indistinguishable between wild-type and PINK1 KO conditions. Similar findings, using the same reporter, were observed in *Drosophila* dopaminergic neurons of PINK1 or Parkin mutant flies [102]. These controversial data suggest that the role of PINK1 in basal mitophagy is minimal and that other quality control pathways are simultaneously functioning to maintain mitochondrial health. Nonetheless, genetic and clinical data clearly demonstrate the significance of PINK1 and Parkin in mitophagy [28,29], so more work is needed to understand the functional role of these proteins as compared to other cellular pathways for mitochondrial quality control in neurons.

### Alternative mitophagy pathways independent of PINK1 and Parkin

While PINK1/Parkin-dependent mitophagy is the best-understood pathway for mitochondrial quality control in neurons to date, several findings summarized above have raised questions as to whether this pathway is functionally dominant *in vivo*. The mild phenotypes displayed by the PINK1 and Parkin KO mice [63,65,67,68] suggest that other compensatory quality control mechanisms are in place. In addition, basal mitophagy in *Drosophila* was suggested to be a rare event [83]. Recently, more focus has been placed on understanding alternative mitophagy pathways and how these mechanisms collectively cooperate to remove damaged organelles (Fig. 2).

#### Receptor-mediated mitophagy

Instead of the requirement of Parkin translocation to damaged mitochondria, receptor-mediated mitophagy utilizes autophagic receptors that contain transmembrane domains, directing integration into the OMM. These receptors contain LIR motifs,

allowing them to independently recruit and bind the autophagic machinery to mediate the engulfment of damaged mitochondria [21,103]. Thus, these receptors directly induce mitophagy independent of PINK1/Parkin. Several receptors of this type have been reported, including NIX (also known as BCL2/adenovirus E1B 19 kDa interacting protein 3-like, BNIP3L). Structurally, NIX/BNIP3L is anchored to the OMM via a C-terminal transmembrane domain and interacts with multiple mATG8s, such as LC3 and GABA type A receptor-associated protein (GABARAP), through an N-terminal LIR domain [22,104]. Phosphorylation of NIX/BNIP3L enhances GABARAP binding [104], but the specific kinases and phosphatases that regulate this process are yet to be identified. The role of NIX/BNIP3L in mitophagy was initially described in erythroblasts where genetic ablation revealed that NIX/BNIP3L plays an important role in programmed mitochondrial clearance and maturation [105,106]. Similar findings for programmed mitophagy were reported in retinal ganglion cells during development and differentiation [107].

Interestingly, in human fibroblasts derived from an asymptomatic carrier of a Parkin mutation, mitochondrial turnover still occurred despite dysfunctional Parkin, suggesting the involvement of an alternative pathway [108]. Mitophagy was shown to be mediated by NIX/BNIP3L; this pathway was unaffected by the additional knock-down of PINK1. The independent role of NIX/BNIP3L in mitophagy was further confirmed in PINK1 and Parkin PD patient cell lines where overexpression or pharmacological activation of NIX/BNIP3L restored mitophagy [108]. In response to hypoxia, NIX/BNIP3L is transcriptionally upregulated by hypoxia-inducible factor (HIF) or forkhead box O3 (FOXO3) to induce mitophagy [109–111]. The functions of NIX/BNIP3L and its homolog BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) are now starting to be investigated in injury and disease models. Current work suggests that NIX/BNIP3L has a neuroprotective role in ischemic stroke [112], despite its homolog having a detrimental role in ischemic-hypoxic brain and spinal cord injury [113,114]. Future work to elucidate these roles could help to provide potential therapeutic targets.

#### Lipid-mediated mitophagy

Lipids can serve as an elimination signal to initiate autophagosome engulfment of damaged mitochondria. Cardiolipin is a mitochondrial phospholipid that is primarily found in the inner mitochondrial membrane [115–117]. The unique conical shape of cardiolipin allows it to interact with proteins located inside and outside of the mitochondria [116,117]. As a result, cardiolipin has been reported to participate



in the maintenance of mitochondrial membrane fluidity/stability and electron transport chain efficiency, fusion/fission, mitochondrial dynamics, cell death, and more recently mitophagy [116,118–122]. In damaged neuronal mitochondria, cardiolipin is externalized to the OMM where it binds LC3 to drive the engulfment of the damaged mitochondria by the autophagosome [122]. In neurons, the redistribution of cardiolipin was facilitated by phospholipid scramblase-3 (PLS3) [122], but other proteins have been reported to mediate cardiolipin externalization, including NDPK-D/NM23-H4 in SH-SY5Y cells [123,124] and Tafazzin (TAZ) in primary MEFs [125]. The interaction between cardiolipin and LC3 is facilitated by the negatively charged head group of cardiolipin and basic residues in LC3. Downregulation of cardiolipin or mutagenesis of LC3 at sites predicted to interact with the phospholipid decreased mitochondrial engulfment [122]. In primary neurons treated with rotenone to induce mitophagy, the fold change of externalized cardiolipin was greater than that seen in HeLa cells, suggesting that cell types have variable responses or thresholds to noxious stimuli and differentially regulate their mitophagy pathways.

#### Ubiquitin-mediated mitophagy

Additional E3 ligases have been identified that can substitute for Parkin in the removal of damaged mitochondria [21]. Mitochondrial ubiquitin ligase 1 (MUL1; otherwise known as MAPL, GIDE, and MULAN) is an E3 ligase that is localized to the OMM and interacts with substrates through a cytosolic C-terminal RING finger [126–128]. In nonneuronal cells, MUL1 was shown to play a role in mitochondrial dynamics, as over-expression caused mitochondria to cluster and fragment [126,127]. Regulation of MUL1 is mediated through interactions with the mitochondrial fission protein dynamin 1 like protein (Drp1) [129] and the mitochondrial fusion protein Mfn [130]; it should be noted that these proteins are also substrates of Parkin. In *Drosophila* and mouse neurons, MUL1 can suppress PINK1 or Parkin mutant phenotypes by negatively regulating Mfn through ubiquitin-mediated degradation [130]. In addition Loss of MUL1 has no effect on Parkin-mediated mitophagy and double mutants of MUL1 with either PINK1 or Parkin had more severe phenotypes than individual nulls. These data suggest that MUL1 functions in a parallel pathway and both MUL1 and Parkin regulate Mfn activity to maintain mitochondrial health. Furthermore, a yeast two-hybrid screen determined MUL1 interacts with GABARAP, and further analysis identified an LIR motif in the MUL1 RING domain [131]. Thus, MUL1 can regulate mitophagy through multiple mechanisms, providing further evidence that the lack of phenotype in PINK1 and Parkin KO

mouse models could be due to compensation by other mitophagy regulators or pathways.

#### Additional aspects of mitochondrial quality control in neurons

##### Nonselective mitochondrial clearance

Besides the selective removal of damaged or aged organelles, mitochondrial fragments are engulfed by autophagosomes in a nonselective manner in the axon terminals of neurons (Fig. 1). This distinct autophagy pathway is a constitutive mechanism that does not appear to involve the selective receptors discussed above [12,13,132]. In cultured neurons, LC3-positive organelles form constitutively in the distal axon and are retrogradely transported to the soma [11–13,133,134]. Similar findings have been reported in other model systems, including *Caenorhabditis elegans* and *Drosophila* [14,15,135]. Autophagosomes mature during trafficking to the soma by fusion with lysosomes, leading to efficient degradation of cargo. The cargos observed in this nonselective uptake include mitochondrial fragments and both aggregated and cytosolic proteins [13,17,35]. In primary dorsal root ganglion neurons, the mitochondria contained in autophagosomes appear fragmented and are morphologically distinct from the rest of the axonal mitochondrial population [13], suggesting that mitochondrial fragments are generated in the distal axon, and are then taken up nonspecifically into constitutively forming autophagosomes. The subsequent breakdown of engulfed mitochondrial fragments was disrupted when the retrograde transport of axonal autophagosomes to the cell soma was blocked [17]. Thus, autophagosome motility is required for effective fusion with lysosomes and cargo degradation. Potentially, this pathway maintains the health of axonal or synaptic mitochondria localized at considerable distances from the soma, but more work is required to clarify the mechanisms involved in the generation and engulfment of mitochondrial fragments within the distal axon.

##### Mitochondrial derived vesicles

Recent work identified mitochondrial derived vesicle (MDV) formation as a novel aspect of mitochondrial cell biology [39]. MDVs are small vesicles that bud off of an organelle and undergo cargo-based targeting to peroxisomes [127] and lysosomes [136] (Fig. 1). The SNARE syntaxin 17 mediates MDV-endolysosomal fusion through interactions with homotypic fusion and vacuole protein sorting (HOPS) tethering complex [137]. MDVs are produced in a constitutive pathway in which vesicles form independent of mitochondrial depolarization, but the mechanism can be further induced by

oxidative stress [136]. MDV biogenesis was disrupted by depletion of PINK1 or overexpression of PD-associated Parkin mutants, indicating that this quality control pathway is also regulated by these proteins [138]. However, it is a distinct pathway from mitophagy because the autophagic machinery is not required [136].

In response to mild perturbations in neurons, syntrophin (SNPH; a mitochondrial anchoring protein [139]) is released from stressed axonal mitochondria, triggering motor-driven motility and the formation of SNPH-positive vesicles derived from mitochondria [140]. These vesicles form independent of Parkin, Drp1, and the autophagy machinery, so they are apparently distinct from the MDVs previously described [127,136–138]. This novel pathway is also upregulated in neurons from early symptomatic stages, but not later stages, of familial ALS-linked and Alzheimer's disease (AD)-mutant mice, suggesting that it is a quality control mechanism that responds quickly to pathological stresses [140]. The generation of SNPH-positive vesicles appears to be an independent pathway that is activated prior to PINK1/Parkin-dependent mitophagy. As a result, damaged proteins and lipids can be removed to preserve the health of the parent organelle before complete removal of the mitochondrion is required [35,39]. This additional quality control mechanism may be critical in the maintenance of axonal mitochondria.

#### *Mitophagy compartmentalization in neurons*

While multiple studies have reported that mitophagy can be induced by mitochondrial damage or stress in neurons, there are discrepancies in regard to the localization of neuronal mitophagy. Since the prompt removal of damaged mitochondria is critical for maintaining cellular health, it has been proposed that local mitophagy occurs throughout the cell. However, primary culture and *in vivo* based studies have suggested otherwise. In cortical neurons, Parkin-positive mitochondria were found to accumulate in the somatodendritic compartment where they underwent LC3 sequestration and lysosomal degradation [61]. Neurons treated with CCCP displayed an increase in retrograde motility of mitochondria, suggesting that damaged mitochondria are trafficked back to the soma for degradation [61]. Conversely, another report described local mitophagy in the axon following treatment of neurons with Antimycin A or upon selective local damage induced by mitoKillerRed [37]. This axonal mitophagy pathway was shown to require PINK1 and Parkin, with clearance mediated by local autophagosome-lysosome fusion.

The spatial aspects of mitophagy have also been examined *in vivo*; these studies suggest that neuronal mitophagy is a compartmentally restricted

mechanism. In PINK1 and Parkin mutant flies, axonal mitochondria displayed normal morphology with a decrease in mitochondrial flux, but no mitochondrial accumulation was observed in the axons of motor neurons [82,83]. However, depletion of either protein resulted in altered somatic mitochondrial morphology, with mitochondria appearing more tubular and reticular than in control flies [82,83]. Additional evidence from examination of Purkinje cells from the *mito-QC* mouse suggests somatodendritic compartmentalization of neuronal mitophagy [99]. It is possible that differences between induced mitophagy in culture and basal mitophagy *in vivo* contribute to the discrepancies reported.

#### *Axonal mitochondrial transport*

Since mitochondrial biogenesis occurs primarily in the soma, newly synthesized organelles need to be trafficked along the axon to replenish mitochondria at sites located distally along the axon [141,142]. Conversely, retrograde transport may play important roles in clearance and repair [61], although there is some debate on the relative contribution of this mechanism. Axonal mitochondria move bidirectionally, with long-distance transport mediated by kinesin and dynein motors. The motility of axonal mitochondria decreases during neuronal maturation, with most organelles generally immobile and localized to presynaptic sites in mature neurons [143]. Mitochondrial motility is regulated by mitochondrial Rho (Miro), a small GTPase localized to the OMM that binds kinesin adaptors, trafficking kinesin-binding protein (TRAK) 1 and 2 (otherwise known as Milton in *Drosophila*) [144–147].

In the axon, there appears to be a relationship between mitochondrial motility and mitophagy, where motility disruptions isolate organelles, facilitating their removal [141,148]. Upon mitochondrial damage, Miro is removed from the OMM, arresting mitochondrial motility. Miro has been linked to PINK1, Parkin, and Leucine-rich repeat kinase 2 (LRRK2; another PD-associated protein encoded by the gene *park8* [149–151]); these proteins promote Miro degradation to halt mitochondria and remove organelles *via* mitophagy [148,152,153]. These observations are consistent with the report that local mitophagy can occur in the axon following mitochondrial damage [37]. Interestingly, mutations in LRRK2 are the most common cause of PD [154]. In LRRK2 mutant neurons, Miro remains on damaged mitochondria, delaying efficient degradation [152]. Furthermore, LRRK2 was still recruited to damaged mitochondria in PD fibroblast lines expressing mutant PINK1 and Parkin, suggesting LRRK2 functions in a parallel pathway to PINK1/Parkin, which converges on Miro [152]. In addition to

regulating Miro, LRRK2 has been implicated in alterations to mitochondrial morphology and function, RAB10 phosphorylation, and modulating Parkin/Drp1 interactions, all of which could potentially play a role in the regulation of mitophagy by LRRK2 [155–159]; more work is clearly needed to clarify the mechanisms involved.

Another axonal quality control mechanism facilitated by transport is fusion/fission events. Through these events, dysfunctional mitochondria can fuse with healthy organelles and subsequently undergo fission to evenly distribute components [160]. Fusion/fission events can also help to isolate damaged mitochondria to facilitate sequestration and elimination.

#### *Interplay of neuronal mitophagy pathways and mitochondrial quality control*

The mild phenotypes displayed in PINK1 and Parkin KO mice *in vivo* suggest that other mitochondrial mechanisms may be at play [63,65,67,68]. This concept is further supported by the findings that loss of PINK1 in mice and loss of PINK1 or Parkin in flies had no effect on basal mitophagy [101,102]. The recent focus on exploring additional mitophagy pathways independent of PINK1/Parkin has increased our understanding of receptor-, lipid-, and ubiquitin-mediated mitophagy [21]. Currently, it is unclear to what extent these mechanisms cooperate to contribute to mitochondrial turnover. It may be that the various mitophagy pathways are developmentally or tissue-specifically regulated or that there is a common dominant pathway and the others are failsafe mechanisms. In addition, it is not understood whether all mitochondria are subject to the same quality control mechanisms. Since neurons are highly polarized, it would be practical to utilize multiple quality control mechanisms to maintain health. For example, in axons, SNPH-positive vesicles formed independent of and prior to PINK/Parkin-dependent mitophagy [140]. This finding supports a model where vesicle budding is an axonal pathway that occurs under low levels of stress to preserve mitochondrial health, whereas the mitophagy pathway is activated under more severe stress or to remove terminal unrecoverable mitochondria. Further work is needed to understand the interplay among the various mitophagy pathways and related mitochondrial quality control mechanisms.

### **Pathophysiological role of mitophagy**

#### *Neurodegenerative diseases*

Mitochondrial dynamics and regulation are important to homeostasis, as disruption of mitophagy is implicated in multiple neurodegenerative diseases, including PD, AD, and Huntington's disease (HD) [161]. PD is characterized by the loss of dopaminer-

gic neurons in the substantia nigra, and proteins that regulate mitophagy have been linked to the disease. As previously mentioned, cell-based experiments directly link PINK1 and Parkin to mitophagy. PD patients also have elevated mitochondrial DNA deletions in the substantia nigra, further associating defects in mitochondrial quality control with PD [95,162]. KO mice do not display PD-phenotypes [63,65,67,68], but *Drosophila* mutant flies have locomotive defects, reduced lifespan, and dopaminergic degeneration [72–74,76,78]. Also, PINK1 KO rats showed dopaminergic loss and motor defects [71]. Both model systems, however, exhibit mitochondrial dysfunction. In mutant flies, swollen mitochondria with disrupted cristae were observed in flight muscle cells which correlated with muscle degeneration [74,75,77,92]. In neurons, mitochondrial impairments and mitophagy defects are upstream of neurodegeneration. Therefore, the lack of phenotype in mice points to compensatory quality control pathways that are sufficient to maintain neuronal health and prevent neurons from crossing a critical threshold that induces degeneration.

Selective autophagy receptors OPTN and p62, as well as their kinase TBK1, are implicated in ALS [26,27,163]; mutations in OPTN and TBK1 are additionally associated with glaucoma [164,165]. It is interesting that some of the disease-associated mutants of PD and ALS converge on a single mitochondrial quality control pathway, yet the disease etiology and symptoms induced by these mutations are diverse. ALS is characterized by the degeneration and loss of motor neurons, whereas PD primarily affects dopaminergic neurons. Since TBK1 phosphorylates OPTN and p62, impaired mitochondrial turnover and protein aggregation may both contribute to neurodegeneration in ALS. Future work to elucidate the role of these proteins *in vivo* will be important in deciphering the molecular signaling necessary for each neurodegenerative disease.

AD is characterized by neuronal degeneration leading to cognitive dysfunction and memory loss [166,167]. The pathophysiology of AD involves formation of intracellular neurofibrillary tangles from tau accumulation and extracellular plaques containing amyloid- $\beta$  (A $\beta$ ) peptides derived from the amyloid precursor protein. This age-related disease is complex, as multiple pathways may contribute to degeneration, such as oxidative stress, mitochondrial impairment, and dysregulation of autophagic and endolysosomal clearing mechanisms [168]. In both familial and sporadic AD, mitochondrial accumulation is observed [167,169–171]. It is thought that reduced mitochondrial clearance contributes to tau and A $\beta$  pathologies that are hallmarks of the disease, but the underlying molecular mechanisms are not well understood [167,171]. Both A $\beta$  fragments and tau have been shown to interact with mitochondrial



proteins resulting in functional impairment [172–175]. In addition, mitochondrial proteostasis induction delayed A $\beta$  toxicity and reduced amyloid aggregation in multiple AD model systems [169]. Recent work reported that mitophagy stimulation reduced A $\beta$  pathology and reversed cognitive decline through the phagocytic activity of microglia in AD mouse models; complementary results were observed in *C. elegans* AD models [171]. The authors proposed a cooperative relationship among damaged mitochondria, mitophagy, tau neurofibrillary tangles, and A $\beta$  plaques whereby defective signaling cascades exacerbate one another, giving rise to AD pathology and neurodegeneration [171].

HD is caused by the abnormal expansion of a trinucleotide (CAG) repeat in the gene that encodes the protein Huntingtin (Htt). Expansion beyond 36 repeats is detrimental, with disease severity correlating with expansion length [176]. HD is characterized by psychiatric disturbances, cognitive decline, and motor dysfunction [168,176,177]. The polyglutamine repeat in the N-terminus of the protein is prone to misfolding and aggregation [178,179]. Due to its unique size and number of interacting partners, Htt is implicated in multiple cellular pathways, many of which have been suggested to contribute to the pathogenesis of HD, such as mitochondrial and autophagosome impairment. In patients and HD model systems, mitochondrial respiration and Ca<sup>2+</sup> buffering are altered, and abnormal mitochondrial dynamics have been observed [180–185]. Morphological alterations have also been observed, including cristae disruption and increased mitochondrial fission through Drp1 interaction and activation [184]. Mitochondrial dysregulation is prevalent in multiple neurodegenerative diseases, but whether dysfunction is causative or correlative is still poorly understood. Further work is needed to elucidate the exact mechanisms that contribute to disease states, which could provide new avenues for therapy.

### *Innate immunity*

The inflammatory signaling pathway can be hyperactivated by impaired removal of damaged mitochondria, eventually leading to chronic inflammation and inflammatory diseases [186]. A microarray to characterize transcriptional alterations in Parkin mutants in *Drosophila* identified activation of genes in the innate immune response [85]. Similar changes were seen in striatal gene expression in PINK KO mice [67]. These observations are consistent with the finding that type 1 interferons (IFNs), cytokines that regulate the innate immune response, are upregulated in post-mortem brains of AD and PD patients and have been implicated in aging. Also, mitochondrial stress induced by rotenone or 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)

treatment upregulates a proinflammatory response in cultured neurons and glia, suggesting mitophagy may alleviate inflammation [187,188]. Specifically, rotenone induces type 1 IFN signaling, which results in a proinflammatory response in glia, resulting in downstream glia-dependent neurotoxicity [188]. Recent work has identified an inflammatory phenotype in PINK1 and Parkin KO mice after acute and chronic mitochondrial stress by excessive exercise and mitochondrial DNA mutations, respectively [189]. This phenotype could be rescued by depletion of stimulator of interferon genes (STING), a regulator of the type 1 IFN signaling response. Together, these data suggest that mitophagy, via PINK1 and Parkin, may alleviate inflammation and neurotoxicity by efficiently clearing damaged mitochondria.

## **Other Forms of Selective Autophagy: ER-Phagy and Aggrephagy in Neurons**

While mitophagy is the best-characterized pathway for selective autophagy in neurons, there are a growing number of cellular cargos known to be selectively targeted for degradation by autophagy, including the ER, peroxisomes, lipid droplets, ribosomes, and protein aggregates. All of these degradative pathways likely contribute to neuronal health by regulating organelle turnover or proteostasis, both critical in terminally differentiated cells that must last the lifetime of the organism. Research on the impact of some of these pathways on neuronal homeostasis is at an early stage, but recently, there has been significant progress in our understanding of ER-phagy (also referred to as reticulophagy) as an essential quality control mechanism to maintain the ER [190]. In contrast to the recent focus on ER-phagy, the clearance of aggregated proteins via aggrephagy has long been considered to be critical to neuronal health, due to the pronounced accumulation of protein aggregates in major neurodegenerative diseases such as HD and ALS.

### **ER-phagy in neurons**

Focused ion-beam scanning electron microscopy of mouse brain tissue has produced 3D reconstructions of the ER in neurons. Images emphasize the extension of ER tubules throughout all neuronal compartments and highlight the numerous contacts made by the ER with organelles, such as mitochondria, and the plasma membrane [191]. Given this extensive ER structure, it is unsurprising that active turnover via autophagy is required to remodel and renew this organelle.

A key pathway for this remodeling involves FAM134B or reticulophagy regulator 1 (RETREG1), an ER-anchored member of the reticulon family of proteins, which binds to both LC3 and GABARAP via

a LIR motif and can remodel proteins through its reticulon homology domain (RHD; [192]). Mutations in FAM134B cause sensory and autonomic neuropathy [32], indicating a key role for this protein in maintaining neuronal health. Consistent with a role in selective autophagy, overexpression of FAM134B causes fragmentation of the ER, followed by autophagosome sequestration and lysosomal degradation [192]. Conversely, depletion of FAM134B or expression of disease-associated mutants causes ER expansion. In mice, disruption of the gene encoding FAM134B induced neurodegeneration of peripheral sensory neurons, similar to the phenotypes seen in human patients [32].

Recent insight into the mechanisms driving ER-phagy came from recent molecular dynamics simulations of the RHD of FAM134B [193]. The RHD domain can induce membrane curvature in a lipid bilayer and clustering of the FAM134B-RHD amplifies membrane deformation. Thus, FAM134B and other reticulon-like ER-phagy receptors facilitate the membrane remodeling necessary for the autophagy of ER sheets [193].

Additional ER-associated receptors involved in the selective removal of ER have been identified, including cell cycle progression 1 (CCPG1; [194]), reticulon 3 (RTN3; [195]), SEC62 [196], atlastin 3 (ATL3; [197]), and testis expressed 265 (TEX264; [198,199]); these proteins contain LIR motifs that link the ER to the growing phagophore. ATL3 is of particular interest, as it is a dynamin-like GTPase from the atlastin family that binds specifically to GABARAP rather than LC3 [197]. ATL3 is mutated in hereditary sensory and autonomic neuropathy type 1 (HSAN1), a neurological disorder characterized by nerve abnormalities in the legs and feet [200,201]. Disease-associated mutations impair binding to GABARAP and disrupt starvation-induced ER-phagy in cell lines [197], although the effects on ER-phagy in neurons have not yet been fully characterized. Furthermore, ATL3 has been shown to affect ER-mitochondrial contacts, while disease-linked mutations affect mitochondrial distribution [202] and axon outgrowth [203] in primary cortical neurons.

TEX264 is a newly identified ER-phagy receptor, a single-pass transmembrane protein localized to the ER with a LIR motif that can bind to LC3B and GABARAP proteins [198,199]. TEX264 has a major role in remodeling the ER proteome in response to nutrient deprivation [199], but the role of this protein in neuronal ER-phagy remains to be assessed. Structural analysis of TEX264 identified a required element, an intrinsically disordered region (IDR) near the LIR motif, that is predicted to extend 25 nm [198]. Similar IDRs were identified in other ER-phagy receptors, including FAM134B, CCPG1, RTN3, and SEC62, leading to the hypothesis that the IDR is required to bridge the gap between the ribosome-

studded ER membrane and the developing autophagosome [198].

The growing number of ER-phagy receptors identified to date raises interesting questions about functional specificity *versus* redundancy. One possibility is that these receptors are selective for the turnover of specific types of ER. For example, FAM134B facilitates the turnover of curved ER sheets [192], while RTN3 and ATL3 regulate the turnover of ER tubules [195,197]. An alternative idea is that the receptors mediate the turnover of different ER-associated proteins [199]. Furthermore, these receptors may be differentially induced by cellular perturbations such as ER stress or nutrient deprivation [197]. Finally, ER-phagy receptors may be differentially expressed within tissues or during development. Thus, some cell types may be uniquely susceptible to mutations in specific components, like the vulnerability of sensory neurons to mutations in either FAM134B or ATL3 [32,200,201]. Further work is clearly required in this emerging area of neuronal cell biology.

### Aggrephagy in neurons

One consistent hallmark of major neurodegenerative diseases, for instance ALS, AD, and HD, is the accumulation of protein aggregates. These observations suggest that neurons are uniquely vulnerable to the accumulation of damaged or misfolded proteins, potentially because of their long-lived nature and highly polarized morphologies. Most cytosolic proteins are cleared by the ubiquitin proteasome system [204], but larger protein aggregates are cleared by autophagy. Some of this clearance is nonselective, as aggregates of either mutant superoxide dismutase (SOD) 1 or mutant Htt are readily engulfed by axonal autophagosomes [13,17]. However, there is strong evidence for the selective recognition and removal of protein aggregates or aggrephagy, which is suggested to be mediated by several receptors, like p62 and autophagy-linked FYVE protein (Alfy).

p62, also known as SQSTM1, is implicated in the selective removal of aggregated proteins. Missense mutations in p62 lead to a range of disorders, including ALS, frontotemporal dementia, and Paget's disease of bone [31]. However, KO of p62 in the mouse does not result in an obvious phenotype of neurodegeneration [5] although there is an accumulation of hyperphosphorylated tau [205]. Behavioral assays indicate increased anxiety and depression in p62 KO mice [205].

In aggrephagy, p62 oligomerizes, mediates phase separation of ubiquitinated/misfolded proteins, and interacts with both LC3B and linear ubiquitin, leading to the bending of membrane around cargo and the effective sequestration of protein aggregates [23,206]. Recently, p62 was shown to directly

interact with FIP200, a scaffolding protein that is part of the autophagic machinery and acts upstream of mATG8 proteins; binding is enhanced by p62 phosphorylation [207]. In reconstituted systems, LC3B can outcompete FIP200 for binding to p62, suggesting that p62 acts upstream during initiation and at later stages during sequestration of ubiquitin condensates in aggrephagy [207]. p62 can also work in concert with other components of the aggrephagy pathway, such as Alf1 [208]. In addition to its functional role in clearing protein aggregates, p62 accumulation is frequently used as a proxy for defective autophagy in cells or tissues.

Alf1, otherwise known as WDFY3, is a molecular scaffold that mediates interactions between cargo and the core members of the autophagy machinery, for example, mATG8 proteins [209]. In stable cell lines, Alf1 acts as an adaptor for aggrephagy [208,210,211], but its involvement in removing cellular debris in neurons is less clear. Alf1 is highly expressed throughout the central nervous system of mice, and deletion of the protein is perinatal lethal; pups are born but exhibit abnormal brain morphology and striking neuronal development defects [209,212]. At the cellular level, there are no widespread autophagy deficits in Alf1 KO mice, but there is a deficit in the selective clearance of ubiquitinated protein aggregates and polyglutamine inclusions in KO MEFs [209].

### Selective autophagy: similar problems, distinct solutions?

There are clear parallels among the three selective autophagy pathways reviewed here. Mitophagy, ER-phagy, and aggrephagy all involve the recruitment of specific receptors that also bind members of the ATG8 family, such as LC3B to facilitate autophagosome formation around selected cargos. In the case of mitophagy and ER-phagy, multiple receptors have been identified that may be partially redundant. For example, mutations in the mitophagy receptor OPTN are sufficient to cause either ALS or glaucoma, but cellular studies suggest that NDP52 can fully compensate for the loss of OPTN when expressed at a high-enough level [52]; however, this has not yet been demonstrated in neurons. Similarly, KO of the specific ER-phagy receptor FAM134B has a surprisingly limited effect in the mouse, but does result in the degeneration of peripheral sensory neurons, consistent with sensory neuropathy seen in human patients with FAM134B mutations [32]. Careful studies will be required to determine whether the multiple receptors mediating either mitophagy or ER-phagy are functionally redundant within neurons, and whether phenotypic differences may result from tissue-specific differences in expression levels. Alternatively, activation of specific receptors may be governed by distinct regulatory control mecha-

nisms, allowing the neuron to react more specifically to metabolic or environmental perturbations. Thus far, neuronal aggrephagy appears distinct from this paradigm, as p62 has a conserved function in the induction of protein aggregates within neurons that does not appear to be redundant with any other receptor.

Several studies have addressed the possible crossover of autophagy receptors between mitophagy and aggrephagy. For example, OPTN was reported to clear protein aggregates in a ubiquitin-independent manner [213]. Similarly, the aggrephagy receptor p62 was initially implicated in mitophagy, although subsequent studies have since shown that while p62 can cluster damaged mitochondria, it does not facilitate their engulfment by the autophagosome [38,52]. The aggrephagy receptor Alf1 has also been implicated in mitochondrial quality control in neurons, although the mechanism involved requires further investigation [214]. In contrast, no crossover of ER-phagy receptors to other selective clearance pathways has been reported. The picture for now remains one in which receptors bind relatively specifically to cellular cargos targeted for degradation, although this may change in future as the clearance of other organelles, such as peroxisomes and lysosomes are explored in more detail. Importantly, there is also a growing literature implicating known mitophagy or aggrephagy receptors such as OPTN or p62 in other cellular pathways, particularly those involved in interferon signaling or inflammation. Exploring the links between autophagy and inflammatory signaling will be an exciting area for future research.

Downstream of receptor recruitment, the pathways for selective autophagy all converge. Binding to LC3B facilitates the formation of an autophagosome around the cargo to be degraded. Some receptors bind equally well or better to other members of the ATG8 family, such as GABARAP; the implications of this selectivity for autophagosome formation deserve further study. Once autophagosomes form, they must fuse with lysosomes to become degradative organelles and this aspect of the pathway does not seem to differ significantly based on the identity of the cargo being degraded. One interesting question is whether different cellular stressors effectively upregulate factors leading to more efficient cargo degradation, such as induction of lysosomal protein synthesis *via* transcription factor EB (TFEB). Again, more work is required to see how this may function in neurons, both *in vitro* and *in vivo*.

### Conclusions

We have highlighted our current understanding of the selective removal of mitochondria, ER, and aggregated proteins in neurons. Although the



signaling cascades are diverse, the pathways discussed here converge on the use of autophagic and endolysosomal machinery to remove cargo, emphasizing the importance of autophagy in the maintenance of neuronal health. Genetic findings continue to identify novel proteins and disease-associated mutations, providing more molecular pieces to the puzzle that is neurodegeneration. For example, our understanding of mitophagy has benefited from the consideration of both PINK1/Parkin-mediated mitochondrial turnover and other complementary pathways that contribute to regulating neuronal mitochondria. In addition, more nuanced approaches are needed to understand the molecular cues and basis for defects. Moving toward *in vivo* studies, it is important to appreciate the molecular overlap that exists among pathways, as mitochondrial dysfunction is implicated in multiple neurodegenerative diseases. It will be important to understand the molecular signaling involved and how that contributes to each disease-state. Mitophagy, ER-phagy, and aggrephagy are the best-characterized selective autophagy pathways in neurons, but other mechanisms like pexophagy, nucleophagy, and lysophagy have been studied in yeast and nonneuronal cell lines. Going forward, it will be of interest to see whether these mechanisms are also conserved in neurons and how they contribute to neuronal homeostasis.

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## Abbreviations used:

KO, knock-out; ER, endoplasmic reticulum; LC3, light chain 3; LIR, LC3-interacting region; ALS, amyotrophic lateral sclerosis; PD, Parkinson's disease; ROS, reactive

oxygen species; OMM, outer mitochondrial membrane; Mfn, mitofusin; OPTN, optineurin; MEF, mouse embryonic fibroblast; MDV, mitochondrial derived vesicle; SNPH, syntaphilin; AD, Alzheimer's disease; HD, Huntington's disease; A $\beta$ , amyloid- $\beta$ ; IFN, interferon; RHD, reticulon homology domain; IDR, intrinsically disordered region.

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