Neuronal Mitophagy: Lessons from a Pathway Linked to Parkinson's Disease

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Abstract

Neurons are specialized cells with complex and extended architecture and high energy requirements. Energy in the form of adenosine triphosphate, produced essentially by mitochondrial respiration, is necessary to preserve neuronal morphology, maintain resting potential, fire action potentials and ensure neurotransmission. Pools of functional mitochondria are required in all neuronal compartments, including cell body and dendrites, nodes of Ranvier, growth cones, axons and synapses. The mechanisms by which old or damaged mitochondria are removed and replaced in neurons remain to be fully understood. Mitophagy has gained considerable interest since the discovery of familial forms of Parkinson's disease caused by dysfunction of PINK1 and Parkin, two multifunctional proteins cooperating in the regulation of this process. Over the past ten years, the molecular mechanisms by which PINK1 and Parkin jointly promote the degradation of defective mitochondria by autophagy have been dissected. However, our understanding of the relevance of mitophagy to mitochondrial homeostasis in neurons remains poor. Insight has been recently gained thanks to the development of fluorescent reporter systems for tracking mitochondria in the acidic compartment of the lysosome. Using these tools, mitophagy events have been visualized in primary neurons in culture and *in vivo*, under basal conditions and in response to toxic insults. Despite these advances, whether PINK1 and Parkin play a major role in promoting neuronal mitophagy under physiological conditions in adult animals and during aging remains a matter of debate. Future studies will have to clarify in how far dysfunction of neuronal mitophagy is central to the pathophysiology of Parkinson's disease.

Introduction

Mitophagy is a form of cargo-selective autophagy removing unnecessary mitochondria, or clearing aged or dysfunctional mitochondria to protect the cells from their deleterious effects. More than fifty years ago, electron microscopy images revealed the presence of altered mitochondrial profiles in atypically enlarged lysosomal vesicles in the liver and kidney, under pathological conditions (Ashford and Porter 1962; De Duve and Wattiaux 1966; Novikoff, 1959; Novikoff and Essner, 1962). This phenomenon was interpreted as progressive mitochondrial degeneration (Novikoff and Essner, 1962). However, the term mitophagy was coined more recently with the emergence of evidence, initially from yeast, indicating that the autophagy of mitochondria does not occur at random, but rather reflects a selective program (Kissova et al. 2004; Lemasters 2005). The term was then used to describe the autophagy-dependent degradation of mitochondria in cultured hepatocytes, following nutrient deprivation or laser-induced photodamage (Kim et al. 2007; Rodriguez-Enriquez et al. 2006). An earlier precursor study in cultured primary sympathetic neurons reported complete removal of mitochondria under conditions of apoptosis induction in the presence of caspase inhibitors, presumably associated with loss of mitochondrial membrane potential and cyrochrome *c* release (Tolkovsky et al. 2002). These studies highlighted a role for mitophagy in the regulation of mitochondrial degradation under conditions of metabolic remodelling or severe mitochondrial damage. Other types of programmed mitophagy were recognized to be central to specific developmental programs: during the maturation of erythrocytes, whereby the cells get rid of their mitochondria to acquire the ability to transport oxygen (Sandoval et al. 2008; Schweers et al. 2007); following fertilization of the oocyte, a stage marked by the removal of paternal mitochondria, underlying the universal principle of maternal mitochondrial inheritance in animals (Al Rawi et al. 2011; Sato and Sato 2011); during cardiomyocyte maturation in the neonatal period, whereby fetal mitochondria are replaced by adult mitochondria to sustain the transition from carbohydrates to fatty acid metabolism (Gong et al. 2015); or during the differentiation of myoblasts into mature myotubes, during which a distinct network of mitochondria replaces the old population to support the metabolic switch from glyocolysis to oxidative phosphorylation (Sin et al. 2016).

It was not until 2008 that mitophagy emerged as a mitochondrial quality control mechanism of potential relevance to neuronal cells, with the groundbreaking discovery of the involvement of the RING-IBR-RING E3 ubiquitin ligase Parkin in the clearance of severely damaged mitochondria (Narendra et al. 2008). Parkin is encoded by the *PARK2* gene, which carries loss-of-function mutations in autosomal recessive forms of Parkinson's disease (PD), a common most often sporadic movement disorder caused by the progressive degeneration of the dopaminergic (DA) neurons in a region of the midbrain termed *substantia nigra pars compacta* (*SNc*). Why these neurons die in PD remains a mystery.

However, mitochondrial dysfunction had been suspected to play a role since the 1980s, when the mitochondrial neurotoxin MPTP was identified as the cause of Parkinsonism in young people consuming drugs (Corti et al. 2011; Exner et al. 2012; Langston and Ballard 1983; Schapira and Gegg 2011). The discovery of Parkin-dependent mitophagy provided a cellular mechanism leading to mitochondrial dysfunction in PD. The role of this mechanism was strengthened a few years later, when work from several laboratories demonstrated that the product of another gene responsible of autosomal recessive PD, the mitochondrial serine/threonine kinase PINK1, was central to the activation of Parkin and its mitochondrial recruitment during mitophagy (Geisler et al. 2010; Matsuda et al. 2010; Narendra et al. 2010). Thanks to an unprecedented collective effort of the scientific community in the field during the past ten years, enlightened by recent descriptions of the crystal structure of Parkin (Byrd and Weissman 2013; Caulfield et al. 2015; Gladkova et al. 2018; Kumar et al. 2017a; Riley et al. 2013; Sauvé et al. 2018; Seirafi et al. 2015; Spratt et al. 2014; Trempe et al. 2013; Wauer and Komander 2013) and PINK1 (Kumar et al. 2017b; Okatsu et al. 2018; Schubert et al. 2017), we have gained a comprehensive view of the molecular mechanisms underlying this specific mitophagy program, which have been summarized in excellent recent reviews (McWilliams and Muqit 2017; Truban et al. 2017). Although other mitophagy programs have been studied at the molecular level (Chu 2018; McWilliams and Muqit 2017), PINK1/Parkin-dependent mitochondrial clearance is undoubtedly the best characterized. Comparatively, however, our understanding of the relevance of PINK1/Parkin-dependent mitophagy to mitochondrial homeostasis in neurons, particularly in those that degenerate in PD, remains poor (Jang et al. 2018; Palikaras et al. 2018).

PINK1/Parkin-dependent mitophagy in brief

Our current understanding of PINK1/Parkin-dependent mitophagy is that it is a program activated by specific stress-related stimuli. Its activation depends on mitochondrial protein import arrest, caused either by toxins leading to mitochondrial depolarization, such as the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Geisler et al. 2010; Matsuda et al. 2010; Narendra et al. 2008; Narendra et al. 2010), by dysfunction of protein import components or proteases involved in PINK1 processing (Bertolin et al. 2013; Greene et al. 2012; Jin et al. 2010), or by unfolded protein stress in the mitochondrial matrix (Fiesel et al. 2017; Jin and Youle 2013). These conditions interfere with the translocation of PINK1 into the organelle, leading to its accumulation in proximity of the translocase of outer mitochondrial membrane (Bertolin et al. 2013; Hasson et al. 2013; Lazarou et al. 2012; Okatsu et al. 2012; Okatsu et al. 2013), its dimerization and autophosphorylation, the phosphorylation of ubiquitin moieties associated with the outer mitochondrial membrane (Koyano et al. 2014; Kane et al. 2014; Kazlauskaite et al. 2014; Matsuda 2016),

the recruitment and activation of the otherwise inactive Parkin ligase (Riley et al. 2013 Trempe et al. 2013; Wauer and Komander 2013), by binding to phospho-ubiquitin, and its stabilization in an active state through the PINK1-dependent phosphorylation of its N-terminal ubiquitin-like domain (Byrd and Weissman 2013; Caulfield et al. 2015; Chaugule et al. 2011; Gladkova et al. 2018; Kazlauskaite and Muqit 2015; Kondapalli et al. 2012; Ordureau et al. 2015; Ordureau et al. 2014; Sauve et al. 2018; Shiba-Fukushima et al. 2012; Spratt et al. 2014; Yamano et al. 2015). As a consequence, Parkin ubiquitylates a number of proteins of the outer mitochondrial membrane by forming different types of ubiquitin chains, which are further phosphorylated by PINK1, recruiting additional Parkin molecules, and thereby feeding an amplification loop (Okatsu et al. 2015; Ordureau et al. 2015; Ordureau et al. 2014; Sarraf et al. 2013). This process is negatively regulated by specific deubiquitylases (Bingol et al. 2014; Cornelissen et al. 2014; Durcan et al. 2014). The ubiquitylation of mitochondrial proteins leads to the recruitment of specific ubiquitin-binding autophagy receptors, including NDP52 and optineurin, which in turn promotes association of upstream autophagy-related proteins, thereby priming mitochondria for autophagy (Heo et al. 2015; Itakura et al. 2012; Lazarou et al. 2015; Wei et al. 2017; Yamano et al. 2018). Evidence has been provided that mitophagy is initiated in proximity of contact sites between mitochondria and the endoplasmic reticulum (ER) (Gautier et al. 2016; Gelmetti et al. 2017; Yang and Yang 2013), and that it requires dissociation between the two organelles to proceed efficiently (McLelland et al. 2018). This is achieved by a rapid burst of ubiquitylation of the Parkin substrate Mitofusin 2 (Mfn2), localized on both outer mitochondrial and ER membranes, which in turn leads to disassembly of Mfn2 complexes and destruction of the mitochondria-ER contact (McLelland et al. 2018).

Neurons: cells on a tight energy budget

The human brain does not exceed 2% of the body's weight but its energy budget represents 20% of that of the body (Harris et al. 2012; Mink et al. 1981). Whilst neural activity drives ATP synthesis by both glycolysis and oxidative phosphorylation, most of the brain ATP is generated by oxidative phosphorylation (Hall et al. 2012; Rangaraju et al. 2014). This makes brain cells highly reliant on mitochondria. Neurons have a uniquely complex cellular architecture intimately linked to their role in information processing and transmission. They are composed of a cell body containing the nucleus and enriched in other essential organelles, including the Golgi apparatus, the endoplasmic reticulum, lysosomes and mitochondria. A system of highly branched dendrites adapted to receive information from other neurons emerge from the cell body, as well as a single extension of variable length, the axon, responsible for transmitting the electrical signal. The axon ramifies into several terminals that pass the signal to the dendrites of other neurons through the specialized compartment of the synapse. In recent years, thanks to the development of techniques for

single neurons tracing and 3-dimensional reconstruction of neuronal morphology, it has been calculated that individual neurons can generate axons of tens of centimeters in length in the rodent brain (Matsuda et al. 2009; Wu et al. 2014). Extrapolations based on these studies and on available morphometric data predict that in the human brain such axons reach lengths of up to tens of meters (Bolam and Pissadaki 2012; Matsuda et al. 2009). DA neurons of the *SNc*, in particular, have an unmyelinated, extremely large and highly branched axonal arbor. In humans, the average total length of the axonal arbor of a single DA neuron of the *SNc* has been estimated to exceed 4.5 m (Bolam and Pissadaki 2012). Such an axon can generate up to 2.4 million synapses in the projection region of the striatum.

Neurons require a lot of energy to preserve their architecture, maintain the resting potential, fire action potentials and ensure neurotransmission in the pre-and post-synaptic compartments (Attwell and Laughlin 2001). Based on a computational model for DA neurons integrating their morphological complexity and electrophysiological properties, it has been calculated that the energy cost associated with axon potential propagation and recovery of resting membrane potential increases exponentially with the number of levels of branches of the axon, and according to a power law of the axonal surface area and number of branch points (Pissadaki and Bolam 2013). The disproportionately higher energy demand associated with the large and highly branched axonal arbor of the *SNc* DA neurons compared to that of smaller axons, such as those of the DA neurons in the less susceptible ventral tegmental area (VTA), may well be one of the determinants of the special vulnerability of these neurons in PD. In a recent study, it was indeed shown that cultured mouse *SNc* DA neuron display larger axonal arbors than cultured DA neurons from the VTA of the olfactory bulb (Pacelli et al. 2015). This property correlated with higher density of mitochondria specifically in the axonal compartment, higher oxygen consumption rates, higher levels of reactive oxygen species and greater sensitivity to mitochondrial neurotoxins. Strikingly, treatment of the *SNc* DA neurons with Semaphorin 7A, a guidance molecule known to modulate axonal arborization in these neurons, reduced the size of the axonal arbour and at the same time diminished oxygen consumption rates and neuronal vulnerability to toxins, highlighting the intimate relationship between the complex morphological architecture of these neurons and their energy requirement and special sensitivity.

Mitochondrial homeostasis in neurons: how are mitochondria rejuvenated?

In contrast to other cell types in the body, neurons do not divide and are therefore destined to last a lifetime. Mitochondria are required in different neuronal compartments and have been shown to accumulate at nodes of Ranvier, in growth cones, axonal branches

and synapses (Hollenbeck and Saxton 2005). How do neurons manage to maintain appropriate pools of healthy mitochondria throughout their complex architecture and the lifespan of the organism? A combination of functionally intertwined mechanisms that are active in other cell types are also at play here (Misgeld and Schwarz 2017; Rugarli and Langer 2012; Shutt and McBride 2013). These include (1) transport from the cell soma to remote peripheral sites in the dendritic arbor, axon and axonal terminals; (2) fusion and fission cycles that shape the morphology and size of the organelle to adapt them to metabolic demand, transport and clearance; (3) pathways for the degradation of individual mitochondrial proteins, portions of mitochondria or whole organelle; and (4) biogenesis of individual components and whole organelles. Studies in cultured neurons and in various *in vivo* models, including C elegans, Drosophila, zebrafish and mouse, have demonstrated that although most mitochondria are stationary, a fraction of about 10%-40% move along axons at any given time (Misgeld and Schwarz 2017). To enter the axon, a mitochondrion has to be separated from the network (Berthet et al. 2014; Verstreken et al. 2005), and in the axon, it can move in both anterograde and retrograde directions. However, the proportion of mitochondria moving from the soma to the peripheral arbor is higher than that transported back from the periphery to the cell body (Misgeld et al. 2007; Pilling et al. 2006; Plucinska et al. 2012; Wang et al. 2011), which lends support to the idea that that “old” stationary mitochondria are, at least in part, turned over at axonal terminals.

Calculations of protein turnover rates based on isotopic labeling at the organism level suggest that mitochondria have an average lifetime of several days, a duration that appears to be conserved during evolution and longer in the brain than in other organs (Price et al. 2010; Vincow et al. 2013). The “rejuvenation” of mitochondria through the replacement of individual components or whole organelles, particularly at distal sites, is probably operated by several parallel pathways. It is admitted that mitochondrial biogenesis is not restricted to the neuronal cell body (Harbauer 2017). Transcripts for nucleus-encoded mitochondrial proteins are present in axons (Aschrafi et al. 2016; Shigeoka et al. 2016), which are endowed with the protein synthesis machinery (Koenig and Giuditta 1999), and there is evidence for replication of mitochondrial DNA (mtDNA) in axons (Amiri and Hollenbeck 2008). Moreover, the smooth endoplasmic reticulum populates even the most remote neuronal compartments, including the thin branches of the axon terminals, often interacting closely with mitochondria, providing the capacity for local membrane lipid biosynthesis (Berridge 1998; Wu et al. 2017). In principle, mitochondrial protein synthesis and mitochondrial biogenesis may thus occur in the neuronal periphery, although the extent to which this is indeed the case has yet to be fully appreciated (Saxton and Hollenbeck 2012). Fusion with younger mitochondria is another key mechanism by which mitochondrial competence is preserved in neurons, through the redistribution of essential mitochondrial constituents and the dilution of damaged components. The relevance of this process to neurons is illustrated by the consequence of mutations in

genes encoding components of the mitochondrial fusion machinery, which cause neurodegenerative diseases in humans (Chen and Chan 2010). Finally, new mitochondria may be supplied in neurons by non-canonical pathways, such as through the transfer of mitochondria from astrocytes (Hayakawa et al. 2016), although the extent to which this occurs awaits further experimental corroboration (Berridge et al. 2016).

How are "old" or damaged mitochondrial components or whole organelles degraded in neurons? Several complementary pathways for the turnover of mitochondrial components have been identified, and again their importance to neuronal homeostasis is underscored by the neurodegenerative diseases engendered by their dysfunction linked to genetic mutations (Misgeld and Schwarz 2017; Rugarli and Langer 2012). These involve the removal of individual proteins by mitochondrial proteases or by the cytosolic proteasome, the vesicular delivery of mitochondrial cargo to the lysosome through mitochondria-derived vesicles (MDVs), intraneuronal mitophagy, and transcellular mitophagy (Table 1). The relevance of each of these pathways, the physiological circumstances in which they are solicited and the specific subcellular compartments in which they take place remain to be fully understood. Notably, in addition to its role in mitophagy, the PINK1/Parkin pathway has also been involved in the regulation of the degradation of outer mitochondrial membrane proteins by the ubiquitin-proteasome pathway (Tanaka and Youle 2010, Karbowski & Youle 2011), and in the delivery of selected damaged mitochondrial components to the lysosome by MDVs (McLelland et al. 2014; McLelland et al. 2016; Sugiura et al. 2014) (Table 1).

Mitophagy in cultured neurons: evidence for or against

Most studies investigated mitophagy in immortalized cell lines, following treatment with chemical uncouplers or inhibitors of the mitochondrial respiratory chain. The question of whether this process is relevant to primary cells, particularly to neurons, under physiological conditions, has been highly debated (Grenier et al. 2013). Relatively quickly after the discovery of Parkin-depedent mitophagy, studies in different primary neuronal models, including DA neurons differentiated from induced pluripotent stem cells (iPSCs), provided evidence for mitochondrial translocation of exogenously expressed Parkin (Cai et al. 2012; Joselin et al. 2012; Seibler et al. 2011; Wang et al. 2011) in cells treated with various mitochondrial toxins. As in other cell types, recruitment of Parkin to mitochondria was dependent on the presence of PINK1 (Seibler et al. 2011). Very recently, it was also shown that exposure to mitochondrial uncouplers stabilizes endogenous PINK1 (Oh et al. 2017; Soutar et al. 2018) and activates endogenous Parkin in primary rodent and human neurons, according to the same molecular mechanisms identified in cell lines overexpressing Parkin (Barini et al. 2018; Oh et al. 2017; Ordureau et al. 2018; McWilliams 2018a).

Some studies provided direct evidence for mitophagic events in neurons. By co-expressing fluorescent proteins targeted to autophagic vesicles (GFP-LC3) or lysosomes (GFP-LAMP1), is was shown that autophagosomes are co-recruited to Parkin-positive mitochondria, and that mitochondria are engulfed by lysosomal vesicles in primary mouse cortical neurons treated with the protonophore carbonyl-cyanide m-chlorophenyl-hydrazone (CCCP) (Cai et al. 2012). These authors also showed accumulation of depolarized mitochondria in cells silenced for endogenous Parkin, strongly supporting the involvement of mitophagy in mitochondrial maintenance in neurons subjected to acute mitochondrial stress. At the same time, however, other researchers reported absence of Parkin tanslocation to mitochondria or recruitment of autophagic vesicles in primary rat cortical neurons exposed to a mitochondrial uncoupler, using similar approaches (Van Laar et al. 2011). These authors proposed that the bioenergetic properties of neurons, their strong reliance on oxidative phosphorylation and reluctance to switch to glyocolytic metabolism under mitochondrial stress, preclude the occurrence of mitophagy. Consistent with this possibility, mitochondrial depolarization triggered by the potassium ionophore valinomycin did not lead to appreciable loss of mitochondrial markers in human iPSc-derived neurons, even following overexpression of Parkin (Rakovic & Klein 2012). However, reductions in mtDNA copy number were reported under these conditions, a phenomenon that was not observed in neurons from patients with *PINK1* mutations (Seibler et al. 2011). Others have confirmed that mitochondrial proteins are also cleared to a certain extent in human iPSc-derived neurons treated with CCCP (Soutar et al. 2018). Careful inspection of the culture conditions across the different studies (Grenier et al. 2013) revealed that authors reporting massive mitochondrial Parkin translocation or signs of mitophagy in neurons had supplemented the medium with apoptosis inhibitors (Cai et al. 2012) or antioxidants (Joselin et al. 2012). Thus, massive neuronal death may mask the occurrence of mitophagy, which is in line with the recent discovery that Parkin not only promotes mitophagy but also sensitized towards apoptosis under conditions of acute mitochondrial stress (Carroll et al. 2014; Zhang et al. 2014).

Global depolarization of the mitochondrial network is unlikely to occur under physiological conditions. In such conditions, mitophagy would rather occur as a local process removing depolarized mitochondrial fragments. To mimic such a situation, some researchers used the mitochondrion-targeted red fluorescent protein mt-KillerRed to photosensitize small subsets of mitochondria in the axonal compartment of rat hippocampal neurons cultured in microfluidic devices (Ashrafi et al. 2014). Using time-lapse video microscopy, they observed local recruitment of GFP-LC3-positive autophagic vesicles and LAMP1-YFP-positive lysosomes to damaged mitochondria as early as 20 minutes following irradiation. In some cases they also reported disappearance of single mitochondria from these structures. These events were observed in the absence of antioxidants in the culture medium, and occurred to a much lesser extent in neurons from Parkin- or PINK1-deficient mice,

demonstrating reliance on the PINK1/Parkin pathway. This study demonstrated that mitophagy occurs locally in the distal axonal compartment. Similar observations were made more recently by Hsieh and colleagues in iPSC-derived neurons exposed to the mitochondrial complex III inhibitor antimycin A (Hsieh et al. 2016). These authors also showed a delay in axonal mitophagy in neurons from PD patients with mutations in *LRRK2* or from sporadic PD patients. This contrasts with the conclusion drawn by Cai and colleagues, who observed accumulation of Parkin-positive mitochondria and associated autophagosomes essentially in the somatodendritic compartment of neurons treated with CCCP for 24 hours (Cai et al. 2012). Based on these observations, it was suggested that autophagic vesicles containing damaged mitochondria are transported back to the soma to be degraded by cytoplasmic lysosomes. This later response may however reflect a compensatory mechanism due the overwhelming of the axonal lysosomes.

All studies mentioned above investigated mitophagy under conditions of acute and severe mitochondrial stress, but what about neuronal mitophagy under conditions of mild mitochondrial stress, which is more relevant to the chronic progressive mitochondrial dysfunction that characterizes neurodegenerative diseases? Lin and colleagues (Lin et al. 2017) recently proposed that mitophagy may not be the main mechanism by which defective mitochondria are removed from axons under conditions of reversible mitochondrial depolarization triggered by low doses of antimycin A. In this case, damaged mitochondria were removed from axons by retrograde transport to the soma, a process that was enhanced by the release from mitochondria of the axonal mitochondrial anchoring protein syntaphilin. Finally, basal mitophagy was explored in cultured hippocampal neurons, using the ratiometric mitochondrion-targeted pH-sensitive biosensor mt-Keima, enabling differentiation between mitochondria in the cytoplasm and mitochondria in the acidic microenvironment of the lysosome (Bingol et al. 2014). This study showed progressive accumulation of the biosensor in lysosomes, mostly in the neuronal soma, consistent with ongoing mitophagy in the absence of Parkin overexpression or toxins. The delivery of mitochondria to lysosomes was reduced upon silencing of PINK1 or Parkin using small hairpin RNAs, suggesting that these proteins are required for basal mitophagy, in addition to stress-induced mitophagy. However, this view has been challenged by recent *in vivo* studies, as will be discussed in the next section.

In vivo mitophagy in the nervous system: a controversial issue

Early attempts to determine the turnover rates of mitochondria in different tissues in the 1960ies were based on the *in vivo* radiolabeling of protein and lipid components in rats. Although some of these reports investigated the turnover of different categories of mitochondrial proteins, e.g. water-soluble, water-insoluble, structural, contractile and/or included the analysis of cytochrome c (Fletcher & Sanadi 1961; Beattie et al., 1967), in

general these studies did not take into account the half-lives of individual mitochondrial proteins. Based on the observation that insoluble and soluble protein, lipid and cytochrome c from rat liver mitochondria turned over at near identical rates, Fletcher and Sanadi proposed for the first time that mitochondria are synthesized and broken down as discrete entities (Fletcher and Sanadi 1961). Later reports of some heterogeneity in the turnover of mitochondrial components in different tissues, including the brain, lend support to the possibility that individual mitochondrial components may be degraded independently, but did not fundamentally challenge the idea that a mitochondrial structural unit is turned over as an entity (Beattie et al. 1967; Cuzner et al. 1966; Gross and Rabinowitz 1968). Together with first observations of mitochondria inside lysosomes by electron microscopy (Ashford and Porter 1962; De Duve and Wattiaux 1966; Novikoff, 1959; Novikoff and Essner, 1962; Swift and Hruban 1964), these pioneering studies anticipated the discovery of mitophagy, and strongly supported the existence of a mechanism for the degradation of mitochondrial entities *in vivo*. This issue was reinvestigated more recently in Drosophila melanogaster, in a proteomic assay based on the feeding of adult flies with deuterated leucine and the use of mass spectrometry analyses to monitor simultaneously the half-lives of numerous mitochondrial and non-mitochondrial proteins (Vincow et al. 2013). Parallel studies in wild type, *parkin* null and autophagy-deficient *Atg7* null flies showed prolonged half-lives for nearly 150 mitochondrial proteins in both mutant strains, and significant correlation between the effects of *Atg7* and *parkin* mutations, specifically for this set of proteins, and not for proteins targeted to other organelles known to be degraded by autophagy. This study provided the first evidence for a role of *parkin* in the regulation of mitophagy *in vivo*. Intriguingly, the turnover of a subset of 40 mitochondrial proteins, including 19 subunits of respiratory chain components representative of the five respiratory complexes, appeared to depend more on Parkin than on Atg7, and there was strong correlation between the effect of *parkin* mutation and the effect of *pink1* mutation on the turnover of these components (Vincow et al. 2013). This suggested the existence of a degradation process dependent on PINK1 and Parkin but independent of autophagy, possibly involving the MDV pathway (Vincow et al. 2013). On the other hand, no change in mitochondrial density was observed in Drosophila larval motor neurons axons or cell bodies, as would have been predicted by the reported role of the PINK1/Parkin pathway in mitophagy (Devireddy et al. 2015). Abnormally large, discrete mitochondria did, however, accumulate in the neuronal cell bodies, suggesting that mitochondrial degradation may be limited to this neuronal compartment in the nervous system *in vivo*.

Several groups made efforts towards clarifying the relevance of PINK1/Parkin-dependent mitophagy to dopaminergic neurons in mouse models, specifically in the context of accelerated accumulation of mtDNA defects (Pickrell et al. 2015; Pinto et al. 2018; Song et al. 2017; Sterky et al. 2011). The rationale for these studies came from the observation that nigral DA neurons in humans are more prone to accumulate mtDNA deletions than

neurons in other brain regions (Bender et al. 2006; Kraytsberg et al. 2006). Such defects are compensated for by an increase in the number of normal mtDNA copies in neurologically healthy individuals but not in PD patients (Dolle et al. 2016), possibly due to alterations in mitochondrial biogenesis (Grunewald et al. 2016). It has thus been suggested that PINK1/Parkin-dependent mitophagy may play a key role in counterselecting mitochondria with high mtDNA mutational loads.

To address the relevance of this mechanism *in vivo*, *Parkin* knockout mice were crossed with different transgenic mice modeling the accumulation of mtDNA deletions or mutations: the MitoPark mouse, with SN DA neuron-specific knockout of mitochondrial transcription factor A, an integral component of the basal mitochondrial transcription machinery with an additional role in the regulation of mtDNA copy number (Sterky et al. 2011); the Mutator mouse, homozygous for a mutation affecting the proofreading activity of DNA polymerase γ, responsible for mtDNA replication (Pickrell et al. 2015); a mouse model expressing a mutant version of the Twinkle helicase, involved in mtDNA replication, specifically in SN DA neurons (Song et al. 2017); and the PD-mito-Pst I mouse, in which a mitochondrion-targeted version of the restriction enzyme PstI is selectively expressed in SN DA neurons (Pinto et al. 2018). While the MitoPark, mutant Twinkle and PD-mito-Pst I models developed age-dependent PD-like phenotpes associated with progressive degeneration of SN DA neurons and defects in locomotor behavior (Ekstrand et al. 2007; Pickrell et al. 2011; Song et al. 2012), the accumulation of somatic mtDNA mutations in Mutator mice led to premature aging in the absence of overt neurodegeneration (Kujoth et al. 2005; Trifunovic et al. 2004). Suprisingly, Parkin-deficiency affected differently the phenotypes in these mice. There was no modification of the mitochondrial morphological alterations or neurodegenerative process characteristic of the MitoPark model (Sterky et al. 2011). In contrast, mtDNA mutation load or predicted pathogenicity, mitochondrial dysfunction, DA neurodegeneration and behavioral defects were anticipated or exacerbated in the three other models. Despite different interpretations about whether the observed effects reflected or not a physiological role of Parkin in the clearance of mitochondria with high mtDNA mutation loads, none of these studies were based on the direct exploration of the mitophagy process.

Direct investigation of mitophagy *in vivo* has only recently been rendered possible by the development of specific fluorescent reporters with acid-labile components, including the already mentioned mt-Keima and mito-QC, a mitochondrion-targeted tandem mCherry-GFP protein (Rodger et al. 2018). These reporters have been used to generate transgenic Drosophila and mouse models and explore the presence of mitochondria within lysosomes under basal conditions across organs and tissues (Cornelissen et al. 2018; Lee et al. 2018; McWilliams et al. 2016; Sun et al. 2015). It should be noted, however, that these reporters cannot formally discriminate between autophagy-dependent and autophagy-independent events, such as those mediated by the MDV pathway, unless systematic parallel analyses

are performed in corresponding autophagy-deficient models (Table 1). Nevertheless, in all these studies, quenching of the acid-labile fluorescent component in the lysosome has been exclusively interpreted in terms of mitophagy. In Drosophila, such structures were abundant in various tissues during development, including in the nervous tissue, and were not observed following deletion of Atg5 or overexpression of a kinase-dead version of Atg1 (Cornelissen et al. 2018; Lee et al. 2018). Importantly, in the adult brain, mitophagy events were readily detected in DA neuron clusters reported to degenerate in *pink1* and *parkin* mutant flies, albeit with some differences depending on the reporter used. For example, using mito-QC, the steady-state levels of the supposed mitophagy were found to be stable during aging in the DA neurons from the PPL1 cluster of the posterior inferiorlateral protocerebrum (Lee et al. 2018), whereas in mt-Keima flies, the mitophagy index increased by 30 % between one and four weeks of age (Cornelissen et al. 2018). In addition, in mito-QC flies mitophagy events were not detectable in muscle tissue, which is also affected in *pink1* and *parkin* mutants (Lee et al. 2018). In contrast, mitolysosomes were observed in the indirect flight muscles of mt-Keima flies, where they were also found to increase fourfold in abundance between week 1 and week 4 (Cornelissen et al. 2018). These discrepancies highlight potential differences in the sensitivities of the biosensors used to track mitochondria in lysosomes. Consistent with this possibility, the use of mt-Keima revealed a 20-fold higher mitophagy index in the DA neurons of the PPL1 cluster than in the indirect flight muscles (Cornelissen et al. 2018), indicating that mitophagy occurs only rarely in muscles, where it may remain below the detection limit when monitored with mito-QC (Lee et al. 2018). Notably, mt-Keima is a mitochondrial matrix protein, whereas mito-QC is targeted to the outer mitochondrial membrane. As reported for proteins of this submitochondrial compartment (Karbowski and Youle 2011), mito-QC may thus by subject to degradation by the ubiquitin-proteasome system in addition to mitophagy, which could act as another source of bias. For example, mitophagy may be artificially underestimated, as a subset of mitochondrial units potentially undergoing mitophagy will be missed because of prior degradation of the fusion protein. On the other hand, mt-Keima cannot be analyzed in fixed samples, because fixation compromises the lysosomal pH gradient, and this may engender signal variability. Moreover, there is partial overlap in the Keima excitation spectrums for red and green fluorescence, which can complicate the interpretation of the data obtained with this probe.

In mice, events interpreted as mitophagy were detected using the same reporters in a range of tissues with high metabolic activity, such as heart and skeletal muscle, kidney, liver, pancreas and brain (McWilliams et al. 2016; McWilliams et al. 2018b; Sun et al. 2015). Here, mitolysosomes were particularly abundant in the Purkinje cell layer of the cerebellum and in regions enriched with neural stem cells, such as the lateral ventricle and the dentate gyrus in the hippocampus (McWilliams et al. 2016; Sun et al. 2015). Moreover, mitolysosomes were abundant in different DA neuron populations, including the

highly vulnerable A9 *SNc* neurons that degenerate in PD, but also the less susceptible A10 DA neurons of the VTA, and the A16 periglomerular DA neurons of the olfactory bulb, known to increase in number in PD (McWilliams et al. 2018b). In these neurons, mitolysosomes were rare in the axonal arbors, whereas they were enriched in the somata and axon initial segments. In slices of the mesencephalon prepared *ex vivo* from mice following viral vector-mediated delivery of mt-Keima, the basal rate of mitophagy turned out to be significantly higher in DA neurons of the *SNc* than in DA neurons of the VTA, or in other basal ganglia neurons (Guzman et al. 2018). Remarkably, chronic treatment of the mice with the Cav1 channel inhibitor isradipine significantly reduced basal mitophagy in *SNc* DA neurons. Cav1 channel-mediated calcium entry into the somatodendritic compartment stimulates mitochondrial intermediary metabolism and oxidative phosphorylation in these neurons to sustain the bioenergetic demand associated with their characteristic autonomous pacemaking activity; this occurs at the cost of an increase in the generation of mitochondrial reactive oxygen species (Dragicevic et al. 2015). In addition to normalizing basal mitophagy, israpidine mitigated oxidative stress in *SNc* neuron, suggesting that this mechanism acts as a key determinant of vulnerability in *SNc* DA neurons (Guzman et al., 2018).

In mito-QC mice, the levels of basal mitophagy did not appear to be affected by loss-of-function mutations of *PINK1* or *PARK2*, supporting the idea that the PINK1/Parkin-dependent mitochondrial clearance program is not active under basal conditions (McWilliams et al. 2018a, b). Alternatively, complementary mitophagy pathways may be activated in the absence of PINK1, as previously suggested (Dagda et al. 2009; Vincow et al. 2013). However, future studies will have to more carefully investigate the possible impact of PINK1/Parkin deficiency on basal mitophagy, particularly during aging and under stress. Sun *et al*. reported a 70% decrease in mitophagy in dentate gyrus neurons in 21-month-old mice compared to 3-month-old animals (Sun et al. 2015), but mitophagy in the absence of PINK1 was only investigated up to 9.5 months of age (McWilliams et al. 2018b). Moreover, although Lee and colleagues did not observe changes in basal mitophagy in the absence of PINK1 in Drosophila (Lee et al. 2018), Cornelissen and his team reported impairment of the age-dependent increase in mitolysosome abundance in flight muscles and dopaminergic neurons of *pink1* and *parkin* mutant flies (Cornelissen et al. 2018). Remarkably,

Conclusions

Since early studies in the 1960s based on classical *in vivo* radiolabeling approaches in rodents to investigate the turnover of protein and lipid components and postulating the existence of a mechanism ensuring the degradation of mitochondria as discrete entities, this hypothesis has been verified in the past fifteen years by the identification of specific programs for the autophagy-dependent destruction of mitochondria. One of these

programs is activated in cell culture by mitochondrial stress and regulated by the protein products of two genes responsible for familial forms of one of the most common neurodegenerative diseases, providing the impetus for the direct investigation of mitophagy in neuronal cells. Despite many debates, fueled by the idea that the metabolic and bioenergetic properties of neurons are not compatible with such a mechanism, several laboratories have provided evidence for its occurrence, not only in cultured neurons exposed to toxins, but also in neurons in the brain of model organisms under basal conditions. Many open questions still persist: what is the relative contribution of mitophagy to mitochondrial quality control in neurons, compared to other mechanisms involving degradation of mitochondrial components by intramitochondrial proteases, or direct delivery to the lysosome through the MDV pathway (Sugiura et al. 2014)? What are the physiological conditions that activate this process in neurons, and what are the mechanisms that regulate basal versus evoked mitophagy? How is mitophagy interconnected with mitochondrial biogenesis, how is it linked to mitochondrial transport fusion and fission in neurons? In which neuronal compartments does it occur primarily? Does it contribute in any way to the modulation of neuronal activity, for example by limiting energy supply to fundamental processes, such as synaptic vesicle recycling?

Finally, with respect to PINK1/Parkin-dependent mitophagy, we still need to understand in how far this pathway is central to neuronal degeneration in Parkinson's disease. This will imply solving present controversies as to the involvement of this specific mechanism in whole organisms, particularly during aging, which is acknowledged to be the greatest risk factor for Parkinson's disease (Collier et al. 2017), or under stress conditions. These studies will have to be paralleled by the in-depth investigation of the mitophagy-independent functions of the PINK1/Parkin signaling pathway (Jang et al. 2018; Palikaras et al. 2018). PINK1 and Parkin have been reported to exert other functions in relation to mitochondrial maintenance, be it cooperatively or independently, including in mitochondrial biogenesis, the derepression of transcripts encoding specific mitochondrial respiratory chain subunits on the outer mitochondrial membrane, the regulation of the activity of mitochondrial respiratory chain complex I, and the MDV pathway (reviewed by Alves Da Costa and Checler, 2012, Charan and LaVoie, 2015, Scarffe et al. 2015, Sugiura et al. 2014, Voigt et al. 2016, Winklhofer 2014, Mouton-Liger F et al. 2017, Chu 2018). Importantly, PINK1 and Parkin play also central roles in maintaining neuronal viability in response to stress by various, only partially elucidated mechanisms, independent of their roles in mitochondrial quality control (Alves Da Costa and Checler 2012, Charan and LaVoie 2015, Winklhofer 2014), and they can even exert proapoptotic functions under specific circumstances (Carrol et al. 2014, Zhang et al. 2014). Considering this complexity, there is still a way to go before we can fully appreciate the relative contribution of each of these specific mechanisms to the pathophysiology of Parkinson's disease.

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Compliance with ethical standards

Conflict of interest

There is no conflict of interest.

Table 1. Pathways for the degradation of mitochondrial components or whole mitochondria.

Pathway	Description and key references
Mitochondrial proteases	Proteolytic systems of the ATP-dependent AAA+ enzyme family, degrading irreversibly misfolded, damaged or oxidatively modified proteins in the mitochondrial matrix and inner mitochondrial membrane. The genetic alteration of these proteases causes neurological disorders in humans (Rugarli et al. 2012; Voos et al. 2016).
Ubiquitin-proteasome system	This pathway is involved in the degradation of proteins of the outer mitochondrial membrane, including pro- and anti-apoptotic proteins, such as Mcl1, and proteins regulating mitochondrial dynamics, such as Mfn2 (Karbowski and Youle 2011). The degradation of some of these proteins is regulated by PINK1 and Parkin (Carrol et al. 2014; McLelland et al. 2018; Tanaka et al. 2010).
MDV pathway	A vesicular mitochondrial quality control pathway involving the PINK1/Parkin-regulated formation of small vesicles emerging from mitochondria (MDVs) under conditions of oxidative stress, and transporting selected oxidized proteins directly to the lysosome for degradation. Contrary to mitophagy, this pathway does not require mitochondrial fission mediated by dynamin-related protein 1 (Drp1), and is independent from canonical autophagy (Soubannier et al 2012; McLelland et al. 2014; McLelland et al. 2016; Sugiura et al. 2014).
Mitophagy	A term designating various mechanisms for the selective delivery of whole mitochondria to the lysosome within autophagic vesicles. This pathway operates under basal conditions, during specific developmental programs or in response to stress or mitochondrial damage (McWilliams and Muqit 2017, Chu et al. 2018). Among the different mitophagy mechanisms described so far, PINK1/Parkin-dependent mitophagy is the most comprehensively characterized at the molecular level (Geisler et al. 2010; Matsuda et al. 2010; McWilliams and Muqit 2017; Narendra et al. 2008; Narendra et al. 2010; Truban et al. 2017).
Transmitophagy	A cell non-autonomous pathway for the autophagy-dependent degradation of neuronal mitochondria by adjacent glial cells. The existence of this pathway has been demonstrated *in vivo* at the optic nerve head, where protrusions shed from axons originating from retinal

ganglion cells were found to be phagocytosed by neighboring astrocytes. Using a mitochondrion-targeted fluorescent reporter combining an acid-labile EGFP and mCherry, it was shown that mitochondria contained in these axonal protrusions were degraded in lysosomal vesicles within the astrocytes (Davis et al. 2014).

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