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Defective Autophagy in Parkinson's Disease: Role of Oxidative Stress

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Abstract Parkinson's disease (PD) is a paradigmatic example of neurodegenerative disorder with a critical role of oxidative stress in its etiopathogenesis. Genetic susceptibility factors of PD, such as mutations in Parkin, PTEN-induced kinase 1, and DJ-1 as well as the exposure to pesticides and heavy metals, both contribute to altered redox balance and degeneration of dopaminergic neurons in the *substantia nigra*. Dysregulation of autophagy, a lysosomal-driven process of self degradation of cellular organelles and protein aggregates, is also implicated in PD and PD-related mutations, and environmental toxins deregulate autophagy. However, experimental evidence suggests a complex and ambiguous role of autophagy in PD since either impaired or abnormally upregulated autophagic flux has been shown to cause neuronal loss. Finally, it is generally believed that oxidative stress is a strong proautophagic stimulus. However, some evidence coming from neurobiology as well as from other fields indicate an inhibitory role of reactive oxygen species and reactive nitrogen species on the autophagic machinery. This review examines the scientific evidence supporting different concepts on how autophagy is dysregulated in PD and attempts to reconcile apparently contradictory views on the role of oxidative stress in autophagy regulation. The complex relationship between autophagy and oxidative stress is also considered in the context of the ongoing search for a novel PD therapy.

Keywords Autophagy · Mitophagy · ROS · Rotenone · Paraquat · MPTP

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Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It is an age-dependent disease characterized by resting tremor, slowed movement, postural instability, and muscle rigidity [1, 2]. The motor symptoms can be treated with dopaminergic drugs, however, the effectiveness diminishes as the severity of the clinical symptoms increases due to progression of the underlying neurodegeneration [3]. The most prominent pathological features are the severe loss of dopaminergic neurons in the *substantia nigra pars compacta* (SN) and the presence of protein inclusions called Lewy bodies primarily composed of fibrillar α-synuclein and ubiquitinated proteins within some remaining nigral neurons and astrocytes [2].

It is well accepted that oxidative stress is not a secondary end-stage epiphenomenon, but has a primary role in PD. There are several different theories on how oxidative stress is generated in SN, including the redox imbalance in dopamine metabolism upon aging, inflammation, and exposure to environmental toxins that likely act in concert with genetic predisposition [3]. During these processes, different reactive oxygen or nitrogen species (ROS/RNS) are formed in excess causing damage to organelles and macromolecules. Specific forms of ROS and RNS include hydrogen

peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), nitric oxide (NO), and reactive lipid species (RLS). NO reacts with $O_2^{\cdot-}$ and generates peroxynitrite ($ONOO^-$). $ONOO^-$ is capable of initiating protein oxidation and nitration [4]. The nitrogen dioxide radical, formed biologically from the reaction of NO with oxygen or decomposition from $ONOO^-$, reacts with tyrosine residues, resulting in 3-nitrotyrosine formation. *S*-nitrosylation results from the addition of NO to thiol groups on proteins. These posttranslational modifications have been detected in a broad range of pathologies, including PD, which is associated with both nitrated α-synuclein and *S*-nitrosylated parkin [5]. Lipid peroxidation is a consistent feature of neurodegenerative diseases and biologically active RLS, such as 4-hydroxynonenal (HNE), accumulates in brains of individuals with PD [6, 7].

ROS represent a link between exposure to environmental factors (e.g., pesticides, herbicides, and heavy metals) and endogenous and genetic risk factors of PD. In recent years, environmental toxins and drugs, including 1-methyl-4-phenylpyridinium (MPP^+) [8, 9], rotenone, paraquat (PQ), and metamphetamine [10], have been implicated in autophagy dysregulation in models of neurotoxin-induced dopaminergic cell death. Data from neuroblastoma cell lines, primary neuronal cultures and, in some cases, from animal models suggest that oxidative stress induces an excessive levels of autophagy leading to apoptotic or nonapoptotic cell death [8, 11, 12]. However, the accumulation of α-synuclein-rich protein inclusions similar to Lewy bodies reported in in vivo models of pesticide-induced parkinsonism (e.g., following exposure to rotenone and PQ) [13] is compatible with an autophagy block, rather than with its excessive stimulation.

Finally, functional analysis of genes mutated in familial forms of PD, including α-synuclein, Parkin, PTEN-induced kinase 1 (PINK-1), and DJ-1, strongly implicate autophagy impairment as the consequence of genetic alterations and cause of the pathology.

This review examines the scientific evidence supporting different concepts on how autophagy is dysregulated in PD and related disorders and attempts to reconcile apparently contradictory views on the role of oxidative stress in autophagy regulation.

Basic Mechanisms of Autophagy

Autophagy is a lysosomal-driven process of self degradation of cellular organelles and ubiqutinated or misfolded protein aggregates [14]. In addition to protein-based substrates, intracellular lipid droplets have been recently recognized as autophagy targets [15]. Selective autophagy of different cargoes is named after the organelle or the type of material ingested, so that mitophagy is used for mitochondria, reticulophagy for endoplasmic reticulum, pexophagy for peroxisomes, lipophagy for lipid droplets, and xenophagy for heterologous substrates (e.g., pathogens).

Autophagy represents an evolutionarily conserved response to nutrient deprivation, as well as to endogenous and exogenous stresses. At optimal physiological conditions in the absence of stressors, basal level of autophagy assures maintenance of cell homeostasis through regular turnover of proteins, lipids, and organelles. Defective or dysregulated autophagy has been associated with several pathologies, including neurodegeneration (for a recent review, see [12, 16]), cancer [17], certain inflammatory and infectious diseases [18], and lipid metabolism disorders [19]. In fact, autophagy helps to get rid of damaged organelles and protein aggregates or lipid droplets, which represent unwanted and usually toxic cargo that may lead to cellular dysfunction. Importantly, defective or dysregulated autophagy has been recognized as a critical pathogenic process in majority of neurodegenerative disorders, including Huntington disease (HD) [12, 16], Alzheimer disease [20], amyotrophic lateral sclerosis [21], and PD as discussed here. In support of the important role of autophagy, particularly in brain development and neuronal quality control, mice engineered for deficiency in key autophagy genes AuTophaGy (Atg)5 and Atg7 exhibit spontaneous neurodegeneration, characterized by motor neuron dysfunction and accumulation of ubiquitinated protein aggregates [22, 23].

The key organelle of the autophagic process is lysosome. We can distinguish different types of autophagy, based on how the cargo is delivered to lysosomes. Macroautophagy involves the engulfment of the cargo into double-membrane autophagosomes, which subsequently fuse with endosomes and lysosomes for cargo degradation. Autophagic activities are mediated by a complex molecular machinery including more than 30 Atg-related proteins and 50 lysosomal hydrolases. As depicted in Fig. 1, the process starts upon activation of cell-specific signaling pathways that suppress mammalian target of rapamycin (mTOR) signaling complex (mTORC1). This is followed by vesicle nucleation at the isolation membrane, most likely at endoplasmic reticulum (ER) level [24]. It involves the recruitment and assembly of several proteins including Vps34, Beclin-1, and UVRAG among others [25]. Vps34 has a PI3K activity and produces phosphatidylinositol 3-phosphate, which is needed for the targeting of Atg family proteins involved in subsequent vesicle elongation.

First, ULK1/2-mAtg13-FIP200 (equivalent to Atg1-Atg13-Atg17 in yeast) complex is build and then two ubiquitin-like conjugation systems (involving Atg12-Atg5-Atg16) are recruited and mediate the lipid conjugation of Atg8/LC3, among other reactions involved in the vesicle elongation step (Fig. 1). LC3 (that arises from microtubular-associated protein-light chain) is first cleaved at its C terminus by Atg4 to produce cytosolic LC3I, which then

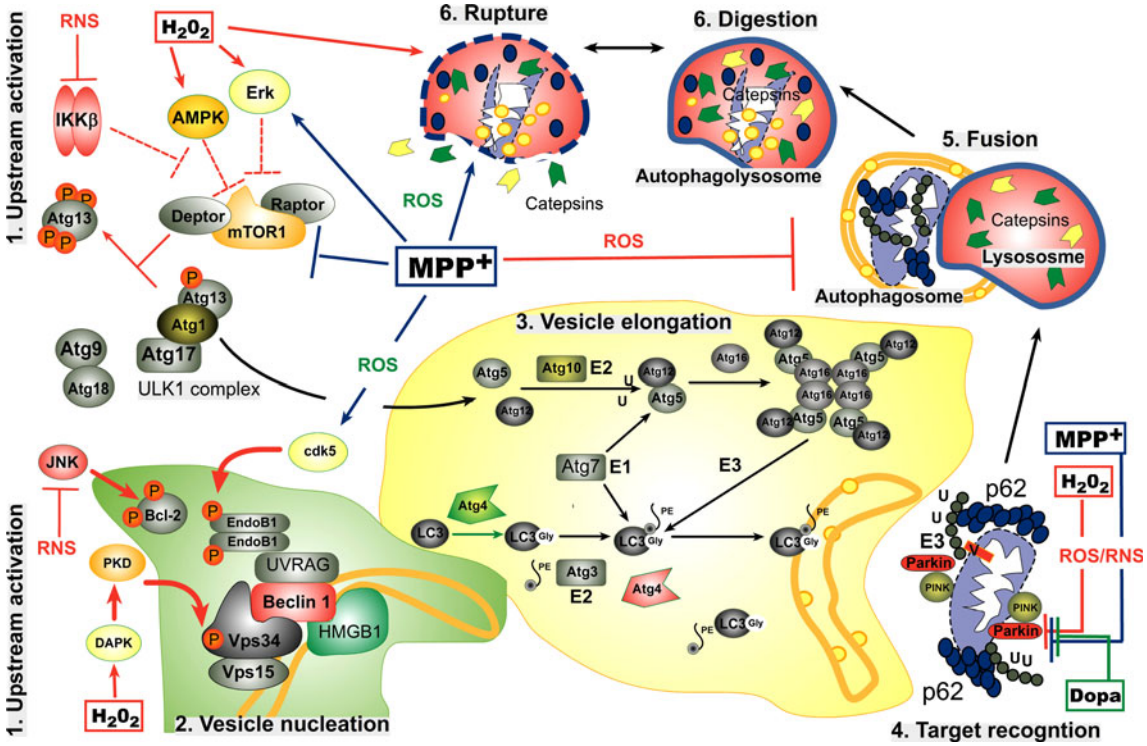

Fig. 1 Signaling pathways and basic molecular machinery of autophagy and mitophagy in dopaminergic neurons: regulation by MPP^+ and H_2O_2 and their intermediates ROS and RNS. For clarity, only representative molecules of most important pathways and pathways cited in the text are depicted. Molecular machinery represents yeast nomenclature and mammalian homologues. Components of autophagic machinery and signaling molecules that are directly regulated by ROS or RNS through adduct formation or oxidation are indicated in *red* (negative) or *green* (positive) regulation. In *yellow*, signal transducers that mediate oxidative stress signals but related mechanisms of redox sensing are unknown. *V* VDAC channel

undergoes phosphatidyletanolamine (PE) conjugation (mediated by Atg7 and Atg3) to form Atg8-PE or lipidated LC3II. The mechanism of autophagosome formation are well understood in yeast but not yet in mammalian cells (for review, see [26]).

Chaperone-mediated autophagy (CMA) and microautophagy are based on the direct transport of the cargo into the lysosomes. CMA is a process operated in a chaperone aided manner. Misfolded proteins bearing the KFERQ sequence at the C terminus are recognized and bound by cytosolic Hsc70 chaperones that finally assist the transfer of the protein inside the lysosome via interaction with the specific membrane receptor LAMP-2A (for review, see [27, 28]). Microautophagy occurs through bulk sequestration of cytoplasmic content by invagination of the lysosomal membrane [29].

Oxidative Stress in PD

Oxidative stress is a common denominator of several neurodegenerative disorders. Accordingly, the brains of patients with PD present, in a post-mortem analysis, a strong depletion of the antioxidant GSH [30, 31], increased protein and DNA oxidation and/or nitration, increased iron levels, and increased SOD2 [32, 33]. The activity of complex I, a major component of the mitochondrial electron transport chain (mETC), is decreased in SN in PD patients [34, 35]. Lipid peroxidation products, such as HNE, are also a prominent feature of PD brains [36]. Oxyradical-mediated DNA damage (increase in 8-hydroxy-deoxyguanosine) occurs to a greater extent in PD patients than in age-matched controls [37]. It is also well accepted that the redox imbalance in PD is not a secondary end-stage epiphenomenon but a driving force of the onset and progression of the disease [3].

The key element of the oxidative stress theory of PD is dopamine metabolism [38]. Neurotransmitter dopamine is oxidized by mitochondrial monoamine oxidase A and B (MAO-A and MAO-B) to a transient neurotoxic 3,4-dihydroxyphenylacetaldehyde or through its auto-oxidation which leads to the formation of reactive dopamine quinones and other compounds and ROS as the by-product. These quinones are also generated by other enzymes such as tyrosinase, lipooxygenese, and cyclooxygenese [39]. ROS can be also generated by tyrosine hydroxylase (TH) itself, the enzyme that normally converts tyrosine to L-DOPA but can also oxidize L-DOPA [40]. Dopamine quinones can be further oxidized to amniochromes and polymerized to form

nontoxic melanin; otherwise, these dopamine metabolites can covalently modify and inactivate several proteins, including TH, dopamine transporter (DAT), and Parkin, which is required for mitochondrial autophagy [39]. Excessive amounts of dopamine quinones are toxic to mitochondria, and they contribute to mitochondrial dysfunction which exacerbate the oxidative stress in dopaminergic neurons [41]. Indeed, the highly abundant mitochondria in SN cells are a major site of generation and action of ROS/RNS. In addition, the increased turnover and cytosolic content of dopamine resulting from L-DOPA therapy may enhance the level of oxidative stress and damage SN neurons [3].

The generation of ROS may also result from a disruption of aerobic metabolism, especially when certain antioxidant enzymes (e.g., Cu/Zn SOD or methallothioneins) are inactivated by genetic mutations. However, among several genes mutated in familial PD, only DJ-1 plays a direct role in oxidative defense mechanisms of SN. DJ-1 controls a transient and mild mitochondrial depolarization or uncoupling, which is necessary to protect the vulnerable SN dopaminergic neurons against oxidative stress during normal autonomous pacemaking of plasma membrane L-type calcium channels [42, 43]. Indeed, the sustained calcium entry into the cytoplasm leads to the uptake of this ion by mitochondria, changes in the mitochondrial membrane potential, increased rate of oxidative phosphorylation, and enhanced ROS production, which can be counteracted by active DJ-1 [42, 44]. Other PD-related gene products, like Parkin and PINK-1, may indirectly control oxidative status of the cell by removing dysfunctional mitochondria, as discussed later.

Alternatively, oxidative stress may result from innate immune defenses of the brain mediated by resident brain macrophages (i.e., microglia) or by astrocytes. Both cell populations play a critical role in homeostatic mechanisms that promote neuronal survival within the microenvironment of the brain. Microglia have a specialized immune surveillance role and mediate innate immune responses to invading pathogens by secreting cytokines, chemokines, prostaglandins, ROS/RNS, and growth factors [45]. Some of these factors have neuroprotective and trophic activities, while others enhance oxidative stress and trigger apoptotic cascades in neurons. Similar pro-inflammatory functions can be mediated by astrocytes under different pathological conditions, including virus infection [46, 47] or rotenone or LPS toxic injury [48–50]. Interestingly, a severe astrocyte inflammation, accompanied by upregulation of α-synuclein and PD-like Lewy bodies, has been observed in patients with diffuse Lewy body disease (DLBD) [51]. In addition, Lewy bodies and other protein inclusions are abundantly present in astrocytes in different synucleinopathies, including PD, DLBD, and amyotrophic lateral sclerosis (ALS) and their pathogenic function is largely unknown [52, 53]. Recently, Gu et al. showed that selective astrocytic expression of PD-related A53T mutant form of α-synuclein causes dramatic activation of astrocytes, leading to a severe astrogliosis and resulting in animal paralysis and death within a few weeks from transgene activation [54]. Therefore, given an important function of astrocytes in regulating blood–brain barrier, redox balance [55], and dopamine detoxification [56] in the neuronal environment, any impairment of their function that could result from toxic protein inclusions or an autophagy defect may have detrimental effects on vulnerable neuronal populations.

Finally, oxidative stress appears to provide a critical link between exposure to environmental factors, such as drugs and pesticides or heavy metals, and genetic factors predisposing to PD [57]. Environmental toxins (e.g., PQ, rotenone, and *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)), that epidemiological studies have shown to be risk factors of PD, are capable of generating reactive intermediates with the ability to directly react with biological macromolecules in processes such as thiol alkylation, carbonylation, nitration, and lipid peroxidation [58, 59]. ROS and RNS production can occur via interference with electron transport chain in mitochondria, as is the case of PQ, rotenone and MPTP [57], or directly by a redox cycling process exploiting cellular oxidoreductases and molecular oxygen (mainly PQ) [57]. In addition, PQ may exacerbate ROS by indirect activation of other metabolic enzymes such as quinone oxidoreductase 2 (QR2) [60], an enzyme with a possible function in dopamine metabolism and PD [61].

Defective Autophagy in PD: Lessons from Genetics

Mutations of at least six genes have been linked with hereditary PD: α-synuclein (SNCA or PARK1), Parkin (PARK2), ubiquitin carboxyhydroxylase L1 (UCH-L1 or PARK5), PTEN-induced putative kinase 1 (PINK-1 or PARK6), DJ-1 (PARK7), and leucine-rich repeat kinase 2 (LRRK2 or PARK8) [62]. Last decade has brought important advances in our understanding of molecular mechanisms of autophagy by elucidating the role of genes mutated in familial forms of PD. The conclusion of the vast majority of these studies is that these genetic defects lead to autophagy impairment [63]. First line of evidence is given by α-synuclein, the principal component of Lewy bodies. Missense mutations in the gene encoding α-synuclein (PARK1) and multiplications of the α-synuclein gene locus (PARK4) lead to familial cases of PD (for review, see [64]). Even sporadic PD cases are genetically linked to α-synuclein polymorphisms, which may modulate α-synuclein transcription [65].

The biological function of α-synuclein in normal neuronal physiology is still poorly understood, but recent findings suggest that it naturally occurs as α-helical tetramer with

high lipid binding capacity [66]. Destabilization of this tetramer has been proposed as an event preceding misfolding and aggregation that occurs in PD and other synucleinopathies [66]. Misfolded α-synuclein oligomers can be degraded by different catabolic pathways including CMA [67] and macroautophagy [68], and it accumulates in different pathological situations underlying PD pathology. Importantly, abnormal expression of α-synuclein can interfere with different types of autophagy. In fact, upregulation of wild-type (wt) α-synuclein leads to significant inhibition of macroautophagy [68] and mutant forms of α-synuclein A30P and A53T have been shown to inhibit CMA [67, 69]. While pathogenic α-synuclein mutations are rare, dopamine-modified α-synuclein, i.e., modified by noncovalent binding of oxidized dopamine, can be a common problem in PD [70]. Such variants of α-synuclein not only are poorly degraded by CMA but also block degradation of other substrates by this pathway [71]. This mechanism suggests an important point of interplay between autophagy and oxidative stress.

Another important breakthrough in the last decade has been the demonstration that Parkin, mutated in autosomal recessive forms of PD [72], is recruited to damaged mitochondria to facilitate the mitochondria-selective type of autophagy, mitophagy [73]. This study stimulated a great interest in mitophagy and initiated a recent wave of studies aimed at further understanding how mitophagy is deregulated in PD and other diseases. Several groups have addressed the mechanisms and role of Parkin in selective targeting and degradation of damaged mitochondria through mitophagy [74–76]. Parkin an E3 ubiquitin ligase expressed in the liver, kidneys, testis, brain, heart, and skeletal muscles [77]. Under basal conditions, Parkin is mainly found in the cytosol where its ubiquitin ligase activity is inhibited by an unknown mechanism [75]. However, under physiologic and pathologic stress, Parkin, but not its PD-specific mutant, associates to damaged mitochondria in a PINK-1-dependent manner to ubiquitinate proteins present on the outer mitochondrial membrane, such as voltage-dependent anion channel 1 and mitofusins 1 and 2 [78–81].

PINK-1 is another protein mutated in familial cases of PD. Loss of mitochondrial membrane potential drives the association of PINK-1 to the mitochondrial outer membrane which signals Parkin recruitment to the mitochondria [73–75]. Mutated forms of PINK-1 are not able to mediate this process, while over-expression of wt forms of PINK-1, as well as wt Parkin, leads to mitochondrial clustering and excessive accumulation of autophagosome-like structures [74]. In addition, PINK-1 significantly enhances basal and starvation-induced autophagy, which is reduced by knocking down Beclin-1 expression or by inhibiting the Beclin partner Vps34 [82]. This is mediated most likely by PINK-1 and Beclin interaction since a mutant PINK-1 unable to interact with Beclin lacks the ability to enhance autophagy [82]. In addition, a Beclin-independent autophagic clearance of defective mitochondria and ubiquitinated β-amyloid is mediated by Parkin in Alzheimer's disease models [83], implying that the protective role of Parkin and PINK-1 is not restricted to PD.

Collectively, these findings suggest that neurodegeneration in PINK-1 and Parkin-positive familial forms of PD may result from a defect in mitophagy, leading to the accumulation of damaged mitochondria and excessive ROS production. In support of this hypothesis, Parkin-deficient mice accumulate dysfunctional mitochondria and oxidative damage [84] while *Drosophila* flies with mutated Parkin are particulary sensitive to oxygen radical stress [85].

Mitophagy is not the only form of autophagy impaired in inherited forms of PD. In rare cases of familial and sporadic PD, mutations in *PARK5* gene coding for UCH-L1, seem to affect CMA [86]. In particular, the I93M mutation has been described in one German family with several cases of PD [87]. The involvement of this mutations in PD etiopathogenesis was further confirmed by UCH-L1^{I93M} transgenic mice exhibiting a progressive dopaminergic cell loss [88]. Several observations indicate that the cause of UCH-L1^{I93M}-associated PD may not be a loss of UCH-L1 function, but an acquired toxicity of the mutated variant of UCH-L1 which may involve protein binding affinity and solubility, rather than its enzymatic activity [89]. UCH-L1 is a deubiquitinating enzyme, but under certain conditions it can also work as an ubiquitin ligase for α-synuclein. This property of UCH-L1 was linked to the regulation of α-synuclein and other protein turnover by proteasome pathway [90]. UCH-L1 activity can be impaired by oxidative modifications, which can occur in sporadic forms of PD in response to oxidative stress [91].

Importantly, UCH-L1 has been shown to regulate CMA. UCH-L1 binds to LAMP-2A, which is the main lysosome receptor mediating the translocatation of ubiquitinated chaperone-bound cargo inside lysosomes [86]. UCH-L1^{I93M} shows aberrant, increased binding to LAMP-2A, which inhibits α-synuclein CMA and leads to its accumulation. UCH-L1^{I93M}-mediated CMA inhibition is independent of UCH-L1 enzymatic activity, partially impaired in UCH-L1^{I93M} and does not lead to the compensatory upregulation of macroautophagy [86].

Although it is not clear if UCH-L1 is a component of CMA machinery, these findings indicate that PARK5 is involved in proteosomal and autophagic protein turnover, which can be impaired by oxidative stress.

Loss-of-function mutations of PARK7/DJ-1 are rare causes of autosomal recessive hereditary PD. DJ-1 is a ubiquitous redox-responsive cytoprotective protein with multiple functions (for review, see [92]). More recent data suggest that many of DJ-1 antioxidant effects might derive

from its ability to mediate a transient and mild uncoupling in mitochondria that is required to protect dopaminergic neurons against L-calcium channels mediated increases in Ca^{++} levels during their normal pacemaking activity [42]. In particular, DJ-1 controls the upregulation of uncoupling proteins UCP4 and UCP5 in response to Ca^{++} and its knockout compromises calcium-induced uncoupling resulting in increased oxidation of matrix proteins specifically in SN dopaminergic neurons [42]. DJ-1 also plays a protective role in autophagy. Loss of DJ-1 has been shown to result in decreased basal autophagy but not in a deficit of induced autophagy, as measured by LC3II levels and autophagic vesicle numbers in wt and DJ-1-deficient myocyte enhancer factor (MEFs) [93]. However, these data were later challenged by studies showing that DJ-1 loss leads to an increased autophagic flux [94]. Although further studies are needed to establish if DJ-1 is directly or indirectly involved in the regulation of autophagic machinery and what is its role in this process in dopamineric neurons, DJ-1 appears as another plausible link between autophagy and oxidative stress [43] as discussed later.

LRRK2, also known as PARK8 or dardarin, is another gene mutated in certain dominant forms of familial PD. Loss of function mutants of LRRK2 have been shown to decrease neuritic arbor and to cause the accumulation of autophagic vesicles and swollen lysosomes containing tau inclusion bodies [95], suggesting some autophagy impairment. In fact, LRRK2 associates with multivesicular bodies, and LRRK2-R1441G mutants disrupt autophagic flux in HEK-293 cells [96]. However, the overexpression of other LRRK2 PD mutants in differentiated SK-SHY5 neuroblastoma cells [97] or in primary cortical neurons caused neurite shortening accompanied by dephosphorylation of LC3 and an increased autophagic flux [98]. Therefore, different dardarin mutants may cause positive or negative dysregulation of autophagy depending on the cellular system. In particular, autophagy-mediated shortening of neurites seem to recapitulate pathological changes in dopaminergic neurons in PD, such as a loss of dendritic spines locating to medium spiny projection neurons in PD-affected brains [99]. It is also very likely that the effects of LRRK2 on autophagy are indirect, as it has been shown to regulate the actin filaments dynamic via small GTP-ase Rac1 [100].

Role of Environmental Factors in Autophagy Dysregulation

Genetic and epidemiological studies support the concept that a concerted interplay of specific genetic susceptibility factors and environmental risk factors concur to the neurodegeneration in PD. However, the role of environment in PD etiopathogenesis must be particularly important since genetically transmitted, familial forms of PD represent only 5 % of all cases.

Accumulating evidence from epidemiological studies and toxin-induced animal models of PD strongly point to environmental toxins as possible triggers of nigrostrial dopaminergic neurons degeneration. The vast majority of epidemiological studies suggests that chronic or frequent exposure to certain pesticides significantly increases the risk of developing PD (risk ratio ranging from 1.2 up to almost 7 in different studies) [1, 101]. While some of the epidemiological data are still inconclusive [1], animal models strongly support the involvement of pesticide toxicity in PD etiopathogenesis (for review, see [13, 102]). Considering epidemiological and experimental evidence, pesticides such as rotenone, PQ, maneb, and ziram have been implicated as possible environmental toxins that might accumulate in brain tissues leading to oxidative stress, neuronal loss, microglia activation, and astrocyte dysfunction in SN. In addition to pesticides, the abuse of recreational drugs (e.g., methamphetamine and analogs) or accidental exposure to toxins such as MPP^+ may result in parkinsonism accompanied by nigrostratial neurodegeneration in humans, and the administration of these drugs to animals recapitulates many neurological features of human PD [103]. Importantly, almost all of these toxic substances have been shown to dysregulate macroautophagy by pathologically enhancing or interfering with autophagic flux as shown in Table 1 and discussed below.

MPTP/MPP^+

MPP^+, the MPTP active metabolite, is the strongest dopaminergic toxin, among the environmental toxins implicated in certain forms of early-onset PD. The administration of MPTP to primates represents the most clinically relevant model of human parkinsonism in which all currently used anti-parkinsonian medications have been shown to be effective [103]. MPP^+ toxicity is mediated by inhibition of mitochondrial complex I and leads to preferential loss of dopaminergic neurons in SN [104]. The selectivity of MPP^+ for dopaminergic neurons is due to the fact that it is an excellent substrate for the DAT [104]. MPP^+ is frequently used in vitro (and MPTP in vivo) to study the mechanistic relationship between oxidative stress and cell death associated with dopaminergic neuronal loss in PD. It is of note that in several cell models, such as human neuroblastoma cell lines SK-SH5Y [9] or BE-M17 [105], PC12 cells [106], as well as in primary mesencephalic [105] or cortical neurons [8] (see Table 1), MPP^+ has been shown to perturb the autophagy flux, as mirrored by an evident increase in the number of LC3-positive autophagic vesicles, in association with apoptotic or nonapoptotic cell death. This observation led to a concept that the activation of autophagy in these

Table 1 Dopaminergic toxins and their effect on autophagy

Neurotoxin	Experimental system	Effect on autophagy	Regulation of autophagy and cell death	References
MPTP/MPP+	SH-SY5Y neuroblastoma cell line	LC3-vesicles ↗↗ and LC3II ↗↗	3-MA no effect; wortmanin no effect, Beclin-1 siRNA no effect, rapamycin auguments cd.	Zhu et al. [9]
	PC12 cells and overexpression of α-synuclein	LC3-vesicles ↗ and LC3II ↗, but accumulation α-synuclein aggregates	Dynein ↗↗ and no colocalization with LAMP-1.	Cai et al. [106]
	Primary midbrain neurons; in vivo C57B/6 mice	LC3-vesicles ↗ and LC3II ↗	3-MA increases viability, Atg5, and Atg12 siRNA protect.	Wong et al. [8]
	Human neuroblastoma cell line BE-M17; primary mesencephalic and midbrain ventral neurons in vivo C57B/6 mice	LC3-vesicles ↗↗ and LC3II ↗ but lysosomes ↗↗, lysosomal membrane rupture	Rapamycin restores lysosomal activity and protects from cd.	Dehay et al. [105]
	MN9D dopaminergic neuronal cell line	LC3-vesicles ↗↗, LC3II ↗ and p62 ↘ but p62 insoluble ↗↗ and ubiquitinated proteins ↗↗	LC3II also precipitated to insoluble fraction dnAtg4B blocks p62 interaction.	Lim et al. [112]
Rotenone	Primary astrocytes	No increase in autophagy	–	Chen et al. [116]
	SH-SY5Y neuroblastoma cell line	No significant change in LC3II levels. No data on LC3-vesicles	Rapamycin activates autophagy, protects against cd and Atg5 siRNA reverse it.	Pan et al. [117]
	SH-SY5Y neuroblastoma cell line	LC3-vesicles ↗	Deferoxamine activates autophagy protects against cd and stabilizes HIF-1a.	Wu et al. [142]
			Carbamazepine, valproic acid and litium activate autophagy.	Xiong et al. [121]
			Resveratrol activates autophagy via AMPK.	Wu et al. [120]
	SH-SY5Y neuroblastoma cell line stably expressing wt or A30P and A53T α-synuclein mutants	Some increase in LC3II levels, mutant α-synuclein cells-LC3-vesicles ↗ and no increase in wt α-synuclein cells	Rapamycin activates autophagy, protects against cd, bafilomycin and 3-MA induce cd.	Dadakhujaev et al. [118]
	Human neuroblastoma cell line BE-M17 stably expressing wt and A53T α-synuclein mutants	24-h treatment LC3II ↗ and 7-day treatment (very low concentration of rotenone) LC3II ↘↘	Rapamycin activates autophagy, protects against cd.	Yu et al. [119]
PQ	SH-SY5Y neuroblastoma cell line	6-h treatment LC3II ↗, LC3 vesicles ↗, mTOR ↘; with DJ-1 siRNA: LC3II ↘, LC3 vesicles ↘, mTOR ↗; with ASK overexpression, LC3II ↗, LC3 vesicles ↗, mTOR ↘, and Beclin-1 ↗	3-MA induces cd, DJ-1 silencing reverses all effects of PQ on autophagy.	Gonzalez-Polo et al. [127, 129]
			Autophagy is protective	Niso-Santano et al. [128]
H_2O_2	SH-SY5Y neuroblastoma cell line stably expressing GFP-LC3	LC3-vesicles ↗↗↗, LC3I ↗↗, mature autophagosomes are formed followed by lysosomal membrane rupture	3-MA and dn-vps34 inhibit apoptosis.	Castino et al. [11]
			Catepsin D release to cytosol in the last step, iron chelating agents protect.	Castino et al. [137]
	SN4741 dopaminergic cell line and SH-SY5Y neuroblastoma cell line	LC3-vesicles ↗↗↗, LC3II ↗↗, mTOR ↘ and lysosomes rupture as above	3-MA inhibits cd rapamycin induces cd, Oxi-α overexpression blocks it.	Choi et al. [134]

"↗↗↗"very strong induction, "↗↗" strong induction, "↗" modest induction, "↘" downregulation or decrease, "↘↘" strong decrease, *dn* dominant negative, *wt* wild-type, *3-MA* 3-methyladenine

systems is part of a cell death program triggered by oxidative stress induced by neurotoxins [12, 107]. Such a cell death mechanism, defined as "autophagic cell death," has been first described in transformed and cancer cell lines, in response to different stimuli, including oxidative stress [108]. The same mechanism of cell death has been proposed to occur in SN in response to excessive ROS and environmental toxins in animal models and also in PD patients [12].

In the neuroblastoma cell line SH-SY5Y, MPP^+ was shown to activate a noncanonical Vps34-Beclin-1-independent autophagy pathway which could be blocked by RNA interference knockdown of Atg7 and LC3/Atg8, as well as by Erk inhibitors, but not by the classical inhibitors used to block starvation-induced autophagy such as 3-methyladenine (3-MA) or wortmannin [9, 109]. Note that this type of autophagy cannot be controlled by Beclin-1 and Bcl-2 [110] or Beclin-1 and Rubicon interaction [25]. In support of a pro-death outcome of abnormal upregulation of autophagy, it has been shown that a general autophagy inhibitor 3-MA as well as silencing of Atg5 or Atg7 expression confer a partial protection from cell death in primary cortical neurons [8]. It was also shown that co-treatment of primary midbrain neurons with MPP^+ and rapamycin, an inhibitor of mTOR and autophagy inducer, led to enhanced accumulation of LC3-positive vesicles and cell death of TH^+ neurons [109].

This latter finding has recently been challenged by Vila et al. who found that inhibition of mTOR activity by rapamycin protects dopaminergic neurons from cell death induced by MPP^+ in cultured cells or MPTP in mice [105]. These authors found a decreased number of lysosomes and LAMP-1 levels in the MPP^+-treated dopaminergic cell line M17 and in the ventral midbrain of MPTP-intoxicated mice, which likely occurred as a consequence of oxidative damage and lysosome membrane rupture [105]. Lysosomal depletion was particularly clear in SN dopaminergic neurons and could be observed at day 0 post-MPTP, thus before the accumulation of autophagosomes. The protective effect of rapamycin in these experimental models was dependent on its ability to induce lysosome biogenesis, beside the induction of autophagosome [105, 111]. The important conclusion of this work was that the accumulation of autophagosomes and LC3II observed following MPTP intoxication is the result of an impaired lysosomal-mediated clearance of autophagosomes, in addition to any potential induction of their formation by MPP^+. In support of these findings, Lim et al. showed that MPP^+ treatment of another dopaminergic cell line induced both accumulation of autophagosomes and LC3II and decreased p62 levels [112]. Further investigation revealed that these phenomena were largely the consequences of blocked autophagic flux. Following MPP^+ treatment, levels of LC3II, p62, and ubiquitinated proteins dramatically increased in the Triton X-100-insoluble fraction [112].

Taken together, it seems safe to affirm that MPP^+ deregulates autophagy by blocking lysosomal activity, rather than excessively enhancing autophagosome formation. Rapamycin can help to get rid of autophagosomes and of damaged mitochondria by stimulating the biogenesis of lysosomes.

Rotenone

Rotenone is a natural insecticide extracted from *Leguminosa* plants. As a potent mitochondrial complex I inhibitor, this compound is one of the best means to induce oxidative stress [113]. Rotenone easily crosses the blood brain barrier and does not need the DAT for cellular entry. Based on the limited environmental use, short half life and poor bioavailability, it is unlikely that the exposure to rotenone alone significantly contributes to sporadic PD induced by environmental factors [13]. However, rotenone is a potent dopaminergic toxin in rodents, causing nigrostriatal dopaminergic loss, accompanied by PD-like behavioral features, including decreased locomotion, flexed posture, and rigidity [114, 115]. In addition, rotenone induces cytoplasmic inclusions containing α-synuclein in neurons, when chronically infused to animals [114, 115], which may implicate a possible defect in protein turnover and autophagy. Accordingly, the effect of rotenone on autophagy was also addressed in in vitro model systems.

In contrast to MPP^+, rotenone does not seem to enhance early steps in autophagy, such as LC3II and autophagosome induction in neurons, astrocytes and neuronal cell lines, but it potently induces autophagic cell death in transformed and cancer cells HEK293 and HeLa cells [116]. This effect is mediated by ROS, as ROS scavengers or Mn-SOD overexpression blocks autophagic response to rotenone. Interestingly, the treatment of primary astrocytes with rotenone does not lead to autophagy induction, an effect that was attributed to very poor induction of oxidative stress by rotenone in these cells [116]. However, in SK-SH5Y neuroblastoma cells, rotenone did not seem to induce any significant autophagic response (only LC3II levels were analyzed), although it caused the accumulation of polyubiquitinated proteins, oxidative stress, mitochondrial dysfunction, and apoptosis [117]. Importantly, the induction of autophagy by rapamycin led to a significant protection against rotenone-induced toxicity and prevented the accumulation of polyubiqutinated aggregates [117]. The accumulation of wt and mutant α-synuclein aggregates enhanced by rotenone was also alleviated by rapamycin in SH-SY5Y. Similar data were obtained in M17 dopaminergic cell line stably overexpressing wt and mutant α-synuclein [118].

In addition, these authors showed that prolonged exposure to nonlethal doses of rotenone actually strongly decreased autophagy levels in this cell system, indicating again that rotenone, rather than inducing autophagy, might interfere with autophagic machinery, possibly by reducing cell metabolism [119]. Accordingly, an inhibition of basal autophagy levels, defined as mild reduction in the number of LC3-positive vesicles and increase in p62 aggregates, was observed in U373 astroglial cells exposed to rotenone concentrations that potently induced oxidative stress (Janda et al., unpublished observations; Fig. 2). In support of a protective function of autophagy against rotenone-induced toxicity, several autophagy-inducing compounds, including deferoxamine, litium, valproic acid, and carbamazepine have been shown to antagonize rotenone-induced apoptosis [120, 121].

Paraquat

PQ (1,1′-dimethyl-4,4′-bipyridinium) is a worldwide-used herbicide. The acute exposure to PQ, upon accidental or suicidal ingestion, causes thousands of deaths each year [122]. The chronic exposure to PQ, which may occur in agricultural communities, has been implicated in the pathogenesis of PD [101, 123], although epidemiological studies related to PQ exposure have been recently questioned [1]. PQ interferes with mitochondrial electron transport, but its most important toxicity depends on its redox-cycling activated by cytoplasmic oxidoreductases such as Nox [113]. In addition, recent findings indicate that PQ-induced toxicity and oxidative stress is mediated by QR2 oxidoreductase [60] expressed in SN dopaminergic neurons and implicated in dopamine quinone metabolism [61], which might suggest a possible mechanism of PQ selectivity for dopaminergic neurons. In fact, systemic PQ administration caused a significant nigrostriatal degeneration and formation of Lewy bodies-like α-synuclein aggregates [124, 125], which was not accompanied by behavioral changes [125]. This can be explained by dosage effect, as a local microinfusion of PQ into SN, leads to potent parkinsonian motor seizures and electrocortical excitatory effects in rats, which can be attenuated by Mn-SOD mimetics [47], as well as by a potent QR2 inhibitor NMDPEF [60].

The mechanisms of protein aggregate accumulation in response PQ in vivo are not clear and several factors such as upregulation and misfolding of α-synuclein and impaired autophagy or defective proteasomal protein clearance may contribute to it. Very recently, these mechanisms have been addressed in vivo in C57B/6 mice treated with nonlethal doses of PQ for 6 weeks [126]. Analysis of autophagy markers in stratial brain samples after chronic PQ administration showed a decrease in total LC3 levels, despite increases in Beclin-1 and Agt12. The experimental conditions used did not allow a convincing separation of LC3I and II, nevertheless the authors were able to detect a reduction in the LC3 II to LC3 I ratio, interpreted as a decreased autophagic flux [126]. This was accompanied by increased levels of the autophagy inhibitor, mTOR, indicative of

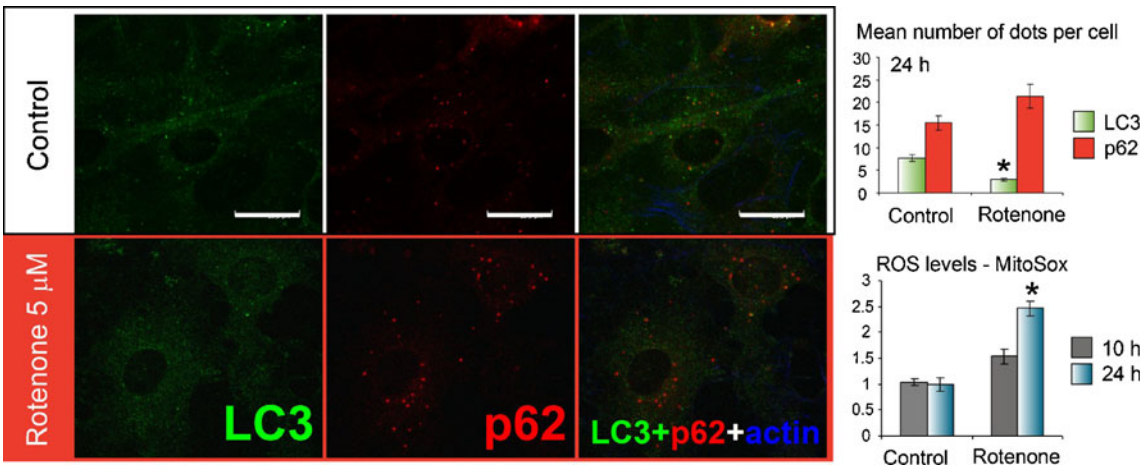

Fig. 2 Rotenone blocks basal autophagy in astroglial cells U373. Twenty-four-hour exposure to rotenone (5 μM) inhibits LC3 vesicle formation and increases the size and number of p62 aggregates. After treatment, the cells were fixed with paraformaldehyde (4 %, 10 min) and stained using standard techniques for LC3 and p62. Confocal analysis was performed. Pictures show representative maximum projection images of 20 images, around the central plane of the cell (4 μm depth in *z*). *White bar*, 20 μm. Number of dots per cell was determined in three independent experiments (at least 50 cells/treatment). Graphs show the mean±SEM. For measurements of oxidative stress, the cells were detached by trypsin and incubated with MitoSox (mitochondrial specific ROS-sensitive fluorescent probe) 10- and 24-h posttreatment and mitochondrial ROS levels were measured by flow cytometry according to Janda et al. [60]. *Bars* represent the mean±SEM of mean fluorescence intensity of MitoSox from three independent recordings. *$p<0.05$, statistically significant difference compared with control

impaired axonal autophagy. Heat shock proteins were either increased or unchanged upon chronic PQ treatment suggesting that CMA is not hampered [126]. In another study, a single injection of PQ resulted in a robust increase in α-synuclein levels, upregulation of LAMP-2A and hsc70 and accumulation of α-synuclein in lysosomes in midbrain regions of treated mice [124]. These findings have been interpreted as a mobilization of CMA to counteract the increased α-synuclein burden [124]. PQ also inhibited soluble proteasomal activity in brain samples, but this was not associated with a decreased expression of 26S proteasome subunits [126]. Thus, in vivo studies suggest an impairment of macroautophagy and proteasome function upon exposure to PQ and either induction or no effect on CMA, depending whether acute or chronic effects of PQ are analyzed. However, the in vivo data are difficult to interpret and need to be carefully verified in vitro.

Up-to-date, only one group investigated the effects of PQ on macroautophagy in dopaminergic neurons in vitro. Gonzalez-Polo et al. reported that a low dose PQ causes a substantial increase in LC3II levels, accompanied by a weak inhibition of mTOR phosphorylation and by some increase in LC3-GFP autophagic vesicles [127]. Interestingly, the Inhibition of autophagy by 3-MA led to acceleration of PQ-induced apoptosis in SH-SY5Y neuroblastoma cells. The authors interpreted these findings as "a protective autophagy" that antagonizes apoptosis [127, 128]. Further studies indicated that autophagy induced by PQ depends on DJ-1, as its knockdown reversed the autophagic response to PQ [129] and on apoptosis signal-regulating kinase 1 since its overexpression upregulates PQ-induced autophagy [128].

These findings, however, contradict what is observed in vivo and may suggest some intrinsic differences between autophagic response to dopaminergic toxins in vivo and in cultured cell lines. It is also possible that the response to PQ depends on culture conditions or is altered in SH-SY5Y cells. Indeed, in contrast to the observations by Gonzalez-Polo et al. [127] in neuroblastoma cells, we found that PQ has a dose-dependent inhibitory effect on basal autophagy in U373 astrocytes. In these cells, PQ reduced LC3II levels and autophagic vesicles, and increased p62 levels and aggregation. This effect was further enhanced in the presence of lysosomal inhibitors (Janda et al., manuscript in preparation). The inhibition of PQ-induced oxidative stress by QR2 inhibitor NMDPEF [60] restored basal autophagy and protected from PQ-induced toxicity more efficiently than rapamycin. Further investigation of signaling pathways induced by PQ in our cell model indicated that PQ induces some positive proautophagic signals (mTOR inhibition and Erk activation), but they fail to stimulate autophagy, suggesting that some inhibitory mechanisms prevail resulting in basal autophagy inhibition (Janda et al., manuscript in preparation).

Hydrogen Peroxide

H_2O_2 is a prototypic ROS generated as a by-product of the normal oxidative metabolism [130] and, in particular, of dopamine metabolism in SN [3]. H_2O_2 represents one of the main ROS species induced by the majority of oxidative insults and stressful conditions, including pesticide intoxication [57], mitochondrial electron transport inhibition and mitochondrial dysfunction [131], and starvation [132]. Primary endogenous source of H_2O_2 is the $O_2^{\cdot-}$ which is converted to H_2O_2 by the $O_2^{\cdot-}$ dismutases SOD1 and SOD2. At low concentrations, H_2O_2 acts as a survival molecule, but at high concentrations, it can lead to irreversible damage, followed by cell death [11, 133]. H_2O_2 invariably induces macroautophagy in many independent cellular systems. In addition, at high levels of oxidative damage, which can be achieved by exogenous administration of H_2O_2, the autophagic response seems to be an initial step of a death program that leads to apoptosis [11, 134] or nonapoptotic cell death [108, 135]. In dopaminergic cell line SH-SY5Y, exposure to 200 μM H_2O_2 leads to apoptosis followed by necrosis, which can be prevented by dominant negative Vps34 or 3-MA [11]. In contrast to the MPP^+-induced autophagic response, hydrogen-peroxide-induced autophagy enhances the formation of LAMP-1-positive autophagolysosomes that appear unstable and leaky [11]. As a consequence, the lysosomal protease catepsin D is released into the cytosol, where it triggers a Bax-dependent activation of the caspase cascade [136]. Thus, the H_2O_2-mediated deregulation of autophagy occurs in the last step of this process, at the level of lysosomal membrane stability [11]. Similar observations have been made in SN4741 dopaminergic cells treated with H_2O_2, though in this system cell death initiated by autophagy was found to be caspase independent [134]. In addition, the inhibition of mTOR activity by rapamycin was shown to exacerbate toxicity while activating mTOR by overexpression of a novel oxidative stress regulator Oxi-α attenuated the neuronal death in this system [134].

However, these findings are difficult to reconcile with the protective effect of rapamycin against MPP^+, rotenone, and PQ-induced cell death as discussed above, in addition to the overwhelming evidence from other neurodegenerative disease models where rapamycin has been shown to correct defective autophagy and protect from cell death [111]. Probably, redundant pathways that cross each other in a complex network of positive and negative signaling are differently regulated in the various cell models, either at genetic and epigenetic levels. For instance, it is assumed that

phosphorylated Akt keeps mTOR in an active state through Rheb, and this results in autophagy suppression (see above). However, in SH-SY5Y cells H_2O_2-induced autophagy was associated with inactivation of mTOR and concomitant (transient) activation of the Akt pathway [137]. A possible interpretation is that H_2O_2 activates both the Akt and the AMP-activated kinase (AMPK)-mTOR autophagy pathways as an attempt to survive, though sustained hyperactivation of autophagy eventually results in autophagy-dependent cell death once the Akt pathway is downregulated.

The role of autophagy in H_2O_2-induced cell death has recently been addressed also in primary cortical neurons. These studies essentially confirmed that the H_2O_2-induced autophagy is a part of the cell death program triggered by excessive oxidative stress [135]. In accordance to the observations in dopaminergic SH-SY5Y cell line [137], 3-MA treatment as well as suppression of Atg7 or Beclin-1, all inhibited cell death triggered by acute oxidative stress in primary cortical neurons [135]. In addition, no caspase activation was detected in these primary neurons following the exposure to H_2O_2. Thus, the nonapoptotic autophagic cell death is not limited to transformed and cancer cells, deficient in apoptotic machinery [108], but it can occur also in primary neurons exposed to H_2O_2. It cannot be excluded that this type of cell death can occur in response to certain oxidative insults in vivo in SN.

Collectively, environmental toxins leading to neurodegeneration of nigrostratial region of the brain, seem to interfere with the autophagic flux at different steps of the process. MPP^+, efficiently induces autophagosome formation, but it also interferes with lysosomal function by promoting lysosomal rupture, which eventually leads to proteolytical damage of the cell, most likely before their fusion with autophagosomes [105]. In contrast, although rotenone is a strong inducer of oxidative stress and has been shown to trigger autophagic cell death in some cancer cells, it does not seem to stimulate autophagy in dopaminergic neurons, suggesting that oxidative damage and misfolded protein aggregates may accumulate due to inefficient autophagy in neurons and astrocytes exposed to rotenone. However, the evidence on how rotenone and PQ dysregulate autophagy is still insufficient. PQ seems to inhibit macroautophagy in stratial regions of the brain, but stimulates autophagy under certain conditions in SH-SY5Y neuroblastoma cells, in a similar fashion to MPP^+. It is possible that PQ can switch from proautophagic to antiautophagic signals depending on the metabolic cell status and presence or absence of regulatory proteins such as DJ-1. Finally, high level of cellular H_2O_2, as a by-product of dysfunctional mitochondria, activates autophagy and causes the release of lysosomal proteases in the final step of autophagy with onset of cell death.

Molecular Mechanism of Autophagy Regulation by Oxidative Stress in PD

Low levels of ROS and RNS play an increasingly recognized role in signal transduction and regulation of physiological processes, including autophagy. The parkinsonian pro-oxidants produce high levels of ROS and RNS that act both positively and negatively on signaling pathways and lipid-protein complexes regulating autophagy. The redox regulation of autophagy may occur on different targets: upstream signal transducers, transcriptional factors, direct regulatory proteins interacting with autophagy machinery, and at the level of autophagy machinery, including lysosome degradative enzymes and lysosomal membrane. The experimental evidence on the cross-talk between autophagy and oxidative stress is vast and comes from basic cellular systems and PD-related experimental settings. Here, we will discuss these evidence in both contexts since several basic aspects of redox regulation of autophagy, discovered in nondopaminergic neurons can be generalized and might also play a role in PD pathogenesis.

Interplay Between Oxidative Stress and Autophagy in PD

Observations from different basic and disease-related systems indicate that autophagy (vesicle-mediated and CMA) and oxidative stress are mutually interdependent. On one hand, defective autophagy leads to oxidative stress, on the other hand the oxidative stress is usually considered a potent activator of autophagy. However, the accumulating evidence suggests a possible inhibitory role of redox signaling in the regulation of autophagic flux. This indicates a complex network of interactions between ROS and RNS and autophagic machinery, which is cell type and status dependent.

First of all, genetically engineered mouse models have provided abundant evidence for the important role of autophagy in the redox balance regulation, via control of mitochondrial integrity and intracellular protein and lipid clearance. For example, $Atg5^{-/-}$ and $Atg7^{-/-}$ or $Beclin\text{-}1^{+/-}$ knockout mice or MEFs derived from these animals accumulate p62, ubiquitinated proteins, and ROS [22, 23, 138]. Cytotoxic effects due to defective autophagy can be suppressed by ROS scavengers, such as *N*-acetyl cysteine (NAC) or by p62 elimination, indicating that p62 aggregates play an important role in the induction of oxidative stress [138]. These studies also evidenced an accumulation of dysfunctional mitochondria with different morphological alterations [22, 23, 138]. Indeed, more recent observations directly implicate p62 in the regulation of mitophagy. p62 is recruited by Parkin and mediates mitochondria aggregation, before they are engulfed by autophagosomes [139]. In addition, more recent findings indicate that AMPK- and ULK1- (homologue of yeast Atg1) knockout liver cells

[140], as well as FIP200- (Atg17 homologue) knockout hematopoietc cells [141] exhibit p62 accumulation, defective mitophagy, and ROS increase. AMPK, ULK1, or FIP200 are ubiquitous upstream regulators of autophagic machinery and it is likely that a genetic deficiency or acquired dysregulation of these proteins play a role in PD etiopatogenesis, as recently suggested [142].

Pharmacological inhibition of autophagy also leads to oxidative stress. Blocking lysosomal activity by the lysosomotropic agent chloroquine or the cathepsin D inhibitor pepstatin A increases the formation of ROS and RNS [143–145].

Importantly, all genetic defects in the mitophagy machinery found in PD lead to the increase in ROS, mainly due to accumulation of dysfunctional mitochondria. In particular, DJ-1 loss-of-function mutations were first identified as generating oxidative stress in mice and *Drosophila* and sensitizing the animals to the toxicity of PQ, rotenone, and MPTP [146, 147], and only recently DJ-1 has been shown to regulate basal autophagy and mitophagy in parallel with Parkin and PINK-1 [43, 93, 94]. Correspondingly, overexpression of DJ-1 protects against oxidative insults. In dopaminergic cell lines, overexpression of wt, but not mutant, DJ-1 was able to protect cells from H_2O_2 and 6-OHDA challenges, leading to reduced levels of reactive species, protein oxidation, and cell death [148, 149]. In animal models, overexpression of wt, but not mutant, DJ-1 was protective against dopaminergic neuronal degeneration in mice exposed to MPTP or rats exposed to 6-OHDA [148, 150], while novel compounds activating DJ-1 protected from 6-OHDA and rotenone-induced degeneration of SN dopaminergic neurons in rat and mice PD models [151]. As mentioned previously, all these effects of DJ-1 can be explained by its ability to attenuate the ROS production in response to local L-calcium channels pacemaking activity in dopaminergic neurons via regulation of mild and transient uncoupling of mitochondria [42].

Similarly to DJ-1, Parkin was first linked to the regulation of oxidative metabolism in mitochondria and, more recently, it was shown to be a critical regulator of mitophagy. *Drosophila* models deficient in Parkin or expressing Parkin with a pathogenic mutation exhibit mitochondrial dysfunction and alterations in oxidative response components [152, 153]. Accordingly, Parkin-deficient *Drosophila* have increased sensitivity to PQ [85]. In Parkin knockout mice, impaired mitochondrial function and decreased antioxidant capacity is accompanied by nigrostriatal defects, synaptic dysfunction, and dopaminergic behavioral deficits [84, 154].

In conclusion, the excessive ROS and RNS are generated when autophagy, and in particular mitophagy, are impaired.

Another platform of interaction between oxidative stress and autophagy is the fact that redox signaling is actually required for starvation-induced autophagy [131] and for the autophagic response to mETC inhibitors [116]. Indeed, an increase in ROS, in particular of $O_2^{\cdot-}$ radicals, can be detected in response to glucose, L-glutamine, pyruvate and serum and amino acid starvation, and ROS scavengers such as NAC and catalase block starvation-induced autophagy [131]. It appears that a complex interplay in signaling pathways involving pro-oxidant target genes of p53, AMPK, and other regulators of glycolysis such as TIGAR and DRAM regulate ROS production during starvation [155–157]. Finally, redox signaling plays also an essential role in the activation of autophagy by pro-oxidant toxins, including rotenone and other mETC inhibitors [116].

There is an overwhelming evidence that oxidative stress can induce autophagy. First indications came from pioneer studies in K562 erythroleukemia cells, in which antioxidant ascorbic acid was shown to inhibit ferritin translocation and degradation into lysosomes [158]. In the following two decades, the induction of autophagy by oxidative stress was shown in several independent systems. Importantly, as discussed previously, majority of dopaminergic and non-dopaminergic neurons exposed to exogenous H_2O_2 and MPP^+ will respond with upregulation of autophagy markers [8, 9, 11, 134, 135, 137]. Endogenous oxidative stress induced by dopamine metabolism in SN is also a strong inducer of autophagy in vivo since dopaminergic cell models [159, 160] and primary TH-positive neurons [161] respond with autophagic cell death to an excessive stimulation with dopamine or 6-OH-dopamine. In addition, 6-OH-dopamine-induced nigrostriatal degeneration in rats is accompanied by upregulation CMA markers [162]. In support of the positive role of ROS in autophagy triggering, thiol antioxidants as well as tocopherols and lipoic acid have been shown to inhibit trehalose and rapamycin-induced autophagy in a number of cell lines and in primary cortical neurons and in *Drosophila* models of PD and HD [163]. As discussed later, these findings may have important implications for designing novel antioxidant therapies for PD.

Another example of positive stimulation of autophagy by oxidative stress comes from a *Drosophila* model of synapse development. During *Drosphila* neuromuscular junction formation, an excessive autophagy stimulation by rapamycin or genetic means, leads to neuromuscular junction overgrowth [164]. Recently, it has been shown that oxidative stress is implicated in hyperactivation of autophagy in the developing synapse resulting in its overgrowth and functional defects. In fact, synapse overgrowth was observed in mutant flies defective for antioxidant protection and in flies subjected to PQ, implicating an excessive ROS burden in autophagy overstimulation [165, 166]. Reducing ROS rescued synaptic overgrowth and electrophysiological deficits [165].

Proautophagic effects of oxidative stress are mediated mainly by the $O_2^{\cdot-}$ anion O_2^- and the contribution of other forms of ROS such as intracellular H_2O_2 are limited to certain forms of autophagy [131]. In addition to ROS, RNS and lipid peroxidation products, such as HNE, are emerging as potential secondary messengers of proautophagic signals [58, 167]. For example, NO has been implicated in autophagy induction, as well as in its inhibition, which probably reflects a well-known duality of NO signaling, depending on the interplay between constitutive and inducible NO synthases (cNOS and iNOS) and different cellular levels of NO and peroxynitrate [46], although this concept has not been yet properly addressed in the context of autophagy. In particular, in primary neurons, NO induces dynamin-related protein 1-mediated mitochondrial fission, which further causes an increase in mitophagy [168], but later cell death. In glioma cells, when combined with hypothermia, NO donors sodium nitroprusside, *S*-nitrosoglutathione, or propylamine propylamine NONOate trigger autophagy, but a prolonged exposure eventually inhibit its completion, as evidenced by LC3II accumulation [169]. In contrast, NO donors such as DEA NONOate, DETA NONOate decreased endogenous LC3II levels in rat primary cortical neurons and HeLa cells, suggesting that NO may be inhibiting basal autophagy and these effects were dependent on *N*-nitrosylation of certain upstream modulators of autophagy as discussed later [170].

Cross-talk between oxidative stress and autophagy is regulated by an intricate network of redox sensitive upstream pathways and by components of autophagic machinery. Positive and negative regulatory mechanisms are now being discovered that could explain the duality of oxidative stress effects on the autophagic flux.

Redox Sensors: Positive Regulation

Virtually, all signal transduction pathways can be modulated by oxidative stress and many of them play a role in the regulation of autophagic machinery, such as AMPK-mTOR, MAPK/Erk, c-Jun N-terminal kinase 1 (JNK), and IκB kinase (IKK)-dependent pathways, but these signaling modules are also involved in hundreds of normal and pathogenic processes, including neurodegeneration in SN. For example, JNK that regulates autophagy by phosphorylating Bcl-2 and liberating Beclin-1 [171] (see Fig. 1), can be activated by starvation and oxidative stress. In particular, oxidative stress generated by defective ROS scavenging mechanisms or PQ in *Drosophila* larvae, mediates JNK activation leading to a potent autophagy stimulation that ultimately results in the neuromuscular synapse overgrowth and functional deficits [165, 166]. The activation of Erk is required for MPP^+-induced autophagy in dopaminergic cell lines [9]. IKKβ, recently implicated in autophagy regulation, plays important roles in mediating immune responses. Both JNK and IKKβ can are negatively regulated by nitrosylation, as discussed later. The negative regulator of mTOR activity AMPK is also an established target of RNS but in contrast to its upstream kinase IKKβ (see Fig. 1) it becomes activated in response to NO, H_2O_2, and oxidative stress induced by starvation in endothelial cells [172–174]. Interestingly, AMPK has been implicated in autophagy induction by antioxidants, such as resveratrol in dopamine cells, suggesting that ROS-independent mechanisms are also involved in AMPK and autophagy activation in dopaminergic neurons [142].

The upstream signals activating autophagy such as JNK, IKKβ AMPK-mTOR, and Erk are not specific for dopaminergic neurons, but play a role in autophagy regulation majority of cells and tissues. This is also true for the transcription factors that regulate the autophagic response to oxidative stress and have been implicated in PD, such as p53 [175], NRF2 [176], and FoxO family transcription factors [177]. Recently, however, a possible neuron specific signal transduction pathway that impacts on the autophagy machinery has been discovered. Cdk5 is a neuronal-specific threonine-serine kinase that is activated in response to MPP^+ and mediates its toxicity through inactivation of survival factor MEF2 [178, 179] and antioxidant peroxiredoxin 2 [180]. More recently, cdk5 has been shown to mediate MPP^+-induced autophagy in primary midbrain neurons [8]. Cdk5 phosphorylates endophilin B1 (EndoB1) that, once phosphorylated, can interact with UVRAG and promote the Vps34-Beclin-1 complex activation on the phagophore [181]. Cdk5 and EndoB1 are required for the autophagic response to MPP^+ in primary midbrain neurons and EndoB1 phosphorylation has been shown to occur also in vivo in response to MPTP intoxication [8]. Thus, cdk5-EndoB1 pathway stimulates autophagy in response to oxidative stress induced by dopaminergic toxins, upstream of Beclin-1 (see Fig. 1).

In addition to this novel pathway, several other oxidative stress-regulated pathways act positively on Vps34-Beclin-1-UVRAG complex and might play a role in dopaminergic neurons. death-associated protein kinase (DAPK) is upregulated in response to PQ-induced oxidative stress in dopaminergic cell lines [182]. DAPK is also activated by H_2O_2 in other systems, where it induces protein kinase D to phosphorylate and activate the lipid kinase Vps34 [183]. Another protein that is positively regulated by ROS to stimulate Vps34-Beclin-1 complex is the nuclear DNA-binding protein high mobility group box 1 (HMGB1). Upon oxidative stress, HMGB1 translocates to the cytoplasm and, subsequently, part of it is released into the extracellular space [184]. Extracellular HMGB1 has been shown to trigger a neurodegenerative process in an in vitro PD model system made up of primary dopaminergic neurons and glial cells in

co-culture [48]. In this system, mesencephalic neurons treated with low-dose parkinsonian toxins (MPP^+ and rotenone) release HMGB1 that stimulates pro-inflammatory cytokines production by microglia. These cytokines in turn increase sensitivity of neurons to neurotoxins and thereby create a "neurodegenerative vicious cycle" [48]. Interestingly, HMGB1 also induces autophagy as shown in other cellular models. Upon oxidative stress, such as exogenous H_2O_2 or SOD1 silencing, HMGB1 protein translocates to the cytoplasm and promotes autophagosome formation by binding to Beclin [185]. As recently shown, HMGB1 knockout in MEFs inhibits oxidative stress-induced autophagy. Importantly, the activity of HMGB1 is regulated by oxidation of several cysteines. For example, the intramolecular disulfide bridge (C23/45) of HMGB1 is required for binding to Beclin-1 and sustaining autophagy during starvation [186], while C106 plays a role in H_2O_2-induced autophagy [185]. Finally, HMGB1 is a regulator of mitophagy. In fact, loss of HMGB1 results in mitochondrial fragmentation with decreased aerobic respiration and adenosine triphosphate production [187]. Thus, HMGB1 is a redox sensor that may play an important role in autophagy deregulation in PD.

Redox sensors can be found also at the level of autophagic machinery. The cysteine protease Atg4 is an important ROS sensing positive regulator of autophagy. Atg4 exerts a dual role in the processing of LC3: it mediates LC3 proteolytic cleavage (priming step), necessary for LC3 conversion into LC3II, and it also acts as a delipidating enzyme in order to recycle LC3. However, this latter function must be temporarily inhibited when it is needed to ensure the conjugation of LC3 to the autophagosomal membrane, and then reactivated once the autophagosome has fused with lysosomes. This regulation of Atg4 activity is achieved through a localized redox reaction that involves Cys 81, which is proximal to the catalytic site. When this cysteine is oxidized, the Atg4 delipidating activity versus LC3II and its homologue GATE-16 is inhibited, while the initial priming activity is unaffected [132]. This may cause the increase in LC3II-PE levels and promote autophagosome formation. Accordingly, H_2O_2 blocks the delipidation activity of Atg4 (following oxidation of Cys81) and stabilizes LC3II, while the potent antioxidant DTT strongly reverses this effect activating the delipidation of GATA-16 and LC3II [132]. As yet, this is the most appealing molecular mechanism of autophagy regulation by ROS, which might also work in other oxidant conditions and for most cell types, as Atg4 is a ubiquitous protease. However, it is not clear why the Cys oxidation does not block the priming activity of Atg4. In addition, in the proximity of the active site of this enzyme different other cysteines and tyrosines are present, that could act as redox sensors to modulate Atg4 activity in both directions. Finally, the autophagy machinery is regulated by a plentitude of other enzymes that can act as redox sensors or can be inhibitory as discussed in the next section.

Redox Sensors: Negative Regulation

Many of posttranslational modifications induced by ROS, RNS, or RLS such as oxidation, nitration, *S*-nitrosylation and others can have inhibitory effects on certain components of the autophagic machinery. Indeed, Parkin can be inhibited by *S*-nitrosylation, which impairs ubiquitination of Parkin substrates [188, 189] and consequently the clearance of damaged mitochondria. Parkin E3 ubiquitin ligase activity can also be inactivated by covalently bound dopamine quinones in living dopaminergic cells [190]. This process increases Parkin insolubility and inactivates its function. Interestingly, Parkin solubility is also significantly reduced in the brains of individuals with sporadic PD [190]. Another oxidative modification of Parkin is sulfonation. MPP^+ treatment of neuroblastoma SH-SY5Y cells induces Parkin sulfonation, decreases Parkin's E3 ligase activity and promotes subsequent self-aggregation [191]. Thus, Parkin may be both a target of RNS signaling and a modulator of mitochondrial ROS generation and oxidative posttranslational modifications of Parkin can phenocopy genetic defects in *PARK2*.

Another example of negative regulation of autophagy machinery by RNS which is relevant for HD and possibly for PD, comes from molecular studies in primary cortical neurons, HeLa and HEK293 cells. These studies have shown that NO-induced *S*-nitrosylation of IKKβ and JNK1 inhibits their activities [170]. JNK1 is an important regulator of the interaction between Beclin-1 and Bcl-2 [171]. It phosphorylates Bcl-2 at multiple sites (T69, S70, and S87) causing Bcl-2 dissociation from Beclin-1. The association Bcl-2-Beclin-1 normally inhibits autophagy by blocking Beclin-1 [110], whereas Bcl-2 phosphorylation by JNK1 inhibits this interaction to stimulate autophagy [171]. On the contrary, *S*-nitrosylation of JNK1 prevents autophagy by negatively controling vps34-Beclin-1 complex formation (Fig. 1) [170]. The second inhibitory mechanism triggered by NO involves IKKβ. IKKβ acts upstream of mTOR to suppress its activity, therefore its *S*-nitrosylation would prevent the inactivation of mTOR and all the cascade of events that leads to the triggering of autophagy machinery while overexpression of NOS would impair autophagosome synthesis [170]. Furthermore, the same author showed that L-NAME (N^G-nitro-L-arginine methyl ester), a NO synthase inhibitor, enhanced the clearance of autophagy substrates in models of HD and PD, such as huntingtin EGFP-HDQ74 mutants and A53T α-synuclein mutants in PC12 cell lines in a way comparable to rapamycin. However, the induction of autophagy by L-NAME was independent of mTORC1 activity or Bcl-2 phosphorylation and had no

effects on Beclin-1 or the lysosomal hydrolase, cathepsin D, suggesting that the depletion of endogenous levels of NO triggers autophagy by different mechanisms then the autophagy signaling pathways perturbed by NO donors [170]. Whether *S*-nitrosylation of JNK and IKKβ are the mechanisms directly involved in the pathogenesis of PD remains to be clarified in the future.

Transglutaminase-2 (TG2)-mediated cross-linking of Beclin-1 induced by ROS is another inhibitory mechanism of autophagy [192, 193]. TG2 is a multifunctional calcium-dependent enzyme which catalyzes the posttranslational protein cross-linking with formation of intra- or intermolecular epsilon(gamma-glutamyl)lysine bonds [194]. TG2 has been suggested to function as a link between oxidative stress, chronic inflammation, and defective autophagy in a cystic fibrosis model. In this model, the levels and activity of TG2 increase in response to oxidative stress triggered by chronic inflammation. TG2-driven cross-linking of Beclin-1 inactivates the Beclin-Vps34 complex and prevents autophagosome formation [193]. It is possible that this mechanism of negative regulation of autophagy by ROS-mediated Beclin-1 cross-linking takes place in SN during pathogenesis of PD and is responsible for autophagy dysregulation and impaired clearance of protein aggregates. Indeed, the increase in TG2 activity together with mitochondrial impairment and collapse of antioxidant cell defenses have been reported in several neurodegenerative disease including PD [194].

Future Perspectives for Antioxidant Therapies: Lessons from Autophagy

The effectiveness of conventional PD therapies based on levodopa is limited to the reduction of PD symptoms. Prescription medicines enhance dopamine neurotransmission, reduce tremors and allow for fluid voluntary movement. Routine adjunct therapies often combine levodopa or dopa-decarboxylase inhibitors with dopamine receptor agonists, catechol-*o*-methyltransferase inhibitors, MAO inhibitors and anticholinergics treatments [195]. While these treatments ameliorate the quality of life of PD patients, they do not address the central etiology of SN degeneration and do not stop the disease course. For this reason, a number of alternative approaches have been considered to slow down the PD progression.

The idea that oxidative stress is pathological in the context of PD and other neurodegenerative diseases and that decreasing oxidative stress is a likely therapeutic strategy, has become a paradigm in the field. Many substances with antioxidant properties have been tested or are currently being tested in preclinical and clinical studies [195, 196]. A number of in vitro and animal studies in different PD model systems have demonstrated protective effects of antioxidants, including thiol antioxidants like NAC [197, 198], glutathione, and cysteamine [199]; vitamins such as ascorbic acid and tocopherol [195]; and plant polyphenols [200], such as resveratrol [142, 201], quercetin, and curcumin [202]. Although, so far the clinical trials of several antioxidants in PD failed to show striking benefit in all primary outcome measures [196], compounds and nutraceutical approaches with antioxidant properties still represent the major hope for a future PD therapy [195, 203].

The lessons learned from basic studies addressing the role of autophagy and the interplay between oxidative stress and autophagy in PD pathogenesis should guide the search for effective drugs. First of all, stimulation of autophagy by drugs such as rapamycin that act both at the level of autophagosome induction and lysosome biogenesis and stability, seems to be a good approach for a number neurodegenerative disorders, also for its potent anti-inflammatory properties [111]. Only in few neurodegenerative disorders, such as ALS, rapamycin should not be considered as a therapeutic approach as it may exacerbate motor neuron degeneration. In fact, the defects in autophagic flux cannot be restored by rapamycin in a transgenic mouse model of ALS bearing SOD1 mutation and further autophagosome induction by rapamaycin is deleterious [204]. In contrast, in majority of PD models discussed previously, rapamycin shows protective effects on dopaminergic neurons [105, 117, 205, 206].

Low doses of rapamycin, adjusted to avoid deleterious side effects on the immune system, could be combined with specific antioxidants to combat the ongoing oxidative damage in SN. However, a plain inhibition of oxidative stress may not necessarily be beneficial for dopaminergic neurons and astroglial cells in SN containing LB inclusions. Indeed, certain levels of oxidative stress are necessary to trigger autophagy, as discussed in "Molecular Mechanism of Autophagy Regulation by Oxidative Stress in PD." Underwood et al. showed that thiol antioxidants can inhibit trehalose and rapamycin-induced autophagy in a number of cell lines and in primary cortical neurons [163]. Importantly, these substances were able to reduce basal autophagy and increase the levels α-synuclein and polyglutamine aggregates in models of PD and HD, respectively. Similar results were obtained with tocopherol and lipoic acid [163].

Therefore, the potential benefits of ROS scavenging may be counterbalanced by an increased load of toxic proteins in these models. The search for ideal antioxidant treatment should consider this complication and concentrate on compounds that show ROS scavenging proprieties, but at the same time stimulate autophagy. These criteria may be fulfilled by several plant polyphenols or related compounds. Indeed, resveratrol, epigallocatechine gallate [207], quercetin [208], kaempferol [209], curcumin [210], and other polyphenols are good anti-inflammatory antioxidants able

to induce autophagy in certain cell systems. For many plant polyphenols, the proautophagic effect has been shown in several cancer cell lines, where these compounds typically induce autophagic cell death, underlying their cytotoxicity for tumor cells [211]. However, in untransformed cells and dopaminergic neurons many polyphenols are cytoprotective in a wide range of concentrations against dopaminergic toxins [195, 203].

As yet, the effect of these compounds on autophagy and protein aggregates in PD model systems has been poorly investigated. There are some indications in literature that protective effects of flavonoids, such as resveratrol, and kaempferol, rely on autophagy induction [142, 209]. Indeed, resveratrol protected against rotenone-induced apoptosis in SH-SY5Y cells and enhanced degradation of alpha-synucleins in alpha-synuclein-expressing PC12 cell lines via autophagy induction [142]. In these PD cellular models, the suppression of AMPK and/or SIRT1 caused decrease of LC3II, indicating that AMPK and/or SIRT1 are required for resveratrol-mediated autophagy induction. Moreover, suppression of AMPK attenuated protective effects of resveratrol on rotenone-induced apoptosis, further suggesting that AMPK-SIRT1-autophagy pathway plays an important role in the neuroprotection by resveratrol [142]. In another study, kaempferol-induced protection against MPP^+ and rotenone in SH-SY5Y cells was shown to depend on its ability to induce mitochondrial turnover via mitophagy. Kaempferol was also protective in vivo and it preserved striatal glutamatergic response in rats, suggesting a more general protection of kaempferol in PD [209]. Thus, resveratrol, kaempferol, and likely other polyphenols with similar characteristics appear as a good candidates for proautophagic antioxidant therapy of PD.

Future studies should address the effects of these compounds in animal models of PD and in clinical trials, both alone or in combination with other drugs, such as conventional therapy or novel drugs targeting calcium channels [44].

Concluding Remarks

The importance of autophagy and in particular mitophagy in PD is underlined by genetic and molecular studies of genes involved in familial forms of the disease. Virtually all known *PARK* genes play some role in the regulation of autophagy and 2 of them Parkin and PINK-1 are specifically involved in the target (damaged mitochondria) recognition phase of mitophagy (Fig. 3). In addition, α-synuclein mutations (*PARK1*) and UCH-L1 mutations (*PARK5*) perturb chaperon-mediated autophagy while increased α-synuclein levels (*PARK4*) impair macroautophagy. Thus, different

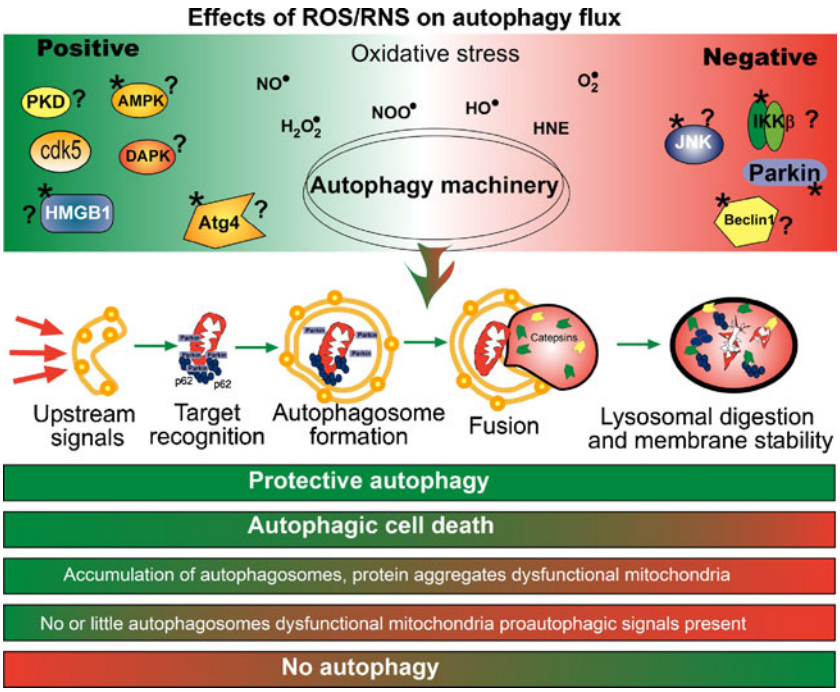

Fig. 3 Oxidative stress regulates macroautophagy by positive and negative signals. The imbalance between positive and negative signals at different points along autophagic pathway may have different outcomes for autophagic flux and different consequences for the cell, such as autophagic cell death or accumulation of defective mitochondria, oxidized protein aggregates, etc., as indicated on the *bottom bars*. ROS or RNS induce posttranslational modifications on different regulators of autophagy: known positively and negatively regulated "redox sensors" are on the *left side* (*green*) or *right side* (*red*), respectively. *Question mark*,the regulation by ROS/RNS has not yet been shown directly in dopaminergic cells. *Asterisk*, autophagy regulators shown to be directly modified by posttranslational modifications

forms of autophagy play a critical role in homeostasis and mitochondrial clearance of dopaminergic neurons in SN while genetic defects in autophagy lead to the disease. Environmental parkinsonian toxins also perturb autophagy, leading to autophagic stress in dopaminergic neurons, but there is no agreement on the role of autophagy in the cellular response to these toxins. In some models, autophagy is deleterious and leads to autophagic cell death (H_2O_2, certain cases MPP^+) while in others it is protective (PQ, rotenone and some cases MPP^+). However, all environmental toxins dysregulate autophagy if we assume that lysosomal function and membrane stability are an integral part of the autophagic process. The major differences stem out of how the autophagic process is perturbed (see Fig. 3). In fact, the autophagic cell death (H_2O_2, MPP^+ certain conditions) results from the interference in the last step of autophagy: lysosomal function. The literature suggests that in other cases (rotenone and MPP^+), pro-oxidants, besides inducing mitochondrial damage may also impair autophagy and mitochondrial clearance, leading to accumulation of dysfunctional mitochondria.

The relationship between autophagy and oxidative stress in PD and other pathologies is very complex and cannot be summarized in one phrase "defective autophagy induces oxidative stress and oxidative stress induces autophagy," mainly because the effects of excessive ROS and RNS on the autophagic machinery can be either activating or inhibiting. Positive and negative sensors of ROS and RNS in the context of signal transduction pathways and autophagy machinery are depicted in Fig. 3.

We postulate that the balance between positive and negative signals will tune the autophagic process in cell type- and oxidant-specific manner and can turn on induced autophagy, as well as turn off the basal autophagy. The actual outcome in terms of the autophagic flux likely depends on the level and duration of oxidative stress and on its source. We can assume that ROS levels generated in neurons and astroglia in SN under normal physiological or certain mild pathological conditions, such as weak inflammation, prolonged excitatory activity or transient nutrient shortage, will activate different autophagic pathways and promote autophagic flux to mitigate oxidative damage. High and acute oxidative stress will strongly upregulate autophagy, but may cause lysosomal leakiness and rupture leading to neuronal death. Finally, as emerged from our review, the oxidative stress triggered by parkinsonian neurotoxins or other PD-related pathogenic mechanisms in SN may have dual effects on the autophagic machinery, either activating or inhibiting at different time points of the autophagic process, leading to autophagic stress, astroglial dysfunction, and neuronal loss. In addition, RNS and different NOS isoforms may play an important role in duality of effects of the oxidative stress in PD and other pathologies and this duality needs to be appropriately addressed in the future. Future studies on relevant cellular models in vitro, such as primary dopaminergic neurons in co-culture with glial cells, and in animal models should clarify the mechanistic aspects of autophagy dysregulation by parkinsonian neurotoxins and their interplay with genetic defects implicated in PD. Hopefully, understanding of these mechanisms will lead to the identification of targets for pharmacological intervention and specific drugs that correct dopaminergic dysfunction and prevent PD progression.

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