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José M. Fuentes *Editor*

Toxicity and Autophagy in Neurodegenerative Disorders

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Toxicity and Autophagy in Neurodegenerative Disorders

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Preface

Autophagy is an intracellular catabolic mechanism mediated by lysosomes, which is responsible for most of the degradation and recycling of cytoplasmic components and intracellular dysfunctional or damaged organelles. Increasing evidences suggest that autophagic deregulation causes accumulation of abnormal proteins or damaged organelles, which is a characteristic of chronic neurodegenerative conditions, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease(HD) o amyotrophic lateral sclerosis. Indeed, promoting the clearance of aggregate prone proteins via pharmacological induction of autophagy has proved to be an useful mechanism for protecting cells against the toxic effects of these proteins in the context of neurodegenerative diseases and protecting neurons from apoptosis.

This book focused is composed of thirteen excellent reviews addressing different aspects of autophagy and its relation with neurodegenerative disorders. Chapters 1–3 are devoted to explain general aspects of autophagy as macroautophagy, chaperone-mediated autophay or mitophagy. Chapters 4–7 are dedicated to detail the role of autophagy in the principal neurodegenerative disorders and finally Chaps. 8–13 show the implication of autophagy in the toxicity of several substances related to the etiology of neurodegenerative diseases.

The compilation of these reviews included in this book show that there is much controversy in this field. Currently there are many research lines that require a lot of work to get there someday to be able to clarify a possible route of finding treatments against neurodegenerative diseases based on the mechanism of autophagy.

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Firstly I extend my gratitude to my two princesses (Andrea and Laura) which brighten my live. They are the essential.

My parents for their constant support.

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I express deep and sincere gratitude to all the authors for their valuable contributions and scholarly cooperation for timely completion of this book. Thanks also to Ms Susan Westendorf whose patience and focus were of immense support in this project.

Finally I am continuously indebted to Scout Movement. I consider that my compromise with Scouts mark and has marked many aspects of my life. Be prepared ¡¡

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Chapter 1
Regulation of Autophagy in Health and Disease

Amal Arachiche and Devrim Gozuacik

Abstract Macroautophagy (autopagy herein) is a cellular stress mechanism characterized by the engulfment of portions of cytoplasm, proteins and organelles in double or multimembrane vesicles. Cargo carried in these autophagic vesicles are then delivered and subsequently degraded by lysosomal/vacuolar systems. Autophagy occurs at low basal levels in all cell types (from yeast to mammals) under non-deprived conditions, performing homeostatic functions. Under conditions leading to cellular stress such as nutrient or growth factor deprivation, autophagy is activated to provide the cell with intracellular building blocks and substrates for energy generation. In addition to the ubiquitin-proteasome system, autophagy is a major degradation pathway for misfolded, mutant or abnormal proteins. Deregulations and abnormalities of autophagy are deleterious for all cell types including neurons. Consequently, autophagy abnormalities are observed in various neuronal diseases. Here, we summarize the basic autophagy machinery and its regulation, and provide a brief summary of the role of autophagy in healthy neurons and in major neurodegenerative diseases.

Keywords Macroautophagy · Microautophagy · Chaperone-mediated autophagy · Basal autophagy in neurons · Neurodegenerative diseases · Autophagy in Alzheimer's disease · Autophagy in Parkinson's disease · Autophagy in Huntington's disease

Abbreviations

ATG	Autophagy related gene
BCL2	B cell lymphoma 2
CBZ	Carbamazepine
CMA	Chaperone mediated autophagy
DEPTOR:	DEP domain containing mTOR-interacting protein

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FAK	Focal adhesion kinase
Foxo3	Forkhead box O3
Hsc70	Heat shock cognate protein 70
Hvps34	Human vacuolar protein sorting 34
LAMP2a	Lysosomal associated membrane protein 2 a
LC3	Microtubule associated protein 1 light chain 3
Mfns	Mitofusins
Mtorc1/2	Mammalian Target of Rapamycin Complex 1 and 2
PEN2	Presenilin enhancer 2
PI3K	Phosphoinositide 3 kinase
PINK1	PTEN induced putative kinase1
PS1/2	Presenilin 1 and 2
Rag Ras	Related GTP-binding protein
TSC	Tuberous sclerosis complex
UPS	Ubiquitin proteasome system

Introduction

To maintain homeostasis, cells must continuously synthesize and degrade their intracellular components. Intracellular protein degradation occurs through two different systems: The ubiquitin-proteosome system and the autophagy-lysosome system. While the ubiquitin-proteosome system degrades soluble short-lived or misfolded proteins, the autophagy-lysosome system degrades insoluble long-lived protein aggregates and damaged organelles. Deregulation of both systems has been associated with several diseases, including neurodegenerative diseases, inflammatory diseases, and cancer [1–3]. In this chapter, we will introduce the molecular basis of autophagy-lysosomal system and its implication in human health and disease. We will provide examples from major pathologies affecting neurons, i.e. Alzheimer's, Parkinson's and Huntington's diseases.

The Autophagy Machinery

The word autophagy "eating self" was first suggested in 1963 by Christian de Duve. Thomas Ashford's and Keith Porter's also made landmark morphological observations about autophagy in 1962 [4]. Autophagy is a conserved biological phenomenon from yeast to mammals. It degrades and recycles intracellular components, such as long-lived proteins and damaged organelles. In addition to its function as a "housekeeping mechanism" that maintains cellular homeostasis and prevents damage during stress, autophagy plays an important role in growth control, cellular differentiation, defense against pathogens, etc. For example, during sexual reproduction, autophagy was shown to be important for the elimination of paternal

mitochondria and their genomes in fertilized oocytes and prevention of the establishment of heteroplasmy [5]. Moreover, autophagy was involved in the maturation of reticulocytes to eryhthrocytes [6]. Indeed organelles, including aberrant or damaged mitochondria, are eliminated through an autophagy-related process in almost all eukaryotic cell types. Autophagy also participates to immune defense. For example, recent studies showed that intestinal autophagic activity is essential for the host defense against *Salmonella typhimurium* infection and autophagy was shown to play a key role in mediating neutrophil responses to bacterial infections [7, 8]. Consequently, autophagic abnormalities were observed in a number of diseases, including cancer, neurodegenerative diseases and metabolic diseases. In this chapter, we will first introduce the basic of autophagy machinery and its signaling pathways. Then we will overview autophagy in neurons and its implication for a number of selected neurodegenerative diseases.

Macroautophagy Depending on the pathways involved in cargo delivery, three types of autophagy were described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy is characterized by the formation of cytosolic double membranes sequestering and delivering cytosolic materials, such as proteins and organelles, into lysosomes for degradation. Whereas, microautophagy and chaperone- mediated autophagy involve direct engulfment of small portions of cytosol by lysosomal membranes or uptake of unfolded proteins through receptor channel complexes, respectively. Here, we will mainly focus on macroautophagy.

Macroautophagy (autophagy herein) is a highly regulated process. To date over 35 autophagy-related (*ATG*) genes have been identified through genetic screens in yeast, and many of them have orthologs in mammalian cells (Table 1.1). The induction of macroautophagy is followed by the formation in the cytosol of autophagosome precursors called isolation membranes or phagophores. Origin of the isolation membranes starts to be revealed. Formerly, membranes were suggested to be synthesized *de novo* [9], yet current evidence indicates that they derive from pre-existing membranes such as the endoplasmic reticulum (ER) [10], mitochondria [11], ER-mitochondria contact sites [12] or the plasma membrane [13]. Once formed, phagophores may engulf portions of cytosol, proteins and organelles, and elongate to form double membrane vesicles called autophagosomes. Eventually, autophagosomes fuse with lysosomes, leading to the formation of lytic compartments called autolysosomes. The process of mammalian autophagosome formation involves several ATG proteins and it is highly regulated by several protein complexes, including uncoordinated-51-like kinase (ULK1/2) complex, class III phosphoinositide 3-kinase (PI3K) complex, ATG9-ATG2-WIPI proteins, and the ubiquitin-like conjugation systems (Atg12–Atg5/LC3–PE) (Fig. 1.1).

Uncoordinated-51-like Kinase (ULK) Complex The ULK complex includes several proteins: ULK1, ULK2, mammalian ATG13, focal adhesion kinase (FAK) family interacting protein of 200 kD (FIP200, which is also called retinoblastoma 1-inducible coiled coil 1, RB1CC1), and ATG101/C12orf44 [1]. ULK1 and ULK2 are closely related members of the ULK family, sharing 78 % homology in their

Table 1.1 List of mammalian autophagy-related (ATG) genes involved in autophagy pathway

Gene	Protein localization	Protein function in autophagy
ULK1/2 (Atg1)	ULK1/2 complex	Serine/threonine kinases activated by mTOR inhibition and mediate PI3K complex activation
ATG2	Peripheral membrane protein, in complex with ATG9 and WIPI proteins	Regulates autophagosome formation
ATG3	LC3-PE conjugation system	E2- ubiquitin-activating enzyme-like that catalyzes the transfer of PE to LC3 to form LC3-PE
ATG4	LC3-PE conjugation system	Cysteine protease that cleaves carboxy-terminal glycine residues from proLC3 to generate LC3-I and lipid deconjugation from LC3-II
ATG5	ATG12-ATG5 conjugation system	Conjugated to ATG12 to generate E3 ubiquitin ligase-like enzyme. Required for targeting the complex to the phagophore
Beclin 1 (Atg6)	PI3K complex	Regulates the activity of hVPS34 a subunit of PI3K complex
ATG7	ATG12-ATG5 conjugation and LC3-PE conjugation system	E1-ubiquitin-activating enzyme-like that activates ATG12 and LC3
LC3 (Atg8)	LC3-PE conjugation system	participates to the expansion and the closure of phagophores to form complete autophagosomes. Mediator of selective autophagy through adaptor protein (e.g. p62) interactions
ATG9	Transmembrane protein which recycle between the Golgi, endosomes and autophagosomes	Interaction with ATG2 and WIPI1 in a ULK1 and PI3K complex dependent-manner to regulate autophagosome formation. Vesicle transfer from near-Golgi compartments
ATG10	ATG12-ATG5 conjugation system	E2- ubiquitin-activating enzyme-like that catalyzes the conjugation of ATG12 to ATG5
ATG12	ATG12-ATG5 conjugation system	In complex with ATG5 and ATG16 forms E3- ubiquitin-activating enzyme-like that catalyzes the transfer of PE to LC3 to form LC3-PE and regulate autophagosome formation
ATG13	ULK1/2 complex	Regulate the activity of ULK1/2 complex
ATG14	PI3K complex	Regulates the activity of PI3K complex
ATG16L1	ATG12-ATG5 conjugation system	Binds to ATG5 in the ATG12-ATG5 complex and olgomerizes to form a larger complex. Role in LC3 targeting to subcompartments
FIP200/RB1CC1 (Atg17)	ULK1/2 complex	Regulates the activity of ULK1/2 complex
ATG101	ULK1/2 complex	Regulates the activity of ULK1/2 complex
WIPI1/2 (Atg18)	In complex with ATG9 and ATG2 proteins	Binds to PI3P and regulates autophagosome formation

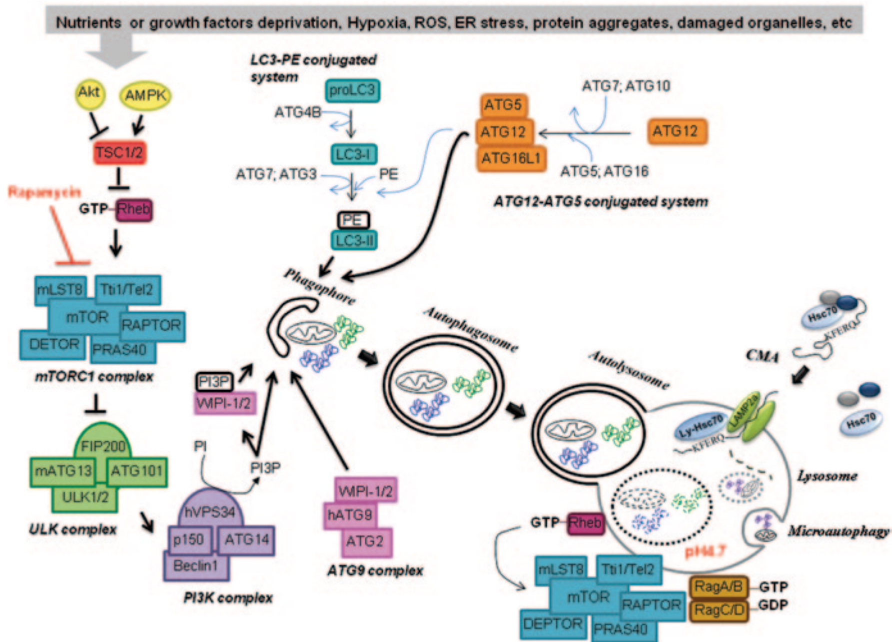

Fig. 1.1 Schematic representation of the autophagy pathway. Under the non-stressed conditions, the mammalian TOR complex 1 (*mTORC1*) is activated by upstream regulators such as Akt or Rag-GTPase in a *Rheb-GTPase dependent-manner, and result in the inhibition of autophagy pathway.* Under stressed conditions, activating pathways are suppressed and mTORC1 is inhibited. Moreover, energy sensor AMPK (*Adenosine monophosphate–activated protein kinase) might inhibit mTORC1 in a TSC1/TSC2 and Rheb-GTPase dependent pathway, resulting in autophagy activation. A*utophagy can also be induced by direct inhibition of mTORC1 with drugs (*e.g. rapamycin*). *Inhibition of mTORC1 results in the activation of ULK1/2 complex which in turn regulates the* initiation of autophagy and activation of the class III PI3-kinase (*PI3K*) complex on the isolation membranes (*phagophores*). Phosphatidylinositol 3-phosphate (PI3P) generated from phosphatidylinositol (*PI*) by PI3K serves as a landing pad, recruiting various autophagy effectors to phagophores. ATG9 participates to phagophore expansion by enrichment of the site with lipids and proteins in a ULK1 and PI3K complex dependent-manner. The activation of two ubiquitination-like conjugation systems ATG12-ATG5 and LC3-PE result in the conjugation of LC3-I to an phosphatidylethanolamine (*PE*) to form LC3-PE (*LC3-II*) which participate to the expansion of double membrane and the closure of the phagophores. Once formed, autophagosomes fuse with lysosomes and form autolysosomes to degrade autophagy cargos. On the other hand, the activation of Chaperone-mediated autophagy (*CMA*) involve the uptake of unfolded proteins through the lysosomal- associated membrane protein 2 a (*LAMP2a*) with the help of chaperone heat shock cognate protein 70 (*Hsc70*) in the cytosol and the intra-lysosomal protein Hsc70 (ly-Hsc70) in the lysosomal lumen. The clearance of protein aggregates and damaged organelle can be induced by a direct engulfment of the cargos by the lysosome, which results in the vesicle scission into the lysosomal lumen and degradation of the contents inside the lysosome

N-terminal kinase domain and the C-terminal region [14]. ULK1/2 proteins are required for autophagy activation under several stress conditions including nutrient starvation [15]. However, mainly ULK1 but not ULK2, was shown to have a cytoprotective function in neurons [16, 17]. Therefore, ULK2's ability to compensate the loss of ULK1 function might be cell-type specific.

ATG13 was conserved as a single homolog in mammals. ULK1/2 are in complex with ATG13, FIP200 and ATG101 even under nutrient- rich conditions [18]. The mammalian target of rapamycin complex 1 (mTORC1) protein, a negative regulator of autophagy, was found to be associated to the ULK1/2 complex under nutrient- rich conditions. mTORC1 regulates ULK1/2 and ATG13 through phosphorylation. Upon nutrient starvation, mTORC1 is inhibited and dissociated from ULK complex, which leads to an increase in ULK1/2 kinase activity [19]. ULK1 and ULK2 are then able to phosphorylate ATG13 and FIP200 to activate autophagy. The conserved carboxy-terminal domain (CTD) which contains a membrane-binding signal of ULK1 and ULK2, controls the regulatory function and localization of both proteins, mediating the recruitment of the complex to the site of autophagosome initiation [20]. ULK1 was also shown to phosphorylate and inhibit mTORC1. This novel negative feed forward loop occurs upon activation of autophagy, and maintains mTORC1 inhibition when nutrient supplies are limiting.

Class III Phosphoinositide 3-kinase (PI3K) Complex Phosphatidylinositol 3-phosphate (PI3P) is an important phospholipid playing a role in the early step of autophagosome formation. PI3P serves as a landing pad, recruiting various autophagy effectors to nucleation sites (e.g. outer membrane of the ER) through their phosphoinositide-binding domains, such as the FYVE finger domains or PH domains [21]. The pool of PI3P necessary for the formation of autophagic membranes is generated by a specific enzyme complex, the class III PI3-kinase (PI3K) complex. In mammalian cells, there are three functional classes of PI3-kinase: Class I, II, and III. The class III PI3K are implicated in intracellular vesicular trafficking including retrograde endosome to Golgi transport, transport at nuclear membrane, and autophagy [22, 23]. Components of the class III PI3K complex are conserved from yeast to mammals. The complex is composed of human vacuolar protein sorting 34 (hVPS34), p150, Beclin1 and ATG14. A serine/threonine kinase protein 150 kDa directly interacts with human VP34 and stimulate its kinase activity leading to the generation of PI3P by the phosphorylation of phosphatidylinositol (PI) [24]. Activity of hVPS34 is also regulated by the autophagy protein Beclin1. In addition to ATG14, AMBRA1 (activating molecule in Beclin-1-regulated autophagy), UVRAG (UV irradiation resistance associated gene) and Bif-1 (endophilin B1) were shown to positively regulate the complex, whereas Rubico and anti-apoptotic proteins B-cell lymphoma 2 (BCL2) family [25] were inhibiting PI3K complex. As ULK1/2 and PI3-kinase complexes are both required to initiate autophagy, their regulation must be coordinated. Indeed, it was shown that recruitment of the PI3K complex to autophagic membranes was dependent on ULK1, placing the ULK1/2 complex upstream to the PI3K complex [26].

ATG9-ATG2-WIPI Proteins Accumulation of PI3P creates a platform to recruit cytosolic autophagic proteins such as ATG2 and WIPI (WD-repeat domain phosphoinositide-interacting protein). The human WIPI protein family consists of at least four members, WIPI-1,-2, −3, and −4. Structural analysis shows that WIPIs proteins possess seven WD40 repeats that fold into an open vector beta-propeller. Detailed analyses showed that both WIPI-1 and WIPI-2 are localized in the membrane of the autophagosome and in the plasma membrane [27]. In addition, WIPI-1

was also localized in the membrane of the endoplasmic reticulum whereas WIPI-2 is present in the Golgi. In autophagy, WIPI-1 and WIPI-2 function upstream to the ATG12-ATG5-ATG16L1 complex and LC3, but downstream to the ULK1/2 and PI3-kinase complexes [28, 29]. WIPI-1 and WIPI-2 bind to PI3P upon the initiation of autophagy and accumulate at the phagophore.

ATG9 is one of the rare transmembrane ATG protein characterized so far [9]. Under nutrient-rich conditions, ATG9 localizes to the trans-Golgi network and partially to endosomes. Whereas under starvation conditions, ATG9 interacts with ATG2 and WIPI, and localizes to autophagosome formation sites in a ULK1 and PI3K complex dependent-manner [30, 31]. The loss of ATG2 was shown to inhibit autophagosome formation [32]. Molecular mechanisms involving ATG9, ATG2 and WIPI are under investigation by several independent groups.

Ubiquitin-like Conjugation Systems (ATG12-ATG5/LC3-PE) Autophagosome formation requires the action of two ubiquitination-like conjugation systems in which several ATG proteins play essential roles [33].

The ATG12-ATG5 Conjugation System In this system, the mammalian ATG7 (E1-ubiquitin-activating enzyme-like) activates the ubiquitin-like protein ATG12, which is then transferred to ATG10 (E2- ubiquitin-activating enzyme-like), and finally covalently conjugated to ATG5 forming the ATG12-ATG5 complex. A carboxyl terminal glycine of ATG12 is essential for the formation of thio-ester bonds with the active site cysteine 572 of ATG7 and cysteine 165 of ATG10 [1]. ATG5 is stoichiometrically conjugated with ATG12 to generate E3 ubiquitin ligase-like enzyme [1, 34]. ATG12-ATG5 conjugation appears to be irreversible, and so far, no enzyme hydrolysing the ATG12-ATG5 conjugate has been identified [35]. It has been shown that the ATG12 C-terminal glycine bind to the Lysine 130 of mammalian ATG5 through an isopeotide bond [36]. ATG5 seems to be the only target of ATG12 and the conjugation of ATG12-ATG5 seems to occur constitutively soon after the proteins are synthesized [36]. The ATG12-ATG5 conjugate further interacts with a small coiled-coil protein, ATG16L1, to form a multimeric complex ATG12-ATG5-ATG16L1 through homo-oligomerization of ATG16 [37]. ATG12-ATG5-ATG16 complex localizes to the isolation membrane and dissociates from the membrane upon completion of the autophagosome. Therefore, ATG12, ATG5, and ATG16 are frequently used as markers for the early steps of autophagosome formation. ATG5 and ATG16, but not ATG12, are required for membrane targeting of the complex [38].

The LC3-PE Conjugation System The second system involves the conjugation of microtubule-associated protein 1 light chain 3 (LC3, homolog of yeast Atg8). Here, LC3 is not conjugated to a protein but to a lipid molecule, namely to a phosphatidylethanolamine (PE) molecule. To date, at least three different mammalian LC3-like proteins have been identified: gamma-aminobutyric acid type A receptor-associated protein (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE-16), and the LC3 (with at least 3 isoforms LC3A, B, C) [39]. LC3 is the best characterized among these LC3-like proteins.

ATG4 proteins are cysteine proteases that cleave proLC3 to generate LC3-I, the cytosolic and non-autophagic form of LC3 with an exposed carboxyl terminal

Glycine. LC3-I is then activated by ATG7 E1-like protein (common to both ubiquitylation-like reactions), and transferred to the E2-like protein, ATG3. LC3-I is finally conjugated to PE to form LC3-PE or autophagosome-associated LC3-II. The lipid-conjugated form of LC3 (LC3-PE) participates to expansion and closure of phagophores to form complete autophagosomes [40]. Because LC3-I conjugation and conversion to LC3-II correlates well with the autophagic activity and autophagosome formation, it is commonly used as an autophagy marker [41].

The LC3-PE conjugation system is also dependent on the ATG12-ATG5 conjugation system. Indeed, once recruited to the membrane, the ATG12-ATG5-ATG16 complex serve as an E3-like complex to mediate the final step of LC3-I conjugation to PE. In other words, the ATG12-ATG5-ATG16 complex is required for LC3 conjugation. In line with this, the attachment of PE to LC3-I was completely impaired in ATG5 knockout cells [42]. Complete autophagosomes, move then rapidly in a microtubule and dynein dependent manner to meet with lysosomes, fuse with them and form autolysosomes where incorporated materials are degraded [43]. In contrast with the ATG12-ATG5-ATG16L1 complex, LC3 stays integrated into complete autophagosomes. Yet, LC3 proteins found on the outer membrane of the autophagosomes are cleaved from the lipid and recycled by the action of ATG4 proteins, an event necessary for autophagosome-lysosome fusion. The lysosomes are acidic organelles with an internal pH of 4.7–4.8 [44]. They contain hydrolytic enzymes that are able to break down macromolecules. The monomers generated by lysosomal degradation such as aminoacids, lipids are recycled and are reused by the cell.

Selective Versus Non-Selective Macroautophagy

Autophagy can be a non-selective or selective mechanism. Non-selective macroautophagy is involved in the degradation of randomly sequestered portions of the cytosol, whereas selective macroautophagy is degrades specific targets such as mitochondria (mitophagy), nucleus (nucleophagy), peroxisomes (macropexophagy) or pathogens (xenophagy) etc. Adaptator proteins implicated in the regulation of selective macroautophagy were identified: The list includes p62/SQSTM1 (sequestosome 1), NDP52, NBR1, NIX, and optineurin. Adaptor proteins interact with ubiquitylated targets via their UBA (ubiquitin-associated) domain and bind to LC3 via their LIR (LC3 interaction region) motif, promoting cargo concentration in autophagosomes and selective cargo degradation through autophagy [45].

The most studied selective autophagy type is mitophagy, a selective elimination of mitochondria by autophagy. Mitophagy implicates an E3 ubiquitin ligase called Parkin, which was originally dicovered as a Parkinson's disease-associated protein. Parkin is recruited to damaged mitochondria and mediates ubiquitination of outer mitochondrial membrane proteins, including mitofusins (Mfns) and voltage-dependent anion channel 1 (VDAC1). The recruitment of Parkin to damaged mitochondria requires PINK1 ((PTEN)-induced putative kinase (another Parkinson's

disease-associated protein), which is localized in the outer mitochondria membranes in stressed mitochondria. Ubiquitinated mitochondria proteins then serve as signal to recruit adaptator proteins such as p62/SQSTM1 which in turn bind to LC3 and recruit the autophagy machinery for the elimination of damaged mitochondria, providing maintenance of healthy mitochondria [46].

Microautophagy Different from macroautophagy, in the microautophagy pathway, lysosomes don't not fuse with autophagic vesicles, but they rather engulf directly cytosoplasmic cargos via invaginations of the lysosomal membrane (Fig. 1.1). Following vesicle scission into the lysosomal lumen and contents degradation are degraded inside lysosomes [47]. In yeast, microautophagy is constitutive but it can also be induced by starvation or treatment with rapamycin (an mTOR inhibitor drug) [48]. Similar to macroautophagy, microautophagy functions also as a "housekeeping" mechanism for the degradation of cytosolic materials. Microautophagy can occur simultaneously with macroautophagy to regulate lysosomal membrane size. As macroautophagy can result in a large flow of membranes to lysosomes, microautophagy can regulate this flow and reduce lysosome size by consuming lysosomal membranes [3]. Additionally, microautophagy was shown to play an important role in early mammalian development. Indeed, microautophagy was used to deliver endosomes to lysosomes in the visceral endoderm of mouse embryos [49]. This process was found to be important for the proper delivery of maternal nutrients as well as signaling molecules to the embryo.

Chaperone-Mediated Autophagy (CMA) Chaperone-mediated autophagy (CMA) differs from the macroautophagy pathway by the absence of vesicular trafficking, instead, only single proteins are delivered to lysosomes for degradation (Fig. 1.1) [50]. Macroautophagy and CMA pathway were shown to be coordinated processes. Indeed, inhibition of macroautophagy induced up-regulation of CMA, even under basal conditions [54].

CMA degrades only unfolded protein substrates with a KFERQ or a KFERQ-like motif which are present in about 30% of all cytosolic proteins [51, 52]. CMA was first described in human fibroblasts cultured under starvation conditions [51]. CMA can also be induced *in vitro* in the presence of isolated lysosomes using a protein substrate with the specific pentapeptide sequence and a molecular chaperone complex [53].

CMA occurs in mammalian cells but not in yeast [55]. In mammalian cells, chaperone heat shock cognate protein 70 (Hsc70) and other cooperating chaperones recognize cytosolic proteins with KFERQ motif, unfold these proteins, and target them to lysosomal membranes. The interaction between chaperone complex and the lysosomal- associated membrane protein 2 a (LAMP2a) channel, present at the lysosomal membrane, facilitate the translocation of unfolded proteins to the lysosomal lumen, where they are rapidly degrade by lysosomal proteases [50]. The translocation of unfolded proteins through LAMP2a requires another chaperon protein, Hsc70 (ly-Hsc70), a protein present in the lysosomal lumen [56]. Once proteins are captured by the lysosome, the Hsc70-cochaperone complex is released from the lysosomal membrane, and is now available to bind other cytosolic proteins with a KFERQ motif [50, 57].

As a variation to the theme, a novel type of autophagy called, RNautophagy was descibed recently [58]. RNautophagy is RNA degradation process by the lysosome. Unlike CMA, RNautophagy is independent of chaperon complexes and use LAMP2-c instead LAMP2-a.

Signaling Pathways Regulating Autophagy Initiation

Regulation of Autophagy by mTOR-Dependent Pathways The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase playing critical role in autophagy. mTOR pathway involves two distinct functional complexes: mTOR complex 1 (mTORC1) controls autophagy (Fig. 1.1), and the mTORC2 complex that mainly regulates cytoskeletal reorganization and migration. Both mTOR complexes are large with mTORC1 having six known protein components and mTORC2 seven protein components. Both complexes share the catalytic mTOR Ser/Thr kinase subunit, mLST8 (mammalian lethal with sec-13 protein 8, also known as GbL) protein, DEPTOR (DEP domain containing mTOR-interacting protein), and the Tti1/Tel2 complex. In contrast, RAPTOR (regulatory-associated protein of mammalian target of rapamycin) and PRAS40 (proline-rich Akt substrate 40 kDa) proteins are specific to mTORC1, whereas RICTOR (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated map kinase-interacting protein 1), and PROTOR1/2 (protein observed with rictor 1 and 2) proteins are only part of mTORC2 [59].

In mammalian cells, autophagy can be induced following nutrient starvation conditions especially by amino acid depletion. Both mTORC1 and mTORC2 are inhibited by starvation [60]. Rapamycin can specifically inhibit mTORC1 [61], whereas mTORC2 is insensitive to this chemical [62]. However, in certain cell types, long term treatment with rapamycin, was shown to block mTORC2 through suppression of its assembly [63]. In mammalian cells, rapamycin inhibits mTORC1 by forming a complex with immunophilin FKBP12 (FK506-binding protein of 12 kDa). Indeed, the FKBP12–rapamycin complex bind to mTOR and blocks substrate recruitment to mTOR complex [64]. The inhibition of mTORC1 with rapamycin enhances autophagy. Under these conditions, a decrease in the phosphorylation and activation of at least two mTORC1 downstream effectors, namely P70S6K (ribosomal protein S6 kinase-1) and 4E-BP1 (translation initiation factor 4E binding protein-1), was observed. These mTORC1 targets play important roles in protein synthesis and cell survival [65]. As rapamycin has limited effect on mTOR kinase activity and is a relatively poor inducer of autophagy, several selective inhibitors for both mTOR complexes (C1 and C2) have been developed. These include Torin 1, Torin 2 and PP242 which are highly potent and ATP-competitive inhibitors. These inhibitors block the active site of the mTOR kinase, leading to the inhibition of both TORC1 and mTORC2 [66–68].

The mTOR signaling pathway is controlled by several upstream molecules such as small GTPases or Tuberous sclerosis complex 1 and 2 proteins (TSC1/2):

Control of mTOR with small GTPases The small GTPases Rag (Ras-related GTP-binding protein) exist as constitutive heterodimers of RagA or RagB, that interact with RagC or RagD, respectively. In response to amino acid stimulus, Rag GTPases become active with RagA and RagB loading with GTP and RagC and RagD loading with GDP. Once activated, Rag GTP bind and activate mTORC1 by inducing its translocation to the lysosomal membrane. There mTORC1 can be activated with a second small GTPase, Rheb (Ras homologue enriched in brain) (Fig. 1.1). Depletion of amino acids in the cytosol results in the inactivation of Rag GTPases favoring GDP- (RagA/B) or (RagC/D) GTP-bound forms. The mTORC1 is then inactivated and autophagy is stimulated [69]. A recent study challenged these results showing that amino acid depletion inhibited Rag-mTORC1 interaction without changing Rag GTPase guanyl nucleotide charging [70]. Nevertheless, all these results indicate that amino acid sensing by Rag-mTORC1 result in alteration of Rag, mTORC1 and modulate cellular anabolic versus catabolic responses.

Control of mTOR with Tuberous Sclerosis Complex 1 and 2 (TSC1/TSC2) The TSC1/TSC2 complex is one of the most important upstream modulators of mTOR (Fig. 1.1). TSC1/TSC2 complex is a GTPase activating protein (GAP) for the mTORC1 positive regulator Rheb protein. Hence, the TSC1/TSC2 complex is a negative regulator of mTORC1. Indeed, once activated, TSC1/TSC2 inhibits the activation of Rheb, inactivating mTORC1. In contrast to mTORC1, mTORC2 is positively regulated by TSC1/TSC2 [71]. Indeed the TSC1/TSC2 complex activates mTORC2 by a mechanism that is independent of its GAP activity toward Rheb, but with a mechanism that is dependent on the direct association with the mTORC2 complex.

TSC1/TSC2 complex activation is regulated by different kinases. Serine/threonine kinase Akt (also known as Protein Kinase B, PKB) which is activated mainly by growth factors and hormones (e.g. insulin-like growth factors), can inhibit autophagy activation via a TSC1/TSC2 dependent mechanism [72]. Indeed, the direct phosphorylation of TSC2 by Akt induces the inactivation of the TSC1/TSC2 complex, consequently relieving Rheb repression and activating of mTORC1. On the other hand, AMP-activated protein kinase (AMPK) phosphorylates TSC2 and enhances its activity, resulting in the inactivation of mTORC1 and initiation of autophagy [73].

Regulation of Autophagy by mTOR-Independent Pathways Various mTOR-independent autophagy pathways have been described, including Inositol, FoxO3 and hypoxia/Beclin1 pathways.

Inositol Signaling Pathway Inositol pathway negatively regulates autophagy. Upon a stimulus activating a G-protein coupled receptor (GPCR), phospholipase C is activated downstream and hydrolyzes phosphatidylinositol 4,5-bisphosphate PIP2 to form inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 acts as a second messenger and induces calcium release from the stores by binding IP3 receptors (IP3R) found on endoplasmic reticulum (ER) membranes [74]. The release of calcium from the ER to the cytosol activates signaling pathways that play important roles in the regulation of autophagy. For example, calpain (a calcium dependent

protease) that is activated by cytosolic calcium was shown to cleave Beclin-1 and deregulate the autophagy pathway [75]. Reduction of intracellular IP3 levels stimulates autophagy, whereas IP3 accumulation inhibits autophagy induced by starvation condition. Indeed, decrease in the level of IP3 by lithium treatment was shown to enhance autophagy through an mTOR-independent manner [76]. In line with these observations, knockdown of the IP3R by small interfering RNAs and pharmacological IP3R blockage resulted in autophagy induction [77].

FoxO3 Signaling Pathway Activation of FoxO3 (forkhead box O3) transcription factors is sufficient for the induction of autophagy in skeletal muscle and result in muscle atrophy *in vivo* [78]. In fact, activated FoxO3 stimulates lysosomal proteolysis in muscle (and other cell types) through autophagy activation. FoxO3 also induces the expression of many autophagy-related genes including LC3, BNIP3 (Bcl-2 adenovirus E1a nineteen kilodalton interacting protein 3) and BNIP3L ((BNIP3 like protein, also known as NIX) [78, 79]. Therefore, transcriptional upregulation of autophagy-related genes through FoxO3 is an important event in the control of autophagic activity. The activity of FoxO3 can be regulated by the serine/threonine kinase Akt. Indeed, Akt was shown to inactivate FoxO3 by phosphorylation, leading to its retention in the cytoplasm in response to insulin or growth factors [80].

Hypoxia/Beclin1pathway In most cases, hypoxia or oxygen deprivation induces autophagy to ensure cell survival rather than cell death. Hypoxia-inducible factor 1α (HIF1α) is a major factor in the cell survival response against hypoxia. HIF1α is a transcriptional factor that upregulates the expression of autophagy proteins, including BNIP3 and BNIP3L [81]. BNIP3 proteins were able to induce autophagy by releasing Beclin 1 from Bcl-2 family proteins, allowing Beclin1 to participate in autophagosome formation as a component of the PI3K complex. HIF1α may play a significant role during hypoxia-induced responses and cell death [81]. However in contrast, hypoxia-induced autophagy via BNIP3 and BNIP3L, might be a survival mechanism in some contexts [82]. Knockdown of BNIP3 or BNIP3L with small interfering RNAs had little effect on autophagy. However the combined silencing of both genes was shown to suppress hypoxia-mediated autophagy and cell survival [82].

Role of Autophagy in Neurons

Basal Autophagy in Neurons Neurons are highly polarized postmitotic cells, consisting of a cell body or soma with branching dendrites and axons forming contacts, i.e. synapses, with other neurons. Soma is the central region of the neuron containing the nucleus and other cellular organelles such as the endoplasmic reticulum, Golgi, mitochondria and most of the lysosomes. The synapse is responsible for the transfer of information from one neuron to another. Soma communicates with synapse via the axon which transfers proteins and organelles over a generally significant distance through axoplasmic transport (Fig. 1.2a). Movement toward the soma is

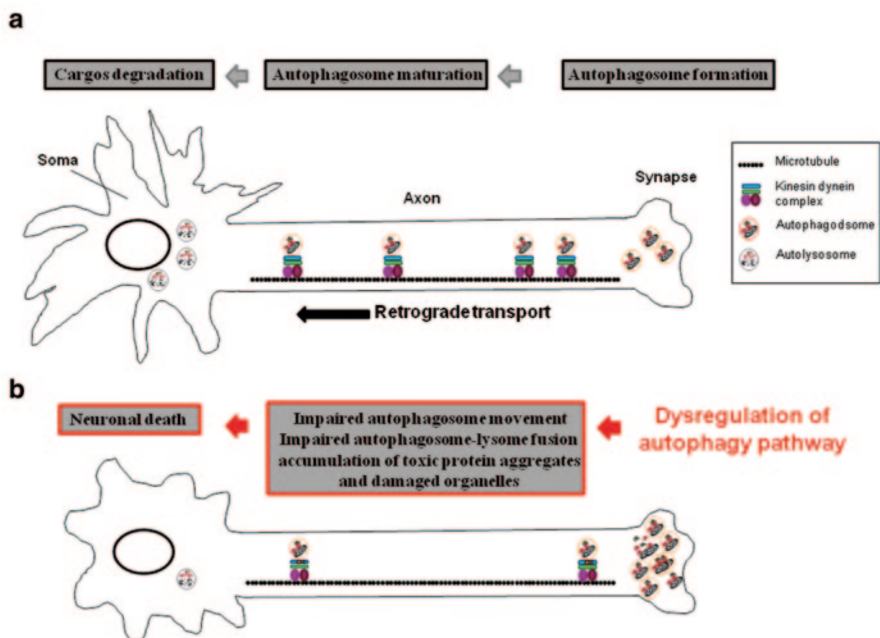

Fig. 1.2 Schematic representation of the autophagy pathway in neurons. **a.** Autophagosomes are constituvely generated near synapses to prevent neuronal proteotoxicity. Upon exit from synapse, autophagosomes follow a unidirectional retrograde transport path, undergo maturation and fuse with lysosomes as they move along the axon toward the soma. When they arrived to the soma, autophagosomes are fully acidified, consistent with the formation of autolysosomes. Degradation of engulfed materials occurs in autolysosomes that are generally found near soma. Digested contents are then recycled. **b.** Dysregulation of autophagy in neuron might lead to the accumulation of toxic proteins, protein aggregates and damaged organelles in the synapse and axon and eventually contribute to neuronal toxicity and cell death

called the retrograde transport while movement toward the synapse is called the anterograde transport.

Because neurons are post-mitotic cells, they are more sensitive to the toxicity of misfolded proteins and/or damaged organelles, than cells that undergo mitosis and that have a chance to dilute them. In neurons, constitutive autophagy has an important task of removing these harmful components and protects neuronal homeostasis. Indeed, it has been shown that suppression of basal autophagy in neural cells caused neurodegeneration in mice [83]. Therefore, by preventing accumulation of toxic materials, basal autophagic activity is important for the prevention of neural function disruption, neurite degeneration and consequent neuronal cell death.

In primary neurons, autophagosomes were shown to be constitutively generated near the synapse [84, 85]. These early autophagosomes were shown to move bidirectionally along microtubules driven by bound kinesin and dynein motors. Upon exit from the synapse, autophagosomes follow a unidirectional retrograde transport and undergo maturation as they move along the axon toward the soma,

carrying engulfed organelles and soluble cargo. Upon arrival to the soma, autophagosomes are fully acidified, consistent with the formation of autolysosomes. The degradation of the cargo occurs near the soma, facilitating efficient recycling of cytosolic contents in the primary site of protein and organelle synthesis (Fig. 1.2a). Constitutive formation of autophagosomes at the synapse, followed by a robust transit to axon constitutes a critical surveillance mechanism for routine maintenance of neurons and for the prevention of degeneration and cell death (Fig. 1.2b). Therefore, it is not surprising that autophagy failure is a general and common observation in a number of neurodegenerative diseases.

Role of Basal Autophagy in the Synapse Basal autophagy is of particular importance in the synaptic compartments of neurons where high energy requirements and protein turnover are necessary to sustain synaptic growth and activity. Autophagy can play a role in synapse plasticity as well. Indeed, a decrease or increase in basal autophagy levels resulted in the aberrations of synaptic size and function in *Drosophila* neuromuscular junctions [86]. Because autophagy is highly conserved, it is likely to play an important role in synapse development in more complex animals as well. Autophagy was shown to play an important role in the degradation of cell-surface neurotransmitter receptors at the presynaptic terminals of the neurons [87]. Indeed, it has been shown that autophagy can function as a selective degradation pathway for the neurotransmitter gamma-aminobutyric acid (GABA) receptors [87]. In line with these studies, recent study showed that Beclin 2 which also participates to autophagy regulation, played an important role in the lysosomal degradation of several G protein-coupled receptors (GPCRs), including the delta opioid receptor (DOR), cannabinoid 1 receptor (CB1R) and dopamine D2 receptor [88]. Finally, macroautophagy is also contributes to synaptic plasticity by regulating the presynaptic structure function in dopaminergic neurons [89]. Mechanism including those cited above might be important for the control of the balance between neuronal excitation and inhibition and consequently synaptic transmission.

Role of Basal Autophagy in the Axon Constitutive autophagy is essential for axon homeostasis as well. Suppression of basal autophagy by either deleting an *ATG* gene or inhibiting autophagic clearance in neurons disrupted axonal transport of autophagosomes, and resulted in axonal swelling and dystrophy [90]. Additionally, mutation of Atg1 and ULK1/2 induced disruptions in the axon ultrastructure and axonal extension, in *C.elegans* and in mice, respectively [91]. Neuron-specific deletion of the mammalian FIP200 (FAK family-interacting protein of 200 kDa), a subunit of ULK complex, implicated in autophagosome biogenesis, caused an increase in neuronal death and axon degeneration [92], Moreover, autophagy was shown to regulate early axonal growth in cortical neurons [93]. These data and others suggest a possible role of autophagy in axon development and homeostasis.

LC3, a key player in the autophagosome formation, was found in association with the microtubule-associated protein 1B (MAP1B), an abundant protein in axons [94]. In fact, MAP1B is involved in the regulation of microtubule stability and in retrograde axonal transport. MAP1B binds both LC3-I (cytosolic form) and LC3-II (lipidated form). Overexpression of MAP1B in non-neuronal cells led to a reduction in the number of LC3-associated autophagosomes [94]. Therefore, MAP1B-LC3

interaction is important for the regulation of autophagosomes in axons and participate to the regulation of the retrograde axonal transport of autophagomes towards the soma allowing autophagosome maturation.

Autophagy in Diseases Autophagy dysfunction is implicated in a wide spectrum of human diseases, including neurodegeneration, infections, cancer and heart diseases and even conditions such as aging. Understanding the molecular pathways and the regulatory network of autophagy is of pivotal importance in the determination of best pharmacological targets for modulating autophagy in each different disease context. Here, we will give a brief overview about the role of autophagy in selected pathologies leading to neurodegeneration.

Autophagy and Neurodegenerative Diseases Autophagy deregulation might result in or contribute to the pathology of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). All of these diseases are characterized by the accumulation of abnormal, misfolded proteins that eventually become toxic for neurons. In an attempt to clear these toxic proteins, neurons activate autophagy, and failure of clearance and/or autophagosome accumulation might in turn have serious consequences for disease progression and neuronal survival [95].

Alzheimer's Disease and Autophagy Patients with Alzheimer's diseases (AD) generally present to the clinic with cognitive impairment and memory problems that are secondary to the degeneration of neurons in the affected brain regions e.g. basal forebrain and hippocampus. AD is characterized by the accumulation of amyloid β-peptides and intracellular neurofibrillary tangles comprising hyperphosphorylated Tau protein in the brain [96, 97]. Accumulation of these amyloid-β peptides is a result of a sequential proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase also called (β-site-APP cleaving enzyme, BACE) and γ-secretase complex which is composed of PS1 (presenilin-1) and PS2, nicastrin, APH1(anterior pharynx-defective 1) and PEN2 (presenilin enhancer 2). APP protein is a member of a family of conserved type 1 membrane proteins which play physiological roles in trafficking, neurotrophic signaling, cell adhesion and cell signaling. After APP is synthesized, the mature glycosylated form is delivered to the plasma membrane, where it is rapidly processed. The cleavage of APP by BACE releases a soluble N-terminal fragment (sAPP-β) and a C-terminal fragment (βCTF) that remains in the membrane. The βCTF fragment is further cleaved by γ-secretase, resulting in a release of a 40-mer peptide (Aβ40) (physiological role in synapse formation) and smaller amounts of a 42-mer peptide (Aβ42) (pathological aggregate-prone forms). The APP in the plasma membrane can also be processed by α-secretase and generate a soluble N-terminal fragment (sAPP-α), which is released from the cell, and a CTF that remains in the membrane which is devoid of the whole Aβ-peptide [98].

Several genetic defects have been identified to cause rare familial forms of AD, such as mutations in the amyloid precursor protein or PS1 and PS2 which are linked to autosomal dominant forms of FAD (familial AD) [99]. Links between Alzheimer's-susceptibility genes/proteins and autophagy-lysosome system were reported. For example, the PS1 subunit of the γ-secretase, was found to be essential

for lysosomal acidification and protein degradation. Indeed in the ER, PS1 induces the N-glycosylation of the V-ATPase V0a1 lysosomal subunit. This posttranslational modification of V-ATPase V0a1 facilitates its translocation to the lysosome where it plays an important role in the acidification of the lysosome [15]. Some PS1 mutations found in the autosomal dominant forms of FAD were shown to impair lysosomal acidification and protein turnover by autophagy, enhancing pathogenic protein accumulations and neuronal cell death. Another link between autophagy and AD was related to the key autophagy protein Beclin1. It was found that, affected brain regions of patients with Alzheimer's disease demonstrated low Beclin1 expression levels, especially in the early stages of the disease. In line with this, heterozygous deletion of Beclin 1 in mice attenuated neuronal autophagy and promoted neurodegeneration [100]. Finally, recent study showed that, the treatment of AD mice model with carbamazepine (CBZ), an anti-epileptic drug that has a potent autophagy enhancement effects, increased autophagic activity and decreased amyloid plaque and amyloid β-peptides levels [101]. Another compound, the mTOR inhibitor temsirolimus, induced autophagy in cellular and animal models of AD, and led to the clearance of amyloid β-peptides through autophagy [102]. Tau might also be targeted by autophagosomes for degradation, yet other reports warned that autophagosomes might contribute to amyloid-beta generation [103]. Moreover, together with abnormalities of endocytosis, lysosomal acidification and intracellular transport that were observed in AD, defective autophagic vesicles incapable of maturation into active autolysosomes might accumulate in AD neurons and contribute to disease progression in many cases.

Parkinson's Disease and Autophagy Patients with Parkinson's disease (PD) suffer from movement disorders, including muscle rigidity, akinesia and resting tremor symptoms caused by the degeneration and loss of dopaminergic neurons in the substansia nigra of the midbrain [104]. Gain of function mutations in the genes encoding α-synuclein (A53T and A30P) or LRRK2 (leucine-rich repeat kinase 2) were found in early-onset autosomal dominant forms of the disease. On the other hand, mutations in the genes encoding parkin, PINK1 (PTEN-induced kinase 1) or DJ-1, causing protein loss of function are associated with recessive forms of the disease [105].

Wild type α-synuclein plays a role in release of vesicles in synaptic junctions [106]. It was shown that both wild type and the pathogenic A53T and A30P α-synuclein mutants could be cleared by the autophagy pathway [107]. However, only the wild type α-synuclein was cleared by the CMA pathway in the absence of a mutant form [108]. Indeed, the α-synuclein mutants were able to bind to lysosomal membranes in the CMA pathway, but they appeared to act as blockers, inhibiting both their own translocation and degradation by the lysosome and that of other substrates [108]. Beclin1 autophagy protein, was shown to play an role in the intracellular degradation of α-synuclein through the autophagy pathway [109]. Coexpression of beclin 1 with α-synuclein activated autophagy, reduced accumulation of the protein, and ameliorated neuron alterations [109].

Several recent studies associated mitophagy abnormality with Parkinson's disease [110]. Pathogenic mutations in proteins that are associated with PD including

parkin, PINK1, DJ-1 and LRRK2 resulted in defects of mitophagy, consequent accumulation of mitochondria and increased neuronal toxicity [111]. As already mentioned above, in the most studied pathway of mitophagy, mitochondrial damage results in the stabilization of otherwise degraded PINK1 on the outer membrane of mitochondria, allowing recruitment of the ubiquitin ligase Parkin. Ubiqutylation and proteasomal degradation of several mitochondrial membrane proteins, primes mitochondria for autophagic sequestration and destruction [112]. LRRK2 protein is predominantly expressed in the cytoplasm but also associates with the mitochondrial outer membrane, plasma membrane and synaptic vesicles in cultured cells including mouse primary neurons [113, 114]. LRRK2 mutants also affected mitochondrial homeostasis in neurons by enhancing constitutive mitophagy in neurons, resulting in mitochondrial degradation and dendrite shortening [115, 116]. Another PD-associated protein, DJ-1 works in parallel to the PINK1/parkin pathway and functions in the maintenance of mitochondrial function during oxidative stress [117]. Moreover, mutations in the *DJ-1* gene lead to mitochondrial depolarization, fragmentation and accumulation of autophagosome in human dopaminergic neurons [117]. In fact autophagy dysregulation by oxidative stress was described as a major contributor to the PD [118–121]. In this context, problems in the autophagic elimination of damaged mitochondria might be among one of the major cellular defects associated with the disease.

Huntington's Disease and Autophagy Patients with Huntington's disease (HD) present with motor dysfunction and cognitive decline symptoms caused by the degeneration of neurons in the striatum and other regions of the brain. HD is an autosomal dominant disorder caused by expansion of polyglutamine (polyQ) repeats in the N-terminus of the Huntington (Htt) protein, altering protein stability. Mutant proteins are resistant to proteolysis, and they are prone to aggregation. Wild-type Htt is mostly found in the cytoplasm, but it might show a nuclear localization as well. Mutant full-length Htt or its N-terminal fragments might accumulate as ubiquitylated inclusions, impairing the UPS system that normally degrades soluble forms of the Htt protein, strongly stimulating autophagy [122]. Furthermore, postmortem analyses revealed a dramatic increase in the number of autophagosome-like structures in the brains of HD patients [123].

Several studies defined autophagy as a Htt clearance mechanism, especially for the insoluble forms creating aggregates. Perturbance of autophagy under these conditions severely compromised cell viability [124, 125]. Consequently, mTOR inhibitors that lead to autophagy activation, including rapamycin derivatives, led to the degradation of Htt aggregates and could treat HD animal models [123]. Additionally, mutant Htt aggregates were shown to sequester mTOR and inactivate it in transgenic mice models and in patient samples, resulting in further stimulation of autophagy [126].

Both wild-type and mutant form of Htt proteins are highly regulated by post-translational modifications including proteolysis, palmitoylation and phosphorylation. Alteration in some of this modification was shown to affect the rate of autophagosome formation in neuronal cell lines under basal conditions [127]. In line with this, autophagic clearance of mutant Htt correlated with its acetylation at

K444 [128]. Overall, autophagy might clear pathological Htt aggregates and alleviate disease burden in patients suffering from HD.

Conclusion

Autophagy is an important process used by the cell to adapt its metabolism to stressfull conditions, such as nutrient starvation, accumulation of abnormal proteins, hypoxia, oxidative stress etc. Strikingly, mere autophagy defects were sufficient to induce neurodegeneration, underlining the importance of this phenomenon for neuronal health and survival [83, 90]. Mutations affecting transport of autophagosomes and those disrupting lysosomal function, caused an increase in autophagosome accumulation and reduced clearance by autophagy leading to neuronal toxicity [129, 130]. Hence, pathologies that proceed through accumulation of mutant proteins and accompanied by protein degradation defects, such as Alzheimer's, Parkinson's and Huntington's diseases also showed autophagy dysregulation. In many cases, autophagy abnormalities are related to neuronal toxicity and cell death. Therefore, autophagy plays a crucial role as a quality-control system in neurons and other cell types, controlling protein aggregate levels and damaged organelles both under basal and stress conditions.

Pharmacological activation of autophagy in neurodegenerative diseases was shown to reduce the levels of insoluble mutant proteins aggregates, such as mutant Htt protein and mutant forms of α-synuclein, and in some cases reduced protein toxicity in animal models of neurodegenerative diseases [130]. On the other hand, defective autophagic vesicle accumulation might be toxic itself under different contexts. In these cases, e.g. AD, activation of autophagy might even deteriorate the situation. Therefore a careful examination of the role of autophagy under specific patological conditions is necessary before designing autophagy-related treatment protocols. Most importantly, more molecular studies are needed to provide details about the connection of autophagy to specific diseases. As our knowledge on the basic machinery of autophagy as well as on the role of autophagy in health and disease expands, more rational and disease-specific approaches will be tailored, sparing normal tissue responses while targeting disease causing phenomena.

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References

1. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451:1069–75.

2. Paul S. Dysfunction of the ubiquitin-proteasome system in multiple disease conditions: therapeutic approaches. Bioessays. 2008;30:1172–84.
3. Todde V, Veenhuis M, van der Klei IJ. Autophagy: principles and significance in health and disease. Biochim Biophys Acta. 2009;1792:3–13.
4. Ashford TP, Porter KR. Cytoplasmic components in hepatic cell lysosomes. J Cell Biol. 1962;12:198–202.
5. Al Rawi S, Louvet-Vallee S, Djeddi A, Sachse M, Culetto E, et al. Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission. Science. 2011;334:1144–47.
6. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci U S A. 2007;104:19500–05.
7. Curt A, Zhang J, Minnerly J, Jia K. Intestinal autophagy activity is essential for host defense against Salmonella typhimurium infection in Caenorhabditis elegans. Dev Comp Immunol. 2014;45:214–8.
8. Chargui A, El May MV. Autophagy mediates neutrophil responses to bacterial infection. APMIS. 2014;122:1047–58.
9. Mari M, Griffith J, Rieter E, Krishnappa L, Klionsky DJ, et al. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. J Cell Biol. 2010;190:1005–22.
10. Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, et al. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol. 2009;11:1433–7.
11. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;141:656–67.
12. Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, et al. Autophagosomes form at ER-mitochondria contact sites. Nature. 2013;495:389–93.
13. Ravikumar B, Moreau K, Rubinsztein DC. Plasma membrane helps autophagosomes grow. Autophagy. 2010;6:1184–6.
14. Wong PM, Puente C, Ganley IG, Jiang XJ. The ULK1 complex sensing nutrient signals for autophagy activation. Autophagy. 2013;9:124–37.
15. Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, et al. (2010) Lysosomal proteolysis and autophagy require Presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 141: 1146–U1191.
16. Lee EJ, Tournier C. The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. Autophagy. 2011;7:689–95.
17. Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, et al. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood. 2008;112:1493–502.
18. Hara T, Takamura A, Kishi C, Iemura S, Natsume T, et al. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. J Cell Biol. 2008;181:497–510.
19. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009;20:1992–2003.
20. Chan EY, Longatti A, McKnight NC, Tooze SA. Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol Cell Biol. 2009;29:157–71.
21. Vergne I, Deretic V. The role of PI3P phosphatases in the regulation of autophagy. FEBS Lett. 2010;584:1313–8.
22. Foster FM, Traer CJ, Abraham SM, Fry MJ. The phosphoinositide (PI) 3-kinase family. J Cell Sci. 2003;116:3037–40.
23. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol. 2008;182:685–701.

24. Panaretou C, Domin J, Cockcroft S, Waterfield MD. Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. J Biol Chem. 1997;272:2477–85.
25. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol. 2010;20:355–62.
26. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol. 2013;15:741–50.
27. Proikas-Cezanne T, Codogno P. Beclin 1 or not Beclin 1. Autophag. 2011;7:671–2.
28. Mauthe M, Jacob A, Freiberger S, Hentschel K, Stierhof YD, et al. Resveratrol-mediated autophagy requires WIPI-1 regulated LC3 lipidation in the absence of induced phagophore formation. Autophagy. 2011;7:1448–61.
29. Bakula D, Takacs Z, Proikas-Cezanne T. WIPI beta-propellers in autophagy-related diseases and longevity. Biochem Soc Trans. 2013;41:962–7.
30. Young ARJ, Chan EYW, Hu XW, Koch R, Crawshaw SG, et al. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci. 2006;119:3888–900.
31. Tanida I. Autophagosome formation and molecular mechanism of autophagy. Antioxid Redox Signal. 2011;14:2201–14.
32. Velikkakath AK, Nishimura T, Oita E, Ishihara N, Mizushima N. Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets. Mol Biol Cell. 2012;23:896–909.
33. Sou YS, Waguri S, Iwata J, Ueno T, Fujimura T, et al. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. Mol Biol Cell. 2008;19:4762–75.
34. Romanov J, Walczak M, Ibiricu I, Schuchner S, Ogris E, et al. Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. Embo J. 2012;31:4304–17.
35. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol. 2000;151:263–76.
36. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem. 1998;273:33889–92.
37. Mizushima N, Noda T, Ohsumi Y. Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. EMBO J. 1999;18:3888–96.
38. Mizushima N, Yoshimori T, Ohsumi Y. Role of the Apg12 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2003;35:553–61.
39. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, et al. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J Cell Sci. 2004;117:2805–12.
40. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2004;36:2503–18.
41. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19:5720–28.
42. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol. 2001;152:657–68.
43. Kimura S, Noda T, Yoshimori T. Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. Cell Struct Funct. 2008;33:109–22.
44. Anderson RG, Falck JR, Goldstein JL, Brown MS. Visualization of acidic organelles in intact cells by electron microscopy. Proc Natl Acad Sci U S A. 1984;81:4838–42.

45. Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. Cell Death Differ. 2013;20:21–30.
46. Scarffe LA, Stevens DA, Dawson VL, Dawson TM. Parkin and PINK1: much more than mitophagy. Trends Neurosci. 2014;37:315–24.
47. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy. 2011;7:673–82.
48. Dubouloz F, Deloche O, Wanke V, Cameroni E, De Virgilio C. The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol Cell. 2005;19:15–26.
49. Wada Y, Sun-Wada GH, Kawamura N. Microautophagy in the visceral endoderm is essential for mouse early development. Autophagy. 2013;9:252–4.
50. Agarraberes FA, Dice JF. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. J Cell Sci. 2001;114:2491–9.
51. Chiang HL, Dice JF. Peptide sequences that target proteins for enhanced degradation during serum withdrawal. J Biol Chem. 1988;263:6797–805.
52. Salvador N, Aguado C, Horst M, Knecht E. Import of a cytosolic protein into lysosomes by chaperone-mediated autophagy depends on its folding state. J Biol Chem. 2000;275: 27447–56.
53. Terlecky SR, Dice JF. Polypeptide import and degradation by isolated lysosomes. J Biol Chem. 1993;268:23490–5.
54. Kaushik S, Massey AC, Mizushima N, Cuervo AM. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Mol Biol Cell. 2008;19:2179–92.
55. Loos B, Engelbrecht AM, Lockshin RA, Klionsky DJ, Zakeri Z. The variability of autophagy and cell death susceptibility Unanswered questions. Autophagy. 2013;9:1270–85.
56. Agarraberes FA, Terlecky SR, Dice JF. An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. J Cell Biol. 1997;137:825–34.
57. Kaushik S, Bandyopadhyay U, Sridhar S, Kiffin R, Martinez-Vicente M, et al. Chaperone-mediated autophagy at a glance. J Cell Sci. 2011;124:495–9.
58. Fujiwara Y, Furuta A, Kikuchi H, Aizawa S, Hatanaka Y, et al. Discovery of a novel type of autophagy targeting RNA. Autophagy. 2013;9:403–9.
59. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149: 274–93.
60. Tato I, Bartrons R, Ventura F, Rosa JL. Amino Acids Activate Mammalian Target of Rapamycin Complex 2 (mTORC2) via PI3K/Akt Signaling. J Biol Chem. 2011;286:6128–42.
61. Nyfeler B, Bergman P, Triantafellow E, Wilson CJ, Zhu YY, et al. Relieving Autophagy and 4EBP1 from Rapamycin Resistance. Mol Cell Biol. 2011;31:2867–76.
62. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6:1122–30.
63. Phung TL, Ziv K, Dabydeen D, Eyiah-Mensch G, Riveros M, et al. Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. Cancer Cell. 2006;10: 159–70.
64. Shimobayashi M, Hall MN. Making new contacts: the mTOR network in metabolism and signalling crosstalk. Nat Rev Mol Cell Biol. 2014;15:155–62.
65. Maiese K, Chong ZZ, Shang YC, Wang S. mTOR: on target for novel therapeutic strategies in the nervous system. Trends Mol Med. 2013;19:51–60.
66. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, et al. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol. 2009;7:e38.
67. Thoreen CC. Many roads from mTOR to eIF4F. Biochem Soc Trans. 2013;41:913–6.
68. Zullo AJ, Jurcic Smith KL, Lee S. Mammalian target of Rapamycin inhibition and mycobacterial survival are uncoupled in murine macrophages. BMC Biochem. 2014;15:4.
69. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, et al. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010;141:290–303.

70. Oshiro N, Rapley J, Avruch J. Amino acids activate mammalian target of rapamycin (mTOR) complex 1 without changing Rag GTPase guanyl nucleotide charging. J Biol Chem. 2014;289:2658–74.
71. Huang JX, Dibble CC, Matsuzaki M, Manning BD. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol. 2008;28:4104–15.
72. Cai D, Wang Y, Ottmann OG, Barth PJ, Neubauer A, et al. FLT3-ITD-, but not BCR/ABL-transformed cells require concurrent Akt/mTor blockage to undergo apoptosis after histone deacetylase inhibitor treatment. Blood. 2006;107:2094–7.
73. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003;17:1829–34.
74. Salido GM, Sage SO, Rosado JA. TRPC channels and store-operated Ca(2+) entry. Biochem Biophys Acta. 2009;1793:223–30.
75. Russo R, Berliocchi L, Adornetto A, Varano GP, Cavaliere F, et al. Calpain-mediated cleavage of Beclin-1 and autophagy deregulation following retinal ischemic injury in vivo. Cell Death Dis. 2011;2:e144.
76. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. J Cell Biol. 2005;170:1101–11.
77. Criollo A, Vicencio JM, Tasdemir E, Maiuri MC, Lavandero S, et al. The inositol trisphosphate receptor in the control of autophagy. Autophagy. 2007;3:350–3.
78. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, et al. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab. 2007;6:458–71.
79. Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ. 2009;16:939–46.
80. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, et al. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell. 1999;96:857–68.
81. Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, et al. Hypoxia induces the expression of the pro-apoptotic gene BNIP3. Cell Death Differ. 2001;8:367–76.
82. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol. 2009;29:2570–81.
83. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441:885–9.
84. Maday S, Holzbaur EL. Autophagosome assembly and cargo capture in the distal axon. Autophagy. 2012;8:858–60.
85. Rixon HW, Brown G, Murray JT, Sugrue RJ. The respiratory syncytial virus small hydrophobic protein is phosphorylated via a mitogen-activated protein kinase p38-dependent tyrosine kinase activity during virus infection. J Gen Virol. 2005;86:375–84.
86. Shen W, Ganetzky B. Autophagy promotes synapse development in Drosophila. J Cell Biol. 2009;187:71–9.
87. Rowland AM, Richmond JE, Olsen JG, Hall DH, Bamber BA. Presynaptic terminals independently regulate synaptic clustering and autophagy of GABAA receptors in Caenorhabditis elegans. J Neurosci. 2006;26:1711–20.
88. He C, Wei Y, Sun K, Li B, Dong X, et al. Beclin 2 functions in autophagy, degradation of G protein-coupled receptors, and metabolism. Cell. 2013;154:1085–99.
89. Hernandez D, Torres CA, Setlik W, Cebrian C, Mosharov EV, et al. Regulation of presynaptic neurotransmission by macroautophagy. Neuron. 2012;74:277–84.
90. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441:880–4.
91. McIntire SL, Garriga G, White J, Jacobson D, Horvitz HR. Genes necessary for directed axonal elongation or fasciculation in C. elegans. Neuron. 1992;8:307–22.
92. Liang CC, Wang C, Peng X, Gan B, Guan JL. Neural-specific deletion of FIP200 leads to cerebellar degeneration caused by increased neuronal death and axon degeneration. J Biol Chem. 2010;285:3499–509.

93. Ban BK, Jun MH, Ryu HH, Jang DJ, Ahmad ST, et al. Autophagy negatively regulates early axon growth in cortical neurons. Mol Cell Biol. 2013;33:3907–19.
94. Wang QJ, Ding Y, Kohtz DS, Mizushima N, Cristea IM, et al. Induction of autophagy in axonal dystrophy and degeneration. J Neurosci. 2006;26:8057–68.
95. Tekirdag KA. Alteration in autophagic-lysosomal potential during ageing and neurological diseases: the microRNA perspective. Curr Pathobiol Rep. 2013;1:247–61.
96. Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, et al. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature. 1992;360:672–4.
97. Scheuner D, Eckman C, Jensen M, Song X, Citron M, et al. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med. 1996;2:864–70.
98. Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci. 2007;120: 4081–91.
99. Selkoe DJ, Wolfe MS. Presenilin: running with scissors in the membrane. Cell. 2007;131: 215–21.
100. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, et al. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest. 2008;118:2190–9.
101. Li W, Tang Y, Fan Z, Meng Y, Yang G, et al. Autophagy is involved in oligodendroglial precursor-mediated clearance of amyloid peptide. Mol Neurodegener. 2013;8:27.
102. Jiang T, Yu JT, Zhu XC, Tan MS, Wang HF, et al. Temsirolimus promotes autophagic clearance of amyloid-beta and provides protective effects in cellular and animal models of Alzheimer's disease. Pharmacol Res. 2014;81:54–63.
103. Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, et al. Macroautophagy-a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol. 2005;171:87–98.
104. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003;39:889–909.
105. Ross CA, Pickart CM. The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases. Trends Cell Biol. 2004;14:703–11.
106. Bellani S, Sousa VL, Ronzitti G, Valtorta F, Meldolesi J, et al. The regulation of synaptic function by alpha-synuclein. Commun Integr Biol. 2010;3:106–9.
107. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem. 2003;278:25009–13.
108. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science. 2004;305:1292–5.
109. Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, et al. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J Neurosci. 2009;29:13578–88.
110. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8:e1000298.
111. Vives-Bauza C, Tocilescu M, Devries RL, Alessi DM, Jackson-Lewis V, et al. Control of mitochondrial integrity in Parkinson's disease. Prog Brain Res. 2010;183:99–113.
112. Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol. 2011;12:9–14.
113. West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sci U S A. 2005;102:16842–7.
114. Hatano T, Kubo S, Imai S, Maeda M, Ishikawa K, et al. Leucine-rich repeat kinase 2 associates with lipid rafts. Hum Mol Genet. 2007;16:678–90.
115. Brice A. Genetics of Parkinson's disease: LRRK2 on the rise. Brain. 2005;128:2760–2.
116. Cherra SJ, 3rd, Steer E, Gusdon AM, Kiselyov K, Chu CT. Mutant LRRK2 elicits calcium imbalance and depletion of dendritic mitochondria in neurons. Am J Pathol. 2013;182:474–84.

117. Thomas KJ, McCoy MK, Blackinton J, Beilina A, van der Brug M, et al. DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Hum Mol Genet. 2011;20:40–50.
118. Dagda RK, Das Banerjee T, Janda E. How Parkinsonian toxins dysregulate the autophagy machinery. Int J Mol Sci. 2013;14:22163–89.
119. Yoo MS, Kawamata H, Kim DJ, Chun HS, Son JH. Experimental strategy to identify genes susceptible to oxidative stress in nigral dopaminergic neurons. Neurochem Res. 2004;29:1223–34.
120. Choi KC, Kim SH, Ha JY, Kim ST, Son JH. A novel mTOR activating protein protects dopamine neurons against oxidative stress by repressing autophagy related cell death. J Neurochem. 2010;112:366–76.
121. Ha JY, Kim JS, Kang YH, Bok E, Kim YS, et al. Tnfaip8 l1/Oxi-beta binds to FBXW5, increasing autophagy through activation of TSC2 in a Parkinson's disease model. J Neurochem. 2014;129:527–38.
122. Holmberg CI, Staniszewski KE, Mensah KN, Matouschek A, Morimoto RI. Inefficient degradation of truncated polyglutamine proteins by the proteasome. EMBO J. 2004;23:4307–18.
123. Sarkar S, Rubinsztein DC. Huntington's disease: degradation of mutant huntingtin by autophagy. FEBS J. 2008;275:4263–70.
124. Bursch W, Ellinger A. Autophagy-a basic mechanism and a potential role for neurodegeneration. Folia Neuropathol. 2005;43:297–310.
125. Santos RX, Cardoso S, Correia S, Carvalho C, Santos MS, et al. Targeting autophagy in the brain: a promising approach? Cent Nerv Syst Agents Med Chem. 2010;10:158–68.
126. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004;36:585–95.
127. Martin DD, Heit RJ, Yap MC, Davidson MW, Hayden MR, et al. Identification of a post-translationally myristoylated autophagy-inducing domain released by caspase cleavage of Huntingtin. Hum Mol Genet. 2014;23:3166–79.
128. Krainc D. Clearance of mutant proteins as a therapeutic target in neurodegenerative diseases. Arch Neurol. 2010;67:388–92.
129. Rubinsztein DC, Ravikumar B, Acevedo-Arozena A, Imarisio S, O'Kane CJ, et al. Dyneins, autophagy, aggregation and neurodegeneration. Autophagy. 2005;1:177–8.
130. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132:27–42.

Chapter 2
Role of Chaperone-Mediated Autophagy in Ageing and Neurodegeneration

J. V. Ferreira, P. Pereira and H. Girao

Abstract Depending on the mechanism and molecular players involved in the targeting of a substrate to the lysosome, autophagy can be divided in three different subtypes: Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy (CMA).

In contrast to other forms of autophagy, in CMA, soluble cytosolic proteins can be targeted selectively for degradation in lysosomes. Selectivity in CMA is conferred by the presence of a pentapeptide motif in the amino acid sequence of the substrate proteins, biochemically related to KFERQ, that is recognized by the cytosolic chaperone Hsc70, which results in the targeting of substrates to the lysosome. Once at the lysosomal surface, the substrate–chaperone complex binds to the membrane, and, after unfolding the substrate, is translocated into the lumen by LAMP2A, that acts as the resident a CMA “receptor”.

CMA has been implicated both in the elimination of parts of the proteome damaged by stressors, as well as, in the selective turnover of substrates directly related with several proteinophaties, most notably in neurodegenerative diseases. In this chapter we will focus on the role of CMA in age-related neurodegeneration and how CMA often becomes the target of the toxic effect of neurodegeneration-related aberrant substrates. The mulfactorial nature of the CMA role in neurodegenerative disorders makes the careful analyses of the evidences gathered thus far instrumental for the understanding of CMA in the context of these diseases.

Keywords Autophagy · Chaperones · Neurodegeneration · LAMP2A · Proteolysis, proteostasis, aging · Lysosome

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Abbreviations

CMA	Chaperone mediated autophagy
Hsc	Heat shock cognate protein
LAMP2	Lysosomal associated protein 2
KDa	Kilodalton
HSP	Heat shock protein
Bag	Bcl 2 associate athanogene
Hop	Hsc70 Hsp90 organizing protein
Hip	Hsc70 interacting protein
GFAP	Glial fibrillary acidic protein
EF1α	Elongation factor 1 alpha
Lys	Lysosomal
UPS	Ubiquitin proteasome system
HD	Huntinton's disease
PD	Parkinson's disease
RNA	Ribonucleic Acid
LRRK2	Leucine rich repeat kinase 2
UCH-L1	Ubiquitin C terminal hydrolase L1
IPS	Induced pluripotent stem cells
MEF	Myocyte enhancer factor
AD	Alzheimer's disease
RCAN1	Regulator of calcineurin 1
HTT	Huntingtin

Introduction

The term Autophagy broadly refers to a catabolic process that is canonically involved in the lyosomal degradation of cytoplasmic components, initially including organelles and soluble proteins, but now also some membrane proteins [1–4]. However, depending on the mechanism and molecular players involved in the targeting of a substrate to the lysosome, autophagy can be divided in three different subtypes: macroautophagy, microautophagy and chaperone-mediated autophagy. Although autophagy was initially thought as a degradation pathway, activated in response to external stimuli, it is now established that it can also act as a mechanism of quality control under basal conditions.

Autophagy plays a vital role in many physiological processes [5], including the response to starvation, cell growth and innate immunity. In contrast to the in-bulk sequestration of cytosolic components characteristic of macro and microautophagy, soluble cytosolic proteins can be targeted selectively for degradation in lysosomes by a process called Chaperone-Mediated Autophagy (CMA). Indeed, what distinguishes CMA from other forms of autophagy is the selective recognition of cargo by cytosolic chaperones and the fact that substrates are not engulfed, but, instead, translocated across the lysosomal membrane in a

receptor-mediated manner [6]. Althought basal CMA activity can be detected in most types of mammalian cells, as in the case of macroautophagy, maximal activation of this pathway is triggered in response to stressors, such as long-term starvation, oxidative stress, or exposure to toxic compounds that induce abnormal conformational changes in cytosolic proteins [7].

Selectivity in CMA is conferred by the presence of a pentapeptide motif in the amino acid sequence of the substrate proteins, biochemically related to KFERQ, that when recognized by a cytosolic chaperone results in the targeting of substrates to lysosomes [8]. Similar to most targeting motifs, the KFERQ motif is degenerated and allows for a series of amino acid combinations as far as they follow this rule: Q should be the flanking amino acid but could be located at the beginning or at the end of the sequence; there could be up to two of the allowed hydrophobic residues (I, F, L or V) or two of the allowed positive residues (R or K), but only one negative charge provided either by E or D [8]. In certain proteins, Q can be replaced with N, but this exchange does not work in all proteins, suggesting that the surrounding amino acid context might be important in this case [9]. The "pure" KFERQ motif is only present in ribonuclease A, the first protein identified as a CMA substrate.

CMA substrates are recognized first in the cytoplasm by the heat shock cognate protein of 70 kD (Hsc70), the constitutively expressed member of the 70-kD family of chaperones [6]. This is actually the same chaperone responsible for disassembly of clathrin from coated vesicles and for assisting in the folding of cytosolic proteins upon recognition of exposed hydrophobic regions. It is unknown what determines the multiplicity of functions of chaperones, but the particular array of cochaperones that bind to Hsc70 in each condition is probably behind the final fate of the substrate protein. A subset of cochaperones, Hsp90, Hsp40, Bcl-2 associate athanogene 1 (Bag-1), Hsc70-Hsp90 organizing protein (Hop), and Hsc70-interacting protein (Hip) have been shown to interact with the CMA substrate–chaperone complex at the lysosomal membrane. Some of the cochaperones may not be directly involved in substrate targeting, but rather participate in the unfolding step, required just before the substrate can translocate across the lysosomal membrane [10].

Once at the lysosomal surface, the substrate–chaperone complex binds to the membrane and, after unfolding the substrate, is translocated into its lumen. This recognition at the lysosomal membrane is mediated by the lysosome-associated membrane protein type 2A (LAMP2A) that acts as the resident a CMA "receptor" [11]. LAMP2A is a single-span membrane protein with a very heavily glycosylated luminal region and a short (12–amino acid) C-terminus tail exposed on the surface of the lysosomes, where substrate proteins bind. LAMP2A is one of the three splice variants of the lamp2 gene, all of which contain identical luminal regions, but different transmembrane and cytosolic tails [12]. The mechanisms behind the translocation of substrate proteins across the lysosomal membrane are, as yet, poorly understood. Much evidence supports direct translocation across the lysosomal membrane, rather than engulfment by invaginations of the membrane. Indeed, invaginations have never been observed when this transport is reproduced in vitro. On the other hand conjugation or cross-linking of substrate proteins to bigger structures, such as gold particles, prevents their uptake. Morover, several studies mainly

carried out by Cuervo and colleagues demonstrated that substrate proteins need to be completely unfolded before reaching the lysosomal lumen [9, 11, 13, 14]. By analogy with other protein translocation systems, it has been speculated that the translocation through the lysosomal membrane might include a multispan membrane protein to create a discontinuity in the lysosomal membrane. However, to date, proteomic analysis of proteins associated with LAMP2A at the lysosomal membrane has not rendered such a partner. Nevertheless, evidences point to the existence of a unique mechanism for translocation of substrate proteins across the lysosomal membrane via CMA that requires multimerization of LAMP2A [15]. In fact, binding of substrate proteins to the cytosolic tail of monomeric forms of LAMP2A drives its multimerization to form a 700-kDa complex at the lysosomal membrane [15]. Moreover, the presence of a lysosome-specific form of Hsp90 on the luminal side of the lysosomal membrane is essential to preserve the stability of LAMP2A while it undergoes these conformational changes at the lysosomal membrane [15]. Also, it seems that the CMA translocation complex forms only transiently and that, once the substrate crosses the membrane, LAMP2A rapidly disassembles in a process mediated by the Hsc70 present on the cytosolic side of the lysosomal membrane [15]. Cytosolic and lysosomal chaperones only associate with lower-order complexes of substrate and LAMP2A, but are no longer present in the 700-kD complex required for translocation. The regulation of CMA through changes in lysosomal LAMP2A highlights the importance of lateral mobility within the membrane, which has been shown to be determined by its dynamic association with lysosomal lipid microdomains [16]. In this context, upon conditions of low CMA activity, part of LAMP2A is recruited into regions of defined lipid composition, whereas the number of LAMP2A molecules in these lipid microdomains is markedly reduced when CMA is activated. Accordingly, an increase in microdomain size, by augmenting lysosomal cholesterol results in reduced CMA, whereas cholesterol-extracting drugs increase membrane levels of LAMP2A, activating CMA [16]. In fact, the regulated degradation of LAMP2A described above occurs in these lipid microdomains, as luminal cathepsin A preferentially associates to the lysosomal membrane in these regions. By contrast, binding of substrates to LAMP2A and its assembly into and disassembly from the multimeric CMA translocation complex only pertains to LAMP2A molecules outside these microdomains [16]. Also, CMA activation includes not only the relocation of LAMP2A outside the lipid microdomains, as well as, a luminal pool of intact LAMP2A that can be retrieved to the lysosomal membrane upon CMA stimulation [17]. Intrinsic properties of LAMP2A are required to modulate its membrane dynamics. In addition to the GxxG motif required for multimerization [15], a proline residue that is present at the interface between its transmembrane and luminal regions is absolutely required for the mobilization of LAMP2A into the lipid microdomains [16]. Other components at the lysosomal membrane that modulate LAMP2A dynamics are the intermediate filament protein glial fibrillary acidic protein (GFAP) and elongation factor 1α (EF1α), a pair of interacting proteins that modify the stability of the multimeric LAMP2A complex and the association of LAMP2A with the lipid microdomains in a GTP-dependent

manner [18]. Concerning GFAP it was shown that a lysosome specific variant of the protein associates with LAMP2A multimers, enhancing the stability of the complex and counteracting the disassembly-promoting effect of Hsc70. Lysosomal GFAP partitions into two subpopulations; unphosphorylated GFAP that binds to multimers of LAMP2A and phosphorylated GFAP (GFAP-P), the latter of which is usually bound to the GTP-binding protein EF1α. Moreover, unphosphorylated GFAP has higher affinity for GFAP-P than for LAMP2A. However, the formation of GFAP–GFAP-P dimers is usually prevented by the presence of EF1α bound to GFAP-P. In the presence of GTP, EF1α is released from the lysosomal membrane allowing the dissociation of GFAP from the translocation complex and its binding to GFAP-P [18]. This dissociation favors the rapid disassembly of the LAMP2A multimeric complex and its active mobilization to lipid microdomains for degradation. Changes in the levels of GFAP–GFAP-P, EF1α present at the lysosomal membrane, as well as of intracellular GTP or intra-lysosomal Ca^{2+} (facilitating association of cathepsin A to lipid microdomains) can all contribute to modulation of CMA activity.

The final step in substrate translocation into the lysosome appears to involve a form of Hsc70 resident in the lysosomal lumen (lys-Hsc70) [19]. Only those lysosomes containing Hsc70 in their lumen are competent for uptake of CMA substrates. Interestingly, the percentage of Hsc70-containing lysosomes, which is no more than 40 % under resting conditions, escalates to 80 % in liver under conditions in which CMA is up-regulated, such as during prolonged starvation or mild oxidative stress [20, 21]. The mechanism by which lys-Hsc70 mediates substrate translocation remains unclear. This chaperone can act either actively, by facilitating substrate internalization in an energy-dependent manner, or passively, by binding the portion of substrate already translocated and preventing its retrograde movement to the cytoplasm. Also unknown is the pathway followed by lys-Hsc70 to reach the lysosomal lumen. It is possible that Hsc70 reaches the lysosome through fusion with late endocytic compartments, where Hsc70 has also been detected. Whether other luminal chaperones are required for substrate translocation is currently unknown.

Sequence analysis of the cytosolic proteome has revealed that about 30 % of cytosolic proteins might be putative substrates for CMA [8]. However, it is possible that this amount is an underestimation, because particular post-translational modifications, such as deamidation, phosphorylation, acetylation, etc., could provide the charge missing in a four–amino acid sequence [10]. This possibility of modulating chaperone recognition of the substrates by post-translational modifications adds an additional level of regulation to CMA. Another interesting fact is the existence of substrates that can be degraded either by the proteasome or by the lysosome, through CMA, suggesting that an interplay between UPS and CMA may exist. Indeed, some canonical proteasomal substrates were shown to have KFERQ motifs and, consequently, get degraded in the lysosome by CMA [4, 22–25]. However the mechanisms and signalling pathways that direct these substrates to either degradative pathway remain unclear.

CMA and Pathology

CMA's Role in Proteostasis

The accumulation of degradation-resistant proteins that become toxic has been consistently associated with various pathological conditions. Moreover, it is consensual that intracellular accumulation of aberrant proteins plays a major role in the aging process and constitutes a key feature shared by a large number of human age-related diseases. The most notorious among these are the neurodegenerative diseases where abnormal proteins accumulate in the form of inclusions in the affected neurons.

In this context CMA has been deemed as an efficient pathway for removing abnormal or otherwise damaged proteins. The first physiological stressor found to activate CMA was prolonged starvation, where the selective breakdown of superfluous proteins through CMA would contribute with the necessary amino acids required for protein synthesis, thus helping cells to cope with nutrient deprivation. Additionally, CMA is activated in circumstances associated with cytosolic protein damage, such as oxidative stress. In fact, exposure of cells to oxidative stress induces a rapid activation of CMA through transcriptional up-regulation of LAMP2A [20]. Moreover, oxidized proteins bearing the CMA-targeting motif are readily identified by Hsc70 and delivered to lysosomes for degradation. Interestingly, uptake of these oxidized proteins is faster than for their unmodified counterparts, suggesting that some oxidative induced unfolding of the substrates promotes its lysosomal targeting in the cytosol [20]. Consistently, experimental blockage of CMA has been shown to lead to the accumulation of oxidized cytosolic proteins in different cellular models [26], thus ascribing a role to CMA in the disposal of a particular subset of proteins that, after their oxidation, have CMA degradation favored over their removal through other pathways. In addition, the type and source of the oxidative stress may also determine the preference for one proteolytic system or another [27].

In the context of the ageing process, CMA-dependent degradation of oxidized proteins has been shown to decrease with age in most organs in rodents, as well as, in primary skin fibroblasts and peripheral leucocytes of healthy human subjects [28, 29], resulting in the accumulation of aggregation-prone oxidized proteins. This decrease was attributed to an age related LAMP2A reduction at the lysosomal membrane [30, 31]. Notably, restoration of normal levels of the CMA receptor in aged animals through genetic manipulation recuperates oxidized proteins levels of aging tissues to pre-ageing levels [32], suggesting a relevant role to CMA in the cellular defense against oxidative damage. These and other evidences strongly support the hypothesis that age is inversely correlated with CMA activity [28]. Indeed it has been suggested that this age-related decline in CMA activity contributes to the accumulation of abnormal and dysfunctional proteins and exacerbates disease progression of age-related pathologies. Logically, growing evidences also support CMA activation in response to oxidative stress in the central nervous system. For example, an increase in CMA markers has been observed in response to

6-hydroxydopamine-induced lesions in the nigrostriatal pathway [33]. Likewise, levels of LAMP2A have been shown to increase in response to seizures and status epilepticus, conditions associated with excessive production of reactive oxygen species [34].

CMA is also part of the cellular response to other stressors such as hypoxia, where CMA up-regulation enhances cell survival [35]. The fact that HIF-1α (hypoxia-inducible factor 1α) has recently been identified as a CMA substrate places this autophagic pathway at the centre of the regulation of the cellular response to hypoxia [22]. On the other hand, up-regulation of CMA components has been described in response to the activation of the pro-apoptotic programme in a common form of rod–cone dystrophy, indicating that CMA is part of the pro-survival response in retinal cells [36]. In fact, the response to stress in cone photoreceptors seems to rely, in a large part, on CMA activity, which explains the increased sensitivity of these cells to different stressors when CMA is compromised [37].

Crosstalk between CMA and Other Proteolytic Pathways

Another important aspect of CMA's role in proteostasis is the fact that its activity is regulated and tightly coordinated, not only with other forms of autophagy, but also with the Ubiquitin Proteasome System (UPS) [38, 39]. Crosstalk between CMA and macroautophagy is exemplified by the observed constitutive activation of CMA in cells deficient in macroautophagy [40]. Conversely, macroautophagy is highly induced in response to CMA blockage [26]. In the case of the CMA and macroautophagy interplay, though the roles of CMA and macroautophagy are considered similar but non-redundant, they can compensate for each other to sustain cell survival, though it may not be sufficient to allow efficient adaptation of cells to a certain stress.

Similarly, many cells respond to chemical blockage of the proteasome by upregulating CMA [22, 41]. On the other hand an impairment of CMA activity perturbs UPS functioning at least during the early stages of acute CMA blockage [42], most likely by affecting the turnover of specific proteasome subunits [43]. In agreement, reports show that CMA blockadge may alter proteasome assembly as exemplified by the fact that the maintenance of CMA efficiency allows for better preservation of UPS activity in old rodents [32]. Strinkingly, as seen in a mouse model of Huntington's disease (HD), such a constitutive upregulation of CMA compensates for the dual failure of macroautophagy [44, 45] and UPS [46]. This activation of CMA in HD is achieved through both enhanced transcription and increased stability of LAMP2A in the affected cells [47]. However, the ability of CMA to compensate for the severe proteolytic deficiency in HD cells is limited by the progressive functional decline in CMA with ageing. In the opposite direction crosstalk between macroautophagy and CMA is crucial in Parkinsons disease (PD) and in certain tauopathies, where the blockage of CMA is often compensated by activation of macroautophagy

[48–51]. In this case, macroautophagy activation is instrumental in promoting the removal of the toxic α-synuclein and tau oligomers. In any case, despite the significant advances in the last few years concerning the elucidation of the mechanisms that govern the crosstalk between CMA and other proteolitic pathways more studies are still needed to establish the molecular determinants that can switch a given substrate from one particular degradative pathway to the other. On this subject we have recently demonstrated that the ubiquitin ligase CHIP can reroute HIF-1A from the proteasome to CMA [22]. This finding opens the way for an involvement of ubiquitination and different ubiquitn chain topologies in diverting the substrates between CMA and other degradative pathways. Additionally, in the future, we may learn to upregulate these specific mechanisms to prevent overloading and subsequent blockage of other components of the proteostasis system.

Ageing and CMA

Reduced or otherwise altered CMA activity is not an exclusive of a pathological condition, but is also closely related with physiological ageing. An age-related reduction in the activity of CMA has been observed in many cell types and tissues [28, 30]. It is known that the transcription, synthesis and lysosomal targeting of the LAMP2A protein to the lysosome remains unchanged throughout lifespan. Nonotheless, the stability of LAMP2A at the lysosomal membrane is severely decreased with ageing [30]. This reduction is most probably linked with changes in the lipid constituents of the lysosomal membrane, which are important in maintaining the dynamics and stability of LAMP2A in the lysosomes. Molecularly, ageing brings changes into the lipid membrane composition of lysosomes, which in turn increases degradation of LAMP2A in the lysosomal lumen [16, 30, 31]. Consequently, the binding and translocation of substrates into the lysosomes by CMA is progressively unpaired during ageing [31]. Similar changes in the lipid composition of lysosomal membranes can be reproduced through diets with high lipidic content, thus underscoring the importance of the diet in the control of this autophagic pathway and the possible acceleration of its decline with age.

Up to this date, the main consequence of the age-related failure of CMA is proteostasis impairment, which induces a defective removal of oxidatively damaged proteins or the ability to respond to stressors [26]. Consequently, it is today largely accepted that age-dependent decline in CMA might constitute a contributing factor in the pathological changes in many age-related disorders. The most striking evidence in support of this theory is that genetic manipulation in old rodents, by expressing an exogenous copy of LAMP2A in mouse liver to preserve CMA function, has proven effective in improving the healthspan of aged animals [32]. Indeed, restored CMA functions in the LAMP2A transgenic animals results in improved cellular homeostasis, enhanced resistance to different stressors and preservation of organ function. Such pronounced beneficial effects in prolonging healthspan makes CMA a potential and promising anti-aging target mechanism.

CMA in Neurodegenerative Diseases

In neurodegeneration CMA is implicated both in the elimination of parts of the proteome damaged by stressors as well as in the selective turnover of substrates directly related with neurodegenerative diseases. Furthermore, CMA often becomes the target of the toxic effect of these aberrant substrates, thus creating a vicious cycle that propagates and aggravates the effect of ageing. The mulfactorial nature of the CMA role in neurodegenerative disorders makes the careful analyses of the evidences gathered thus far instrumental for the understanding of CMA in the context of these diseases.

Parkinson's Disease

Since LAMP2A suffers an age-related reduction at the lysosomal membrane it is conceivable that CMA can play a role in neurodegenerative disorders that have ageing as a risk factor. Up until now, the most compelling evidences on the role of CMA malfunction in neurodegenerative diseases have arisen from PD related research. Indeed, several studies have consistently suggested that the gradual decrease in LAMP2A levels with age constitutes a major contributor to PD progression in older patients [28, 52]. In agreement, very recent data showed that some of the microRNAs that are deregulated in PD brains may underlie the down-regulation of some CMA components in the affected neurons [53]. Furthermore, a sequence variation in the promoter region of LAMP2 has been recently identified in a PD patient [54], which might indicate that genetic induced variations in CMA components could be a causative factor for some forms of the disease. The association between PD and CMA is further suggested in studies in which both chemical [55] and genetic [56] upregulation of CMA were demonstrated to be sufficient to alleviate cellular toxicity associated with pathogenic forms of α-synuclein in a model of dopaminergic neuronal degeneration. Interestingly, CMA seems to be involved both in the familial and the sporadic forms of PD. In the familial form of PD [24, 57–59] sequence analysis reveals the presence of CMA-targeting motifs in the majority of PD-related proteins (i.e. α-synuclein, the ubiquitin C-terminal hydrolase L1 (UCH-L1) and the leucine-rich repeat kinase 2 (LRRK2)). Consistently, using a number of experimental approaches such as isolated lysosomes, primary mouse neuronal cultures, mouse models of PD, neuronal-differentiated induced pluripotent stem cells as well as brains from familial and sporadic PD patients, it was shown that the two most commonly mutated proteins in patients with familial PD, α-synuclein and LRRK2, are *bona fied* CMA substrates. Moreover, several pathogenic mutants of such proteins as α-synuclein (A30P and A53T) and of LRRK2 (G2019S and R1441C), have a direct toxic effect in CMA through aberrant interactions of these mutants with LAMP2A, such that these interactions induce CMA inhibition. In fact, these mutants bind to LAMP2A with an abnormally high affinity, which prevents their translocation across the lysosomal membrane [60], thus precluding access of other substrates to the lysosome to get degraded. Consequently, not only the mutant

and toxic forms of α-synuclein and LRRK2 will have decreased degradation but they will also impair other CMA substrates degradation [60]. In accordance, it was shown that accumulation of α-synuclein can be caused by an aberrant interaction of mutant LRRK2 with LAMP2A. In fact, LRRK2 mutants impedes LAMP2A organization into complexes and leaves increased levels of monomeric forms of LAMP2A at the lysosomal membrane, increasing lysosomal association with α-synuclein that cannot be degraded [57]. Even with wild-type α-synuclein, the presence of mutant LRRK2 is sufficient to promote toxic oligomerization of α-synuclein at the lysosomal membrane due to a compromise in lysosomal translocation. The presence of these oligomeric forms of α-synuclein stuck at the lysosomal membrane further impairs CMA activity and might even contribute to the seeding of protein aggregates characteristic of this disorder [57]. Interestingly, artificial mutations that prevent delivery of α-synuclein to the lysosomal membrane improve cell survival, partially because it no longer impedes CMA function [59]. These dual pathogenic effects of reduced elimination of the pathogenic protein and additional CMA blockage should contribute significantly to a more severe PD pathology in LRRK2-mediated PD cases.

In addition to LRRK2, also mutated forms of UCH-L1 have been associated with CMA impairment. Wild-type UCH-L1 binds to CMA-related chaperones and LAMP2A. But similarly to LRRK2, the levels of these interactions are abnormally increased by the PD-linked I93M mutation in UCH-L1, leading once again to blockage of α-synuclein degradation by CMA [61].

Besides the familiar form of the disease, there are also evidences linking CMA to sporadic PD [57, 60]. In general, an inhibition of α-synuclein degradation by post-translational modifications caused by stressors such as pesticides or oxidative stress, are instrumental in the development of sporadic PD and other synucleinopathies [62]. In this context, dopamine-modified α-synuclein shows reduced susceptibility to CMA degradation similarly to that of hereditary α-synuclein mutants [60], where the tight binding but inefficient translocation by CMA of dopamine-modified α-synuclein inhibits its degradation, alongside other CMA substrates. A perpetuation of this scenario will promote the formation of highly toxic α-synuclein oligomers or protofibrils at the lysosomal membrane [60]. Also, very recently, reports showed that even in the absence of post-translational modifications, increased levels of either α-synuclein [24] or LRRK2 [57], beyond a certain threshold, are sufficient for CMA inhibition in a manner where either one of these proteins can potentiate each other toxic effect on CMA [57].

Compelling evidences of the involvement of CMA in PD have also arisen from post-mortem brain samples from PD patients. The analysis of theses samples has made clear that in the early stages of PD, LAMP2A is increased [57]. Furthermore, studies in iPS (induced pluripotent stem) cells from a PD patient have also revealed that the initial up-regulation of CMA is followed by a marked decrease in CMA activity as the disease progresses [57]. Late down-regulation of this pathway is also supported by the reduced expression of LAMP2A and Hsc70 identified in peripheral leucocytes in idiopathic forms of PD [63] and in the substantia nigra pars compacta of PD brains [64].

The involvement of CMA in PD has also been associated with the myocyte enhancer factor 2D (MEF2D) since it is a substrate for CMA [65]. MEFs transcription

factors have been shown to play an important role in the survival of different types of neurons. In fact, a genetic polymorphism of the MEF2A gene has been linked to the risk of late onset of Alzheimer's disease (AD) [66–69] and in cellular models, inhibition of MEF2s contributes to neuronal death. In contrast, enhancing MEF2 activity protects neurons from death in vitro and in the substantia nigra pars compacta in a mouse model of PD [69]. Additionally neurotoxic insults cause MEF2 degradation in part by a caspase-dependent mechanism [70]. In this context, MEF2D was observed to be continuously shuttle to the cytoplasm, interact with the chaperone Hsc70, and undergo degradation. Interestingly, high levels of α-synuclein cause the disruption of this process, which leads to accumulation of MEF2D in the cytoplasm [65] and may underlie the pathogenic process in PD.

Alzheimer's Disease

As the list of newly found CMA substrates was growing, so was the list of proteins implicating CMA in neurodegeneratice diseases, such as PD. Soon enough tough, proteins involved in the pathogenesis of Alzheimer's Disease (AD) were included in this list. Reports showed that CMA blockage also played an important role in the levels of neurofibrillary tangles arising from the aggregation of mutant tau proteins associated with Alzheimer's disease and tauopathies [48]. In this case, normal tau protein has two KFERQ motifs on its C-terminus and one of them is recognized by hsc70 for degradation by CMA [48]. Interinstingly, the mutant tau variants of the protein have an aberrant CMA degradation mechanism where, once bound to LAMP2A, only a part of tau is internalized. The portion of the protein that gains entry into the lysosomal lumen is trimmed, resulting in the formation of smaller amyloidogenic tau fragments at the lysosomal membrane [48]. Futhermore, these fragments oligomerize directly at the surface of lysosomes, resulting in disruption of the lysosomal membrane integrity and blockage of normal CMA function. It seems conceivable that the release of these toxic mutant tau oligomers from the lysosomes, upon membrane rupture, might contribute even further in seeding tau aggregation in the cytosol and increase AD progression.

The involvement of CMA in AD may also occur via the regulator of calcineurin 1 (RCAN1), that has been established as a CMA substrate. RCAN1 is a protein whose high expression in AD brains has been linked to neuronal demise [23]. In this context it is likely that, as it happens with the interplay between α-synuclein and LRRK2 in PD, mutant tau in AD might contribute to the blockage of CMA and therefore increase RCAN1 levels in the affected neurons.

Huntington's Disease

Huntington's Disease (HD), is a neurodegenerative disease that is caused by an CAG expansion of the huntingtin (HTT) protein, that leads to the accumulation of an abnormally long version of HTT, that can be further cleaved into smaller fragments. Although full-length huntingtin is not a particular good substrate of CMA, cleavage

products of this protein are [71]. In this context, phosphorylation and acetylation of the N-terminal fragment encoded by exon 1 of huntingtin drives its degradation through CMA. In fact, genetic up-regulation of CMA in brain slices overexpressing this HTT fragment has an antitoxic effect [72]. The degradation of HTT fragments by CMA has arisen as one of the more compelling evidences of the protective role of this proteolytic pathway in neurodegenerative disorders. Thus far, HD is the only neurodegenerative disorder where stimulation of mutant HTT degradation by CMA has been demonstrated both in neuronal cultures and in mouse models in vivo [47]. In agreement, artificial enhancement of CMA targeting of mutant huntingtin with adaptor molecules efficiently induces its degradation and improves neuronal pathology in HD mouse models [73]. Strikingly, recent data obtained in an HD mouse model revealed that CMA is constitutively upregulated [47]. Moreover, it has been demonstrated that activation of CMA in these circumstances can compensate for the dual failure of macroautophagy and UPS [44, 45]. This example highlights the importance of the crosstalk between degradation pathways in development of HD.

In HD, activation of CMA can be achieved through both enhanced transcription and increased stability of LAMP2A in the affected cells [47]. Since CMA is constitutively up-regulated in HD-affected neurons, artificial enhancement of CMA targeting of mutant huntingtin might have some therapeutic potential. However the window of therapeutic opportunity could be narrow, since the ability of CMA to compensate for the severe proteolytic deficiency in HD cells is limited by the progressive functional decline in CMA with age [47].

Concluding Remarks

Understanding the role of CMA in neurodegeneration is a hot topic in the field right now. Although there is still an enormous amount of work to be done, it is incontrovertible that in a few years the scientific community has already found very important and compeling evidences on the role of CMA in the brain, that span way beyond the sole elimination of damage proteins. As we continue to explore both the molecular mechanisms and substrates of CMA and its implications in the brain we might just learn how to modulate these pathways and ameliorate neurodegenerative disorders.

References

1. Okiyoneda T, Barriere H, Bagdany M, Rabeh WM, Du K, Hohfeld J, et al. Peripheral protein quality control removes unfolded CFTR from the plasma membrane. Science. 2010;329(5993):805–10.
2. Bejarano E, Girao H, Yuste A, Patel B, Marques C, Spray DC, et al. Autophagy modulates dynamics of connexins at the plasma membrane in a ubiquitin-dependent manner. Mol Biol Cell. 2012 ;23(11):2156–69.

3. Pedrozo Z, Torrealba N, Fernandez C, Gatica D, Toro B, Quiroga C, et al. Cardiomyocyte ryanodine receptor degradation by chaperone-mediated autophagy. Cardiovasc Res. 2013;98(2):277–85.
4. Shen S, Zhang P, Lovchik MA, Li Y, Tang L, Chen Z, et al. Cyclodepsipeptide toxin promotes the degradation of Hsp90 client proteins through chaperone-mediated autophagy. J Cell Biol. 2009;185(4):629–39.
5. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004;6(4):463–77.
6. Chiang HL, Terlecky SR, Plant CP, Dice JF. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science. 1989;246(4928):382–5.
7. Massey AC, Zhang C, Cuervo AM. Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol. 2006;73:205–35.
8. Dice JF. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci. 1990;15(8):305–9.
9. Cuervo AM, Terlecky SR, Dice JF, Knecht E. Selective binding and uptake of ribonuclease A and glyceraldehyde-3-phosphate dehydrogenase by isolated rat liver lysosomes. J Biol Chem. 1994;269(42):26374–80.
10. Dice JF. Chaperone-mediated autophagy. Autophagy. 2007;3(4):295–9.
11. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. Science. 1996;273(5274):501–3.
12. Gough NR, Hatem CL, Fambrough DM. The family of LAMP-2 proteins arises by alternative splicing from a single gene: characterization of the avian LAMP-2 gene and identification of mammalian homologs of LAMP-2b and LAMP-2c. DNA Cell Biol. 1995;14(10):863–7.
13. Cuervo AM, Knecht E, Terlecky SR, Dice JF. Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. Am J Physiol. 1995;269(5 Pt 1):C1200–8.
14. Terlecky SR, Dice JF. Polypeptide import and degradation by isolated lysosomes. J Biol Chem. 1993;268(31):23490–5.
15. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. Mol Cell Biol. 2008;28(18):5747–63.
16. Kaushik S, Massey AC, Cuervo AM. Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy. EMBO J. 2006;25(17):3921–33.
17. Cuervo AM, Dice JF. Regulation of lamp2a levels in the lysosomal membrane. Traffic. 2000;1(7):570–83.
18. Bandyopadhyay U, Sridhar S, Kaushik S, Kiffin R, Cuervo AM. Identification of regulators of chaperone-mediated autophagy. Mol Cell. 2010;39(4):535–47.
19. Agarraberes FA, Terlecky SR, Dice JF. An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. J Cell Biol. 1997;137(4):825–34.
20. Kiffin R, Christian C, Knecht E, Cuervo AM. Activation of chaperone-mediated autophagy during oxidative stress. Mol Biol Cell. 2004;15(11):4829–40.
21. Cuervo AM, Dice JF, Knecht E. A population of rat liver lysosomes responsible for the selective uptake and degradation of cytosolic proteins. J Biol Chem. 1997;272(9):5606–15.
22. Ferreira JV, Fofo H, Bejarano E, Bento CF, Ramalho JS, Girao H, et al. STUB1/CHIP is required for HIF1A degradation by chaperone-mediated autophagy. Autophagy. 2013;9(9): 1349–66.
23. Liu H, Wang P, Song W, Sun X. Degradation of regulator of calcineurin 1 (RCAN1) is mediated by both chaperone-mediated autophagy and ubiquitin proteasome pathways. FASEB J. 2009;23(10):3383–92.
24. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science. 2004;305(5688):1292–5.
25. Cuervo AM, Hu W, Lim B, Dice JF. IkappaB is a substrate for a selective pathway of lysosomal proteolysis. Mol Biol Cell. 1998;9(8):1995–2010.
26. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. Consequences of the selective blockage of chaperone-mediated autophagy. Proc Natl Acad Sci U S A. 2006;103(15):5805–10.

27. Wang Y, Singh R, Xiang Y, Czaja MJ. Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress. Hepatology. 2010;52(1):266–77.
28. Cuervo AM, Dice JF. Age-related decline in chaperone-mediated autophagy. J Biol Chem. 2000;275(40):31505–13.
29. Huang J, Xu J, Pang S, Bai B, Yan B. Age-related decrease of the LAMP-2 gene expression in human leukocytes. Clin Biochem. 2012;45(15):1229–32.
30. Kiffin R, Kaushik S, Zeng M, Bandyopadhyay U, Zhang C, Massey AC, et al. Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age. J Cell Sci. 2007;120(Pt 5):782–91.
31. Rodriguez-Navarro JA, Kaushik S, Koga H, Dall'Armi C, Shui G, Wenk MR, et al. Inhibitory effect of dietary lipids on chaperone-mediated autophagy. Proc Natl Acad Sci U S A. 2012;109(12):E705–14.
32. Zhang C, Cuervo AM. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. Nat Med. 2008;14(9):959–65.
33. Marin C, Aguilar E. In vivo 6-OHDA-induced neurodegeneration and nigral autophagic markers expression. Neurochem Int. 2011;58(4):521–6.
34. Cao L, Chen R, Xu J, Lin Y, Wang R, Chi Z. Vitamin E inhibits activated chaperone-mediated autophagy in rats with status epilepticus. Neuroscience. 2009;161(1):73–7.
35. Dohi E, Tanaka S, Seki T, Miyagi T, Hide I, Takahashi T, et al. Hypoxic stress activates chaperone-mediated autophagy and modulates neuronal cell survival. Neurochem Int. 2012;60(4):431–42.
36. Metrailler S, Schorderet DF, Cottet S. Early apoptosis of rod photoreceptors in Rpe65(-/-) mice is associated with the upregulated expression of lysosomal-mediated autophagic genes. Exp Eye Res. 2012;96(1):70–81.
37. Rodriguez-Muela N, Koga H, Garcia-Ledo L, de la Villa P, de la Rosa EJ, Cuervo AM, et al. Balance between autophagic pathways preserves retinal homeostasis. Aging Cell. 2013;12(3):478–88.
38. Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harb Perspect Biol. 2010;2(12):a006734.
39. Park C, Cuervo AM. Selective autophagy: talking with the UPS. Cell Biochem Biophys. 2013;67(1):3–13.
40. Kaushik S, Massey AC, Mizushima N, Cuervo AM. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Mol Biol Cell. 2008;19(5):2179–92.
41. Koga H, Martinez-Vicente M, Macian F, Verkhusha VV, Cuervo AM. A photoconvertible fluorescent reporter to track chaperone-mediated autophagy. Nat Commun. 2011;2:386.
42. Massey AC, Follenzi A, Kiffin R, Zhang C, Cuervo AM. Early cellular changes after blockage of chaperone-mediated autophagy. Autophagy. 2008;4(4):442–56.
43. Cuervo AM, Palmer A, Rivett AJ, Knecht E. Degradation of proteasomes by lysosomes in rat liver. Eur J Biochem. 1995;227(3):792–800.
44. Ravikumar B, Duden R, Rubinsztein DC. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum Mol Genet. 2002;11(9):1107–17.
45. Martinez-Vicente M, Talloczy Z, Wong E, Tang G, Koga H, Kaushik S, et al. Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. Nat Neurosci. 2010;13(5):567–76.
46. Hipp MS, Patel CN, Bersuker K, Riley BE, Kaiser SE, Shaler TA, et al. Indirect inhibition of 26S proteasome activity in a cellular model of Huntington's disease. J Cell Biol. 2012;196(5):573–87.
47. Koga H, Martinez-Vicente M, Arias E, Kaushik S, Sulzer D, Cuervo AM. Constitutive upregulation of chaperone-mediated autophagy in Huntington's disease. J Neurosci. 2011;31(50):18492–505.
48. Wang Y, Martinez-Vicente M, Kruger U, Kaushik S, Wong E, Mandelkow EM, et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. Hum Mol Genet. 2009;18(21):4153–70.

49. Wong ES, Tan JM, Soong WE, Hussein K, Nukina N, Dawson VL, et al. Autophagy-mediated clearance of aggresomes is not a universal phenomenon. Hum Mol Genet. 2008;17(16):2570–82.
50. Wong E, Bejarano E, Rakshit M, Lee K, Hanson HH, Zaarur N, et al. Molecular determinants of selective clearance of protein inclusions by autophagy. Nat Commun. 2012;3:1240.
51. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem. 2003;278(27):25009–13.
52. Cuervo AM, Wong E. Chaperone-mediated autophagy: roles in disease and aging. Cell Res. 2014;24(1):92–104.
53. Alvarez-Erviti L, Seow Y, Schapira AH, Rodriguez-Oroz MC, Obeso JA, Cooper JM. Influence of microRNA deregulation on chaperone-mediated autophagy and alpha-synuclein pathology in Parkinson's disease. Cell Death Dis. 2013;4:e545.
54. Pang S, Chen D, Zhang A, Qin X, Yan B. Genetic analysis of the LAMP-2 gene promoter in patients with sporadic Parkinson's disease. Neurosci Lett. 2012;526(1):63–7.
55. Anguiano J, Garner TP, Mahalingam M, Das BC, Gavathiotis E, Cuervo AM. Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives. Nat Chem Biol. 2013;9(6):374–82.
56. Xilouri M, Brekk OR, Landeck N, Pitychoutis PM, Papasilekas T, Papadopoulou-Daifoti Z, et al. Boosting chaperone-mediated autophagy in vivo mitigates alpha-synuclein-induced neurodegeneration. Brain. 2013;136(Pt 7):2130–46.
57. Orenstein SJ, Kuo SH, Tasset I, Arias E, Koga H, Fernandez-Carasa I, et al. Interplay of LRRK2 with chaperone-mediated autophagy. Nat Neurosci. 2013;16(4):394–406.
58. Mak SK, McCormack AL, Manning-Bog AB, Cuervo AM, Di Monte DA. Lysosomal degradation of alpha-synuclein in vivo. J Biol Chem. 2010;285(18):13621–9.
59. Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. J Biol Chem. 2008;283(35):23542–56.
60. Martinez-Vicente M, Talloczy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, et al. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J Clin Invest. 2008;118(2):777–88.
61. Kabuta T, Furuta A, Aoki S, Furuta K, Wada K. Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. J Biol Chem. 2008;283(35):23731–8.
62. Oueslati A, Fournier M, Lashuel HA. Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. Prog Brain Res. 2010;183:115–45.
63. Wu G, Wang X, Feng X, Zhang A, Li J, Gu K, et al. Altered expression of autophagic genes in the peripheral leukocytes of patients with sporadic Parkinson's disease. Brain Res. 2011;1394:105–11.
64. Alvarez-Erviti L, Rodriguez-Oroz MC, Cooper JM, Caballero C, Ferrer I, Obeso JA, et al. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch Neurol. 2010;67(12):1464–72.
65. Yang Q, She H, Gearing M, Colla E, Lee M, Shacka JJ, et al. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. Science. 2009;323(5910):124–7.
66. Mao Z, Bonni A, Xia F, Nadal-Vicens M, Greenberg ME. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science. 1999;286(5440):785–90.
67. Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, et al. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron. 2003;38(1):33–46.
68. Gonzalez P, Alvarez V, Menendez M, Lahoz CH, Martinez C, Corao AI, et al. Myocyte enhancing factor-2A in Alzheimer's disease: genetic analysis and association with MEF2A-polymorphisms. Neurosci Lett. 2007;411(1):47–51.
69. Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, et al. Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci. 2006;26(2):440–7.

70. Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, et al. Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J Neurosci. 2005;25(19):4823–34.
71. Qi L, Zhang XD, Wu JC, Lin F, Wang J, DiFiglia M, et al. The role of chaperone-mediated autophagy in huntingtin degradation. PLoS ONE. 2012;7(10):e46834.
72. Thompson LM, Aiken CT, Kaltenbach LS, Agrawal N, Illes K, Khoshnan A, et al. IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. J Cell Biol. 2009;187(7):1083–99.
73. Bauer PO, Goswami A, Wong HK, Okuno M, Kurosawa M, Yamada M, et al. Harnessing chaperone-mediated autophagy for the selective degradation of mutant huntingtin protein. Nat Biotechnol. 2010;28(3):256–63.

Chapter 3
Interaction Between Mitochondria and Autophagy

Matthew E. Gegg

Abstract Mitochondria play a critical role in controlling both cellular metabolism and the initiation of apoptosis. Therefore for cell survival, the maintenance of a healthy pool of mitochondria is essential, particularly for cell types such as neurons that are very reliant on ATP generated by oxidative phosphorylation. Damaged/dysfunctional mitochondria are degraded by macroautophagy. This pathway has been termed mitophagy. There is increasing evidence that impaired mitophagy contributes to the mitochondrial dysfunction associated with several diseases, and in particular neurological disorders such as Parkinson's disease. Given the central involvement of mitochondria in cellular metabolism it is emerging that these organelles also appear to play a role in starvation-induced macroautophagy, ranging from providing membrane for synthesis of new phagophores to acting as hubs for autophagic proteins to initiate and control macroautophagy. The mechanisms by which damaged mitochondria are selectively removed by mitophagy and how impairment of this process may contribute to neurodegenerative disorders, in addition to the putative role of healthy mitochondria integrating cellular metabolism with autophagy will be described in this chapter.

Keywords Mitophagy · PINK1 · Parkin · Parkinson's disease · Neurodegeneration · Mitochondria · Autophagy

Abbreviations

AD	Alzheimer's disease
ALP	Autophagy lysosomal pathway
AMPK	Adenosine monophosphate activated kinase
AP	Autophagosome
CCCP	Carbonyl cyanide *m*:chlorophenyl hydrazine
DLB	Dementia with Lewy bodies

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ER	Endoplasmic reticulum
ETC	Electron transport chain
GCase	Glucocerebrosidase
GD	Gaucher disease
KD	Knock down
KO	Knock out
LIR	LC3 interacting domain
LSD	Lysosomal storage disorder;
Mfn	Mitofusin
mtDNA	Mitochondrial DNA
NPC1	Niemann pick type C1
OMM	Outer mitochondrial membrane
PD	Parkinson's disease
ROS	Reactive oxygen species
TOM	Translocase of the outer membrane
ψm	Mitochondrial membrane potential

Introduction

Healthy mitochondria are necessary for the correct function and survival of neurons. The central nervous system accounts for 20% of the body's total oxidative metabolism, with neurons in particular highly dependent on ATP generated via oxidative phosphorylation [1]. A by-product of oxidative phosphorylation is the generation of reactive oxygen species (ROS) which are highly damaging to DNA, proteins and lipids. These ROS not only damage mitochondria but also other structures and organelles in the cell [2]. Damage to mitochondria will exacerbate ROS generation producing an increasingly vicious cycle of damage and also promote the release of pro-apoptotic proteins such as cytochrome c. Therefore the maintenance of a healthy pool of mitochondria is vital for the survival of neurons. The post-mitotic nature of neurons and their unique morphology provide extra complexity when compared to other eukaryotic cells.

Damaged mitochondria can be selectively degraded by macroautophagy, also termed mitophagy [3–5]. The function of both mitochondria and the autophagy-lysosome pathway (ALP) deteriorates with age and both are implicated in aging and the pathogenesis of common age-related neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [2, 6]. It is becoming increasingly evident that functional mitochondria also play a key role in other forms of autophagy, and in particular starvation-induced macroautophagy.

In this chapter I will focus on how mitochondrial dynamics are required for mitophagy in neurons and starvation-induced macroautophagy. The mechanisms by which damaged mitochondria are selected for mitophagy will be described and how failure of various components of this pathway might contribute to the pathogenesis of neurodegenerative disorders such as PD, lysosomal storage disorders (LSDs) and dementia.

Mitochondrial Dynamics

Mitochondria are not static but dynamic organelles that can either fuse with each other to form a reticular network or undergo fission depending on the physiological context (reviewed by [1, 7]). Mitochondria undergo fission prior to degradation by mitophagy or the induction of apoptosis. Fission is also required for the movement of mitochondria around the cell. Neurons are both long and highly compartmentalized so mitochondria have to be dynamic to maintain cellular function. Mitochondria are predominantly made in the soma, whereas the need for mitochondria to synthesise ATP and buffer calcium is particularly important at the synaptic terminals [8, 9]. Neurons rely on the microtubule network to transport mitochondria from one compartment to the other. 'Plus' end microtubules direct traffic towards the terminals (anterograde), while 'minus' ends direct towards the soma (retrograde). Mitochondria in the soma undergo fission and are transported to the synaptic terminals via microtubules and the kinesin motor KIF5 (reviewed in [9]). Two other important proteins are required for the anterograde transport of mitochondria: milton and miro. Milton acts as an adaptor between KIF5 and miro which is associated with the outer mitochondrial membrane (OMM) of mitochondria. Miro has been proposed to stop mitochondrial movement when it binds calcium, and this is likely to be the process by which mitochondria are unloaded from the kinesin motors once they reach the synaptic terminal [9]. Mitochondria can move towards the soma along axons using predominantly dynein as a motor. Damaged mitochondria in the synapse undergo fission prior to their recruitment to autophagosomes (APs) and return to the soma for mitophagic degradation via microtubules and will be discussed further below [10–12].

Fusion of mitochondria is generally thought to improve the efficiency of oxidative phosphorylation and the maintenance of the mitochondrial DNA (mtDNA) pool [7, 13]. Thirteen subunits of the mitochondrial electron transport chain (ETC) are encoded for by mtDNA, and thus mutations in mtDNA can have a significant effect on oxidative phosphorylation. Mutations and deletions of mtDNA are most likely a combination of mitochondrial ROS damage and less sophisticated DNA repair mechanisms [2]. This is a particular problem for neurons as they are post-mitotic and therefore cannot dilute the amount of mitochondria with mutated DNA by cell division. Instead mtDNA mutations are thought to accumulate in neurons as they age [14, 15]. Neurons unable to fuse their mitochondria have aberrant mitochondrial distribution, ultrastructure, and oxidative phosphorylation [13]. The fusion of mitochondria has been proposed as a mechanism by which a heteroplasmic mix of wild-type and mutant mtDNA can be achieved in neurons, thus prolonging normal mitochondrial function [1, 7].

The fission and fusion of mitochondria in eukaryotes is regulated by several proteins (see Fig. 3.1). Fission is mediated via the dynamin related protein Drp1. Drp1 is primarily localized in the cytosol and is recruited to mitochondria for fission, where it forms oligomers that wrap around the mitochondria and constricts the outer and inner mitochondrial membranes [7, 16]. The fusion of the OMM is controlled by mitofusins 1 and 2 (Mfn1, Mfn 2), while Opa1 is responsible for the fusion of the inner mitochondrial membrane [7, 17].

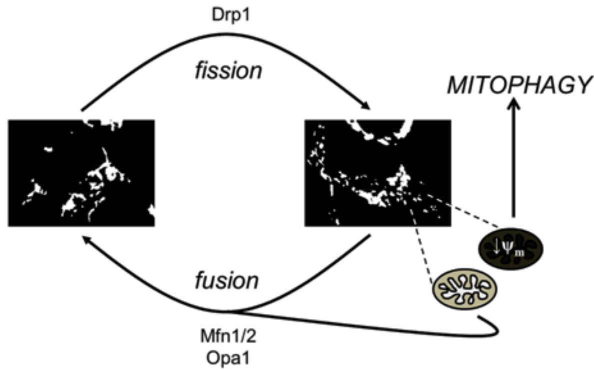

Fig. 3.1 Fission and fusion of mitochondria. Mitochondria are dynamic organelles that undergo both fusion and fission with each other. Under basal conditions cells tend to form a network of connected mitochondria (*left* picture; mitochondria (*white*) in SH-SY5Y cells detected by immunofluorescence with antibody against cytochrome *c* oxidase subunit 1). Fission of this network is mediated by Drp1 yielding smaller segregated mitochondria (*right picture*). If the mitochondrial membrane potential (Ψm) is maintained in these mitochondria they can undergo fusion back in to the mitochondrial network via Opa1 and Mfn1/2. If the mitochondria are damaged and have significantly reduced Ψm they are degraded via mitophagy

Mfn1 and Mfn2 are transmembrane proteins that form homotypic or heterotypic dimers between two opposing OMM, and mediates their fusion via the hydrolysis of GTP [17]. While Mfn1 and Mfn2 are ubiquitously expressed, Mfn2 expression is greater in high energy demanding tissue such as skeletal muscle, heart and brain [18]. Mfn1 or Mfn2 knockout (KO) mice are embryonic lethal. However, conditional Mfn1 KO mice are viable but conditional KO of Mfn2 affects movement and cerebellar development. Mutations in Mfn2 also cause the neurodegenerative disorder Charcot-Marie-Tooth 2A disease [7, 17, 18]. These observations suggest that Mfn2 is more essential in the brain.

Mitochondrial dynamics play a key role in mitophagy. The fission of damaged mitochondria from the network is likely the first step in the mitophagy process, while the Mfns are also regulated at a downstream stage and both will be described in the following section.

Mitophagy Mechanisms

Loss of Mitochondrial Membrane Potential

The mitochondrial membrane potential (Ψm) is generated by the passing of electrons from NADH and $FADH_2$ (generated by the tricarboxylic acid cycle, β-oxidation) along the ETC located on the inner mitochondrial membrane. The Ψm is then used by ATP synthase (complex V) to drive the phosphorylation of ADP to form ATP.

Damaged mitochondria often exhibit a reduced Ψm and are described as being depolarised.

The concept that depolarised mitochondria are susceptible to degradation by autophagy had been proposed for many years [3, 4, 19]. Then an elegant study using fluorescently labeled mitochondria showed that when mitochondria underwent fission in a Drp1-dependent fashion, unequal daughter mitochondria were quite often generated, with one having a high Ψm, while the other had a reduced Ψm (see Fig. 3.1). The mitochondria with decreased Ψm had a lower probability of fusing back with the mitochondrial network and were subject to elimination by macroautophagy [5]. This paper suggested that fission of mitochondria followed by selective fusion segregates dysfunctional mitochondria, which are then removed by mitophagy.

Mitochondria can be depolarised using proton ionophores such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). When Narendra et al. [20] used CCCP to depolarise mitochondria in Hela cells over expressing the E3 ubiquitin ligase parkin, they found that mitochondria rapidly fragmented and were almost entirely eliminated after 48 h. This removal of mitochondria was prevented in cells lacking the key autophagic protein ATG5, or when the autophagy inhibitors 3-methyladenine or bafilomycin A1 were used. Parkin is predominantly a cytosolic protein; however, following CCCP treatment for 1 h, parkin was clearly recruited to depolarised mitochondria. Use of dominant-negative Drp1K38A prevented mitochondrial fragmentation following CCCP treatment, yet still exhibited mitochondrial accumulation of parkin. Conversely inducing fragmentation of mitochondria without noticeably depolarising mitochondria (by expressing viral mitochondrial associated inhibitor of apoptosis), failed to recruit parkin [20]. These experiments indicated that the recruitment of parkin to mitochondria is dependent on loss of Ψm, rather than fragmentation.

Pink1/Parkin-Mediated Mitophagy

Mutations in the *parkin* gene are a cause of early onset familial forms of PD and loss of parkin function results in mitochondrial dysfunction [21, 22]. *Drosophila* models of parkin deficiency exhibit morphological and functional defects of mitochondria and are associated with degeneration of indirect flight muscle and dopaminergic neurons [23, 24]. It was observed that the phenotype of this fly model was very similar to that of flies that were *PINK1*-null. Mutations in human *PINK1* gene are also a cause of early onset familial PD and encodes a mitochondrial serine/threonine kinase [22, 25]. Genetic manipulation of *Drosophila* indicated that expression of human parkin could rescue the *PINK1-null* phenotype, but expression of human PINK1 could not rescue the *parkin* deficient flies, strongly suggesting that PINK1 was upstream of parkin in a common pathway [23, 24]. These observations lead to the logical hypothesis that PINK1 was responsible for the recruitment of parkin to depolarised mitochondria.

Full length (FL) PINK1 is a 581 amino acid protein that is predominantly localised to mitochondria [25, 26]. Under basal conditions, PINK1 is cleaved by mitochondrial proteases such as PARL at the mitochochondrial inner membrane and is then rapidly degraded by the proteasome [26–28]. Depolarisation of mitochondria by CCCP results in the rapid accumulation of FL-PINK1 (within 30 min) on the OMM in Hela cells and the human neuroblastoma SH-SY5Y cell line [29–31]. The early accumulation of FL-PINK1 is because the loss of Ψm prevents FL-PINK1 from being imported to the inner mitochondrial membrane and being cleaved. However, prolonged depolarisation of mitochondria also leads to increased transcription of FL-PINK1 [32].

Knock down of PINK1 by siRNA in Hela and neuroblastoma cells, or studies in cortical neurons and mouse embryonic fibroblasts (MEFS) derived from PINK1 KO mice, were the first to indicate that PINK1 was required for the recruitment of parkin to depolarised mitochondria [29–31]. *In vitro* and cell assays then convincingly showed that FL-PINK1 can directly phosphorylate parkin following depolarization of mitochondria [33, 34]. It is becoming evident that PINK1 not only needs to phosphorylate parkin at serine 65 in the ubiquitin-like domain to fully activate the E3 ubiquitin ligase enzyme activity, but also requires PINK1 to phosphorylate ubiquitin at serine 65 as well [35–37]. While it is clear that PINK1 phosphorylates and activates parkin, the precise details of how parkin is recruited to the mitochondria remains unclear. The translocase of the outer membrane (TOM), which is involved in the import of proteins in to mitochondria, has been postulated to stabilise FL-PINK1 on the OMM of depolarised mitochondria [38, 39].

Several proteins have been reported to facilitate the recruitment of parkin to the mitochondria. Fbx07 is a substrate-recruiting subunit of Skp1-cullin1-FBP-type E3 ubiquitin ligases and is normally located in the cytosol. Upon depolarisation of mitochondria with CCCP, Fbx07 was found to relocate to mitochondria [40]. Furthermore, it was found that Fbx07 can directly bind to parkin and facilitates the recruitment of parkin to depolarised mitochondria and the ubiquitination of Mfn1 (see below) in SH-SY5Y cells and human fibroblasts. Over expression of Fbx07 also rescued the phenotype of mutant *parkin* flies. However, Fbx07 could not rescue the phenotype in PINK1-deficient flies or mammalian cells indicating that PINK1 is upstream of Fbx07 [40]. The mechanism by which Fbx07 is recruited to the mitochondria remains unresolved. Co-immunoprecipitation studies showed that PINK1 can bind Fbx07 suggesting that PINK1 phosphorylation of Fbx07 might be a possibility, but has yet to be directly shown.

A genome-wide RNAi screen in Hela cells did not identify Fbx07 as one of the top hits required for parkin translocation to depolarised mitochondria. A candidate identified in this study to promote parkin translocation was the heat shock protein 70 family member HSPA1, while BAG4, which can inhibit chaperone activity, was a negative regulator of parkin accumulation on depolarised mitochondria [39].

The accumulation and activation of parkin on depolarised mitochondria results in the ubiquitination of a large number of mitochondrial proteins in Hela and SH-SY5Y cells (see Fig. 3.2) [29, 41–45]. The majority of these proteins are resident in the OMM [44, 45]. In Hela cells K48-linked ubiquitin chains increased by 9-fold,

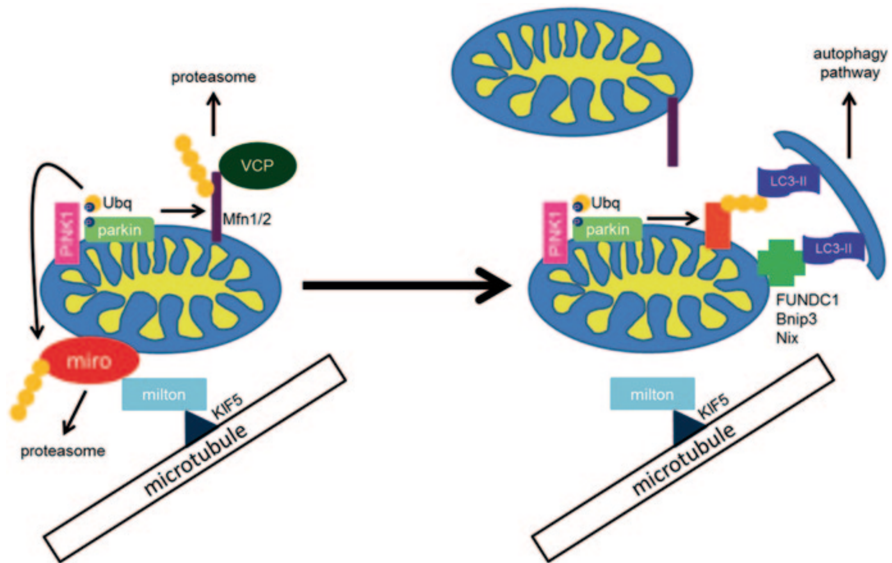

Fig. 3.2 Mitophagy mechanisms. FL-PINK1 accumulates on the OMM of depolarised mitochondria. Parkin is recruited to depolarised mitochondria in a PINK1-dependent manner. PINK1 then phosphorylates (*P*) both ubiquitin (*Ubq*) and parkin to fully activate the E3 ubiquitin ligase activity of parkin. Parkin rapidly ubiquitinates Mfn1/2, which are then degraded by the proteasome with the aid of VCP. It has been proposed that degradation of the Mfns prevents segregated mitochondria destined for mitophagy from undergoing fusion with the healthy mitochondrial network. Miro is also ubiquitinated by parkin and degraded by the proteasome. This is thought to disconnect mitochondria from the adaptor milton and the kinesin motor KIF5, which transport mitochondria along microtubules. Depolarised mitochondria can then be sequestered in to APs for mitophagic degradation. The parkin-dependent ubiquitination of other OMM proteins (*dark orange rectangle*) allows the binding of autophagic proteins such as LC3 and p62/SQSTM1, which enable the sequestration of cargo to the isolation membrane, which then matures to an AP and then fuses with lysosomes for degradation. Other mitophagy receptors (*green cross*) located on the OMM such as FUNDC1, BNIP3, Nix and cardiolipin have also been shown to bind LC3

while K63-linked ubiquitin chains were increased by 20-fold following CCCP treatment [45]. Traditionally K48-linked ubiquitination is associated with degradation of proteins by the proteasome, while K63-linked chains are degraded via autophagy. Therefore, some of these OMM ubiquitinated proteins are most likely bound by the ubiquitin-binding autophagic proteins LC3-II and p62/SQSMT1[31, 41, 43, 45, 46], thus helping the recruitment of the damaged mitochondria to isolation membranes, which then mature in to APs and then fuse with lysosomes for degradation (See Fig. 3.2). In cancer cell lines such as Hela and SH-SY5Y cells, mitochondria destined for degradation by mitophagy form perinuclear clusters, and p62/SQSMT1 and HDAC6 are required for transport to this location [30, 41]. In neurons, depolarised or damaged mitochondria also co-localise with LC3 [11, 47]. The majority of lysosomes in neurons are resident in the soma, and therefore mitochondria have to be transported back to the soma in APs for degradation [6, 11]. The movement

of mitochondria in neurons with respect to mitophagy and the role of additional mitophagy receptors will be discussed below.

The first OMM substrate of parkin was shown to be VDAC [43], and was then rapidly followed by the Mfns, originally in *Drosophila* models [48, 49], and then in SH-SY5Y and Hela cells [42, 45, 50]. The ubiquitination of Mfn1 and Mfn2 in mammalian cells was found to occur within 1 h of mitochondrial depolarization and then rapidly degraded. The kinetics of Mfn degradation was greater than mitochondrial proteins resident in the inner mitochondrial membrane or matrix suggesting that these proteins were not being degraded via autophagy [42, 45, 50]. The degradation of the Mfns and VDAC was prevented in these cells in the presence of inhibitors of the proteasome (see Fig. 3.2) [45, 50]. Furthermore it was found that the extraction of these proteins from the OMM and proteasome-dependent degradation required valosin-containing protein (VCP), also known as p97, an AAA+ATPase [50, 51]. Although it is widely accepted that the ubiquitination of Mfns results in proteasomal degradation, the exact nature of this ubiquitination is far from clear. In mammalian cells Mfn1 and Mfn2 were ubiquitinated at 8 and 15 sites, respectively [44]. In vitro ubiquitin ligase assays suggested that Mfn1 showed both K48 and K63-linkage [52]. Conversely, Mfn1 was reported to show atypical lysine linkage independent of K48 or K63 in SH-SY5Y [53]. It should be noted that there are a variety of E3 ubiquitin ligases associated with mitochondria such as MARCH5 and MULAN [40, 54], so it is likely that some of the ubiquitination of OMM proteins following depolarisation is downstream or independent of parkin. In parkin deficient flies, Mfn protein levels are increased under basal conditions [48, 49]. Expression of human Fbx07 in these flies decreased Mfn protein levels back to steady-state, suggesting that Skp1-cullin1-FBP-type E3 ubiquitin ligases can also ubiquitinate Mfns during mitophagy [40].

Given the role the Mfns play in the fusion of mitochondria, it has been proposed that the rapid degradation of the Mfns on depolarised/damaged mitochondria is a mechanism by which the cells ensure that the mitochondria identified for mitophagy remain segregated and are unable to undergo fusion back in to the mitochondrial network containing healthy mitochondria [50]. Mitochondria also form many connections with the endoplasmic reticulum (ER) throughout the cell, and one of the proteins that is involved at these contact sites is Mfn2 [55, 56]. Therefore the rapid degradation of Mfn2 may also be required for disconnecting damaged mitochondria from the ER. Indeed in Hela cells where mitochondrial damage is induced by mitochondria generated ROS, parkin-positive mitochondrial tubules became devoid of Mfn2 before acquiring LC3, with ubiquitination foci being notable where ER and impaired mitochondria overlapped [57]. It should be noted that PINK1 has also been reported to directly phosphorylate Mfn2, and that this is required for the recruitment of parkin to depolarised mitochondria [58]. If Mfn2 is acting as a mitochondrial receptor for parkin, degradation of Mfn2 may also act as an off-switch to prevent further recruitment of parkin to damaged mitochondria. It should be noted that while *Drosophila* lacking Mfn partially impaired parkin translocation [48], MEFS lacking both Mfn1 and Mfn2 could still recruit parkin to mitochondria and have a similar rate of mitophagy as wild-type MEFs [20, 45].

Proteomic analysis of CCCP-treated mitochondria also identified miro to be ubiquitinated in a parkin-dependent manner and then degraded by the proteasome [44, 45]. Miro is a Rho-like GTPase that is located at the OMM and is required for the association of mitochondria with the adaptor protein milton and the kinesin motor KIF5 [9, 12]. Kinesin motors mediate anterograde transport (soma to synapse transport), while dynein motors drive retrograde mitochondria movement from synapses back to the soma. Over expression of PINK1 or parkin in either Drosophila or rat neurons decreased the mitochondrial movement in axons [12, 59]. Upon depolarisation of mitochondria, PINK1 was found to phosphorylate miro, and is then bound by parkin. Miro was then ubiquitinated and degraded via the proteasome [12, 59], leading to the release of milton and KIF5 from mitochondria (see Fig. 3.2). It has been proposed that the prevention of mitochondrial movement along microtubules is another mechanism by which cells can quarantine damaged mitochondria, with immobilisation allowing damaged mitochondria to be recruited to APs, which then acidify as they are transported back to the soma for degradation [10, 60]. Depolarised mitochondria which have recruited parkin exhibit reduced anterograde and increased retrograde transport in neurons, and accumulate in the soma [11]. Down regulation of kinesin motors on early APs may account for the retrograde movement of APs back towards the soma [60]. Furthermore, inhibition of lysosomal proteases with pepstatin A and E64D increased the co-localisation of mitochondria and LAMP1-positive lysosomes in the soma of CCCP-treated neurons [11].

The vast majority of work to dissect PINK1/parkin-mediated mitophagy has been performed in proliferating mammalian cells and translation of these findings in to neurons has been less clear. The neuronal study described above investigating mitochondrial movement [11] noted that CCCP-induced parkin translocation to mitochondria was detected 12–18 h after treatment, compared to less than 1 h in transformed cell lines such as Hela and SH-SY5Y. Robust co-localisation of VCP with mitochondria was observed in primary neurons treated with CCCP for 48 h, and was parkin dependent [51]. PINK1/parkin-dependent Mfn1 ubiquitination has also been detected in another primary culture model, but was quite weak, and the authors noted that very particular culture conditions had to be employed [61]. Some neuronal models have not detected any involvement of parkin in the clearance of damaged mitochondria [62, 63]. The different methods to damage mitochondria and model systems employed may well account for the differing contribution of PINK1/parkin-mediated mitophagy. However there is growing evidence that alternative mitophagy mechanisms could also be employed by neurons and are described next.

The Role of FUNDC1, Bnip3, Nix and Cardiolipin in Mitophagy

FUNDC1 is an integral OMM and has recently been implicated in both CCCP and hypoxia induced mitophagy [64–66]. Similar to CCCP, hypoxia induces fragmentation of the mitochondrial network [65, 66]. FUNDC1 contains the classical LC3-interacting domain (LIR) similar to that found in other LC3-binding proteins such

as p62/SQSMT1 [64]. Notably over expression of FUNDC1 co-localised LC3 to mitochondria and led to the sequestration of mitochondria in to characteristic double membrane APs and the eventual decrease in cellular mitochondrial content. Inhibition of autophagy with bafilomycin A1 prevented the decrease in mitochondrial content [64]. Two alternative mechanisms by which FUNDC1 activates mitophagy have been identified. The first is that ULK1, a serine/threonine kinase involved in early AP formation, is recruited to fragmented mitochondria and phosphorylates FUNDC1 at Ser17, and thus increasing LC3 binding to FUNDC1 [65]. Alternatively, the serine/threonine phosphatase PGAM5, also an OMM resident, dephosphorylates FUNDC1 at Ser13, thus increasing the interaction between LC3 and FUNDC1 [66]. FUNDC1 has also been reported to be ubiquitinated in SH-SY5Y cells following CCCP treatment, and this post-translational modification might also contribute to the regulation of mitophagy [44].

Two other OMM resident proteins thought to act as mitophagy receptors are Bnip3 and its homolog Nix, also known as Bnip3L [67]. Nix is required for the elimination of mitochondria during erythrocyte maturation, and is also expressed with Bnip3 in high energy requiring tissues such as muscle, heart, liver and brain. It is highly induced following hypoxia [67]. Over expression of Nix or Bnip3 activates autophagy and both contain LIR domains enabling them to bind LC3 [68, 69]. Phosphorylation of serine residues flanking the Bnip3 LIR increased LC3 binding, while phosphorylation-mimicking LIR mutations increased mitochondrial sequestration in to APs, and delivery to lysosomes for degradation [68]. Parkin recruitment to depolarised mitochondria and the number of LC3 puncta are both significantly decreased in Nix deficient MEFS treated with CCCP [46]. Nix and Bnip are also capable of depolarising mitochondria by associating with the pro-apoptotic proteins Bax and Bak. Therefore it is possible that Nix and Bnip3 are intricately involved in deciding cellular fate during stress: either to remove damaged mitochondria via mitophagy or allow depolarised mitochondria to initiate an apoptotic cascade. Increased binding of Bnip3 to LC3 has been shown to concurrently increase mitophagy and reduce the amount of cytochrome c released by mitochondria [68].

Another mitochondrial component that has been found to directly bind LC3 is cardiolipin. Cardiolipin is a phospholipid normally found in the inner mitochondrial membrane. Treatment with the mitochondrial toxin rotenone, and to a lesser extent CCCP, caused cardiolipin to translocate to the OMM [47]. In vitro studies suggested that N-terminal of LC3 could directly bind cardiolipin and that the externalization of cardiolpin correlated with increased colocalisation of mitochondria and LC3.

Mitochondria as Autophagy Hubs

In the previous section the mechanisms by which damaged mitochondria might be segregated and identified for recruitment to isolation membranes/APs and then degraded by mitophagy were highlighted. However, mitochondria appear to play key roles in other aspects of the autophagy, that are not only restricted to mitophagy but also starvation-induced macroautophagy.

Macroautophagy is initiated by the formation of isolation membrane, which then expands to engulf macromolecules and organelles destined for degradation by lysosomes (reviewed in [70]). This biogenesis of APs is mediated by a succession of autophagic proteins. Autophagy is initiated by ULK1/2 and ULK1 has been reported to be recruited to mitochondria following CCCP and hypoxia induced mitochondrial damage [65, 71]. The autophagy specific phosphatidyl inositol-3-OH kinase (PI(3)K) complex is equally important for AP biogenesis. This complex contains Beclin1, VPS34, p150 and ATG14. ATG14 has been shown to be recruited to depolarised mitochondria [71], while FL-PINK1, which rapidly accumulates on mitochondrial following CCCP treatment, has been shown to interact with Beclin1 and promote autophagy [72]. Cleaved isoforms of PINK1 did not interact with Beclin1. These data imply that the initiation and expansion of the isolation membrane can be directed from mitochondria. This is reinforced by the observation that the source of the isolation membrane and the site of its expansion might be at ER-mitochondria contact sites [55, 56].

The location of AP biogenesis is controversial with the ER, Golgi, endosomes, plasma membrane, and mitochondria all proposed to provide material for the isolation membrane [56, 70]. The source of the membrane might be dependent on the type of autophagy stimuli and cell type. The transfer of mitochondrial lipids to newly forming APs has been observed in rat kidney cells following starvation-induced autophagy [56]. This was coincident with the co-localisation of mitochondria and the AP-formation marker ATG5. Notably, this AP formation was impeded in a cell line lacking Mfn2, which is localised at ER-mitochondria contact sites [56]. Starvation of monkey kidney COS7 cells was then shown to result in the localisation of the early-AP biogenesis marker ATG14 at ER-mitochondria contact sites [55]. ATG5 also localised to this site until AP formation was complete. While the source of the isolation membrane for mitophagy is yet to be determined, the co-localisation of ULK and ATG14 to depolarised mitochondria suggests that ER-mitochondria contact sites could be involved [71].

Mitochondria, Metabolism and Autophagy

To achieve homeostasis and balance tissue degradation by autophagy with synthesis of new macromolecules requires significant energy expenditure. It has been estimated that for a human to rebuild 10% of the body's protein content requires 20% of the daily dietary calorie intake [73]. Therefore the supply of ATP by mitochondria is crucial to maintain autophagy. Mitochondria can also utilise the metabolites generate by autophagy to generate ATP. A key activator of autophagy is the enzyme adenosine monophosphate-activated kinase (AMPK). As the name suggests, the enzyme is activated when the AMP:ATP ratio is increased, a signal that the cell is becoming energy depleted. AMPK inhibits mTORC1, thus activating autophagy [73]. The glucose generated by autophagy can be utilized by glycolysis to produce pyruvate, which can then enter the tricarboxylic acid cycle to generate NADH and

$FADH_2$ for mitochondrial ATP production. Amino acids and fatty acids generated by the turnover of proteins and lipids that are not used to synthesise new macromolecules can also enter the tricarboxylic acid cycle.

Given the crucial nature of mitochondria producing energy from the metabolites of macroautophagy, it is not surprising that mitochondria undergo morphological changes during starvation-induced macroautophagy that allow them to not only maintain cellular ATP levels under stress conditions but also prevents themselves from being degraded by macroautophagy. Studies in MEFs have shown that during starvation, mitochondria elongate due to unopposed fusion of mitochondria [74, 75]. Starvation results in a decrease in the mitochondrial localisation of the pro-fission protein Drp1. This is coupled with a change in the phosphorylation status of the Drp1 [74, 75]. As discussed above, formation of mitochondria in to tubular networks ensures that the mitochondria cannot be recruited in to APs and degraded by macroautophagy. Furthermore, wild-type MEFs that had elongated mitochondria were able to maintain mitochondrial Ψm for at least 5 h of starvation, which meant that total cellular ATP levels were constant during this time [75]. MEFs lacking the pro-fusion proteins Opa1 or both Mfn1 and Mfn2 could not elongate during starvation and were unable to maintain Ψm. This resulted in decreased cellular ATP levels and a much greater loss of cell viability [75].

Neurodegeneration and Impaired Mitophagy

Parkinson's Disease (PD)

The identification of PINK1, parkin and Fbx07 involvement in the clearance of depolarised/damaged mitochondria means that autosomal recessive early-onset familial PD has the strongest association with impaired mitophagy of all the neurodegenerative disorders.

Mutations in the *parkin* (*PARK2*) and *PINK1* (*PARK6*) genes were identified in 1998 and 2004, respectively [21, 25]. Since then mitochondrial dysfunction such as decreased oxidative phosphorylation and perturbed mitochondrial morphology has consistently been shown to occur in both cellular and animal models of PINK1 and parkin deficiency (reviewed in [22]). In cellular and animal models of PINK1 deficiency both oxidative stress and impairment of oxidative phosphorylation progressively worsened with time [76, 77]. These observations fit well with the idea that the loss of PINK1 activity results in inhibition of mitophagy and thus the accumulation of damaged mitochondria. Furthermore, the inhibition of oxidative phosphorylation in SH-SY5Y cells following PINK1 knockdown (KD) can be reversed by over expression of parkin [42]. This restoration of mitochondrial function by exogenous parkin was coincident with an increase in autophagic flux. Mutations in the *Fbx07* (*PARK15*) are a cause of very rare early onset PD. The observation that Fbx07 participates in the recruitment of parkin to depolarised mitochondria, and subsequent

parkin-dependent ubiquitination of Mfn1 in both mammalian cells and *Drosophila* is a further link between mitophagy and PD, especially as PD-associated Fbx07 mutations interfered with this process [40].

Other forms of familial PD are likely to contribute to impairment of mitophagy in a less direct manner. Mutations in the *ATP13A2* (*PARK9*) gene cause Kufor-Rakeb syndrome, an autosomal recessive form of parkinsonism with dementia and a juvenile onset. ATP13A2 is a lysosomal P-type ATPase whose function is still unknown. Given the lysosomal location of ATP13A2 it was not surprising that KD of the protein in primary mouse cortical neurons and SH-SY5Y cells resulted in an inhibition of macroautophagy flux [78]. Lysosomal degradation of long-lived proteins was also inhibited in fibroblasts with *ATP13A2* mutations [79]. In KD neurons α-synuclein protein levels were increased. This protein is normally degraded by autophagy and aberrant protein quality control of this protein is a hallmark of PD. Increased mitochondrial fragmentation, cellular mitochondrial content and mitochondrial ROS production, were observed in SH-SY5Y cells with ATP13A2 KD, suggesting that damaged mitochondria were accumulating, presumably due to inhibition of mitophagy [78]. Fragmented mitochondria and abnormal oxidative phosphorylation has also been observed in *ATP13A2* mutant fibroblasts [80].

Another lysosomal enzyme associated with PD is glucocerebrosidase (GCase), an enzyme involved in sphingolipid metabolism. Mutations in the *GBA1* gene, which encodes GCase, are the greatest known genetic risk factor for developing PD [81]. Lewy body pathology and the pattern of neuronal loss is similar to sporadic PD, but the age of onset is approximately 5 years earlier than sporadic PD [81, 82]. Homozygous mutations in *GBA1* cause the lysosomal storage disorder Gaucher disease. The impairment of macroautophagy and mitochondrial function will be discussed in the lysosomal storage disease section below. Post-mortem analysis of PD brains with heterozygote *GBA1* mutations showed a 60 % decrease of GCase activity in the substantia nigra [83], the area where most dopaminergic neuronal loss occurs in PD. Notably GCase activity and protein expression was decreased by approximately 40 % in the substantia nigra of sporadic PD brains [83]. This lysosomal deficiency is likely to contribute to the impairment of the protein and organelle quality control mechanisms linked with PD pathogenesis.

The majority of PD cases (~ 90 %) have no apparent genetic cause and are termed sporadic or idiopathic [84]. Impairment of the ALP and mitochondrial dysfunction are both heavily implicated in the pathogenesis of sporadic PD, and is supported by the observation that many of the genes causing familial PD are associated with these two processes (reviewed in [6, 22]). However, the lack of animal models that replicate the disease in humans and robust mitophagy assays that can be used in post-mortem tissue means that at the time of writing, the link between mitophagy and sporadic PD remains circumstantial.

The greatest risk factor for developing PD is aging. The function of mitochondria and the autophagy-lysosome system declines with age [2, 6] and therefore it is logical to envisage that the development of mitochondrial dysfunction is due, at least in part, to the accumulation of damaged mitochondria that are unable to be degraded by mitophagy.

Analysis of mtDNA deletions in the neurons of aged human substantia nigra neurons indicated that a high proportion of neurons contained significant levels of deletions [14, 15]. These deletions were different among neurons and were largely clonal (e.g. a single mtDNA deletion was clonally expanded within each neuron). Furthermore, the level of mtDNA mutations was greater in the substantia nigra of PD brains, compared to age-matched controls [14]. Oxidative phosphorylation was no longer able to be maintained when the amount of mtDNA mutations reached a particular threshold [15]. Therefore, to maintain mitochondrial function it is likely that neurons need to eliminate mitochondria with mtDNA mutations by mitophagy.

This is supported by cellular models of mtDNA mutations that cause human disease. The first was a study in which long term expression of parkin rescued mitochondrial function in a cell line with 75% mutant mtDNA (G>A m.6930 in cytochrome *c* oxidase subunit I gene). Expression of parkin for 60 days in this cell line resulted in the vast majority of mitochondria containing wild-type mtDNA and restoration of cytochrome c oxidase activity to normal levels [85]. The mtDNA mutation m.3243A>G causes mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome and predominantly results in a defect of complex I of the ETC. Inducible pluripotent stem cells were derived from fibroblasts containing this mtDNA mutation and then differentiated in to neurons [86]. Neurons with high mutant mtDNA load exhibited co-localisation of complex I with LC3, parkin and PINK1 at a perinuclear location. Intriguingly, the authors stated that while other complexes of the ETC were also co-localised to APs, this occurred after depletion of complex I, and suggested that this might be an explanation why complex I defects are commonly associated with mitochondria-associated neurodegeneration [86]. Complex I activity is specifically decreased by 40% in the substantia nigra of sporadic PD brains [87].

A *Drosophila* study has also suggested that increasing parkin expression during aging improves mitochondria function. Mfn protein expression was found to increase with age in flies, and this could be reversed by over expression of parkin [88], consistent with the idea that mitophagy is being increased. Elevation of parkin levels in *Drosophila* also increased lifespan and markers of mitochondrial function [88]. However, it should be noted that some of the increase in mitochondrial activity may have been a result of increased mitochondrial synthesis, as mRNA levels of the master regulator of mitochondrial biogenesis, PGC1α, was also increased by induction of parkin expression.

Lysosomal Storage Disorders (LSDs)

LSDs are a group of about 50 genetic metabolic disorders that are characterised by lysosomal dysfunction. Consequently, these disorders are increasingly associated with impaired macroautophagy and mitochondrial dysfunction [89–91]. The most common LSD is Gaucher disease (GD), which is caused by autosomal recessive mutations in the *GBA1* gene. Type II and Type III GD are neuronopathic [82, 92].

GBA encodes for the lysosomal enzyme GCase, and is involved in sphingolipid metabolism. Loss of GCase activity results in the accumulation of glucosylceramide/glucosylsphingosine and impairment of the ALP, resulting in the accumulation of α-synuclein in neuronal models and the mouse brains (reviewed in [82]). Inhibition of GCase with the toxin conduritol B-expoxide in SH-SY5Y cells resulted in decreased mitochondrial Ψm, impaired ETC and increased oxidative stress [93]. Furthermore, neuronopathic mouse models of GD, where macroautophagy was impaired, also exhibited loss of mitochondrial Ψm, decreased oxidative phosphorylation and changes in mitochondrial morphology [90, 94]. Experiments suggested that the increase in mitochondrial dysfunction was because damaged mitochondria were not being degraded by mitophagy and were accumulating in GCase deficient neurons [90].

Niemann Pick type C1 (NPC1) is a progressive neurodegenerative disorder where cholesterol and other lipids accumulate in lysosomes. Mitochondrial fragmentation was increased in NPC1 patient fibroblasts and human neurons with KD of NPC1 [91]. Macroautophagy flux was decreased, while mitochondrial content was increased, in both models, suggesting mitophagy was impaired [91]. Depletion of cholesterol in these cells rescued autophagic flux and mitochondrial length.

The neuronal ceroid lipofuscinoses (NCLs) are neurodegenerative disorders with onset in childhood. Although there are ten genetic loci linked to NCL, they all have lysosomal storage of ceroid lipofuscin (proteolipid and dolichols), and typically, the mitochondrial ATP synthase subunit c protein [95]. Murine cerebellar cells lacking *CLN3* or *CLN6* both accumulate subunit c, and exhibit changes in mitochondrial morphology and decreased ATP levels [95]. Previously CLN3 deficient cells were shown to have impaired maturation of APs and altered mitochondrial morphology [96].

Alzheimer's Disease (AD), Dementia with Lewy Bodies (DLB) and Multisytem Proteinopathy

Similar to the LSDs and some forms of PD, it is likely that the contribution of impaired mitophagy to AD and DLB is a consequence of a broad impairment of the ALP. Autolysosomes have been identified as the principal component in the neuritic swellings observed in the dystrophic neurites of AD brains and an AD mouse model [6, 10]. Similar swellings could be mimicked by inhibiting the cathepsins or microtubule transport implying that it was the clearance of APs by lysosomes that is the problem, rather than initiation of autophagy [10]. Indeed, the AD-associated protein presenilin-1 has been shown to affect the acidification of lysosomes in neurons and fibroblasts [97, 98].

PD patients with *GBA1* mutations in addition to an earlier age of onset are known to have a greater incidence of cognitive decline [82]. A recent multicentre study has shown a significant association between *GBA1* carriers and PD with dementia or DLB. The odds ratio for PD with dementia was 6.5, while it was 8.3 for DLB [99]. This is compared to an odds ratio of 5.4 in PD [81, 82]. Given the evidence of ALP

impairment in PD cases with *GBA1* mutations described above, it is likely that a similar scenario applies to DLB.

Mutations in VCP cause dominantly inherited multisystem degenerative disorders that affect muscle, bone and brain. The wide spectrum of VCP-related diseases has led to the term multisystem proteinopathy [51]. Neurological disorders include frontotemporal dementia, sporadic and familial amyotrophic lateral sclerosis and hereditary spastic paraplegia [51]. VCP has a wide range of described functions, with two of them having particular emphasis for mitophagy. The first is that VCP is required for the proteasomal degradation of mitochondrial proteins that have been ubiquitinated by parkin following its recruitment to depolarized mitochondria [50, 51]. The second is that VCP appears to be necessary for AP maturation following inhibition of the proteasome, but not starvation [100]. This suggests that VCP is involved with the autophagic degradation of ubiquitin containing substrates. Pathogenic mutations of VCP have been shown to cause mitochondrial dysfunction and impede the removal of damaged mitochondria by mitophagy [51, 101].

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References

1. Van Laar VS, Berman SB. The interplay of neuronal mitochondrial dynamics and bioenergetics: implications for Parkinson's disease. Neurobiol Dis. 2013;51:43–55.
2. Terman A, Kurz T, Navratil M, Arriaga EA, Brunk UT. Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. Antioxid Redox Signal. 2010;12:503–35.
3. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys. 2007;462:245–53.
4. Tolkovsky AM, Xue L, Fletcher GC, Borutaite V. Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? Biochimie. 2002;84:233–40.
5. Twig G, Elorza A, Molina AJA, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008;27:433–46.
6. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013;19:983–97.
7. Zorzano A, Liesa M, Sebastián D, Segalés J, Palacín M. Mitochondrial fusion proteins: dual regulators of morphology and metabolism. Semin Cell Dev Biol. 2010;21:566–74.
8. Friedman JR, Nunnari J. Mitochondrial form and function. Nature. 2014;505:335–43.
9. Cai Q, Davis ML, Sheng Z-H. Regulation of axonal mitochondrial transport and its impact on synaptic transmission. Neurosci Res. 2011;70:9–15.
10. Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, Nixon RA. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci. 2008;28:6926–37.
11. Cai Q, Zakaria HM, Simone A, Sheng Z-H. Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. Curr Biol. 2012;22:545–52.
12. Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, Rice S, Steen J, LaVoie MJ, Schwarz TL. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell. 2011;147:893–906.
13. Chen H, McCaffery JM, Chan DC. Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell. 2007;130:548–62.

14. Bender A, Krishnan KJ, Morris CM, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38:515–7.
15. Kraytsberg Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, Khrapko K. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet. 2006;38:518–20.
16. Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK. ER tubules mark sites of mitochondrial division. Science. 2011;334:358–2.
17. Hoppins S, Edlich F, Cleland MM, Banerjee S, McCaffery JM, Youle RJ, Nunnari J. The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Mol Cell. 2011;41:150–60.
18. Dorn GW 2nd. Mitochondrial dynamism and cardiac fate-a personal perspective. Circ J. 2013;77:1370–9.
19. Elmore SP, Qian T, Grissom SF, Lemasters JJ. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. FASEB J. 2001;15:2286–7.
20. Narendra D, Tanaka A, Suen D-F, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol. 2008;183:795–803.
21. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;392:605–8.
22. Schapira AHV, Gegg M. Mitochondrial contribution to Parkinson's disease pathogenesis. Park Dis. 2011;2011:159160.
23. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, Yoo SJ, Hay BA, Guo M. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature. 2006;441:1162–6.
24. Park J, Lee SB, Lee S, et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature. 2006;441:1157–61.
25. Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;304:1158–1160.
26. Lin W, Kang UJ. Characterization of PINK1 processing, stability, and subcellular localization. J Neurochem. 2008;106:464–74.
27. Deas E, Plun-Favreau H, Gandhi S, et al. PINK1 cleavage at position A103 by the mitochondrial protease PARL. Hum Mol Genet. 2011;20:867–79.
28. Takatori S, Ito G, Iwatsubo T. Cytoplasmic localization and proteasomal degradation of N-terminally cleaved form of PINK1. Neurosci Lett. 2008;430:13–7.
29. Matsuda N, Sato S, Shiba K, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol. 2010;189:211–21.
30. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, Cookson MR, Youle RJ. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8:e1000298.
31. Vives-Bauza C, Zhou C, Huang Y, et al. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc Natl Acad Sci U S A. 2010;107:378–83.
32. Gómez-Sánchez R, Gegg ME, Bravo-San Pedro JM, et al. Mitochondrial impairment increases FL-PINK1 levels by calcium-dependent gene expression. Neurobiol Dis. 2014;62:426–40.
33. Kondapalli C, Kazlauskaite A, Zhang N, et al. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biol. 2012;2:120080.
34. Shiba-Fukushima K, Imai Y, Yoshida S, Ishihama Y, Kanao T, Sato S, Hattori N. PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. Sci Rep. 2012;2:1002.
35. Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ. PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. J Cell Biol. 2014;205:143–53.

36. Kazlauskaite A, Kondapalli C, Gourlay R, Campbell DG, Ritorto MS, Hofmann K, Alessi DR, Knebel A, Trost M, Muqit MMK. Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. Biochem J. 2014;460:127–39.
37. Koyano F, Okatsu K, Kosako H, et al. Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature. 2014. doi:10.1038/nature13392.
38. Lazarou M, Jin SM, Kane LA, Youle RJ. Role of PINK1 binding to the TOM complex and alternate intracellular membranes in recruitment and activation of the E3 ligase Parkin. Dev Cell. 2012. 22:320–33.
39. Hasson SA, Kane LA, Yamano K, et al. High-content genome-wide RNAi screens identify regulators of parkin upstream of mitophagy. Nature. 2013 504:291–5.
40. Burchell VS, Nelson DE, Sanchez-Martinez A, et al. The Parkinson's disease-linked proteins Fbxo7 and Parkin interact to mediate mitophagy. Nat Neurosci. 2013;16:1257–65.
41. Lee J-Y, Nagano Y, Taylor JP, Lim KL, Yao T-P. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. J Cell Biol. 2010;189:671–9.
42. Gegg ME, Cooper JM, Chau K-Y, Rojo M, Schapira AHV, Taanman J-W. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet. 2010;19:4861–70.
43. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol. 2010;12:119–31.
44. Sarraf SA, Raman M, Guarani-Pereira V, Sowa ME, Huttlin EL, Gygi SP, Harper JW. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature. 2013;496:372–6.
45. Chan NC, Salazar AM, Pham AH, Sweredoski MJ, Kolawa NJ, Graham RLJ, Hess S, Chan DC. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. Hum Mol Genet. 2011;20:1726–37.
46. Ding W-X, Ni H-M, Li M, Liao Y, Chen X, Stolz DB, Dorn GW 2nd, Yin X-M. Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. J Biol Chem. 2010;285: 27879–90.
47. Chu CT, Ji J, Dagda RK, et al. Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. Nat Cell Biol. 2013;15:1197–205.
48. Ziviani E, Tao RN, Whitworth AJ. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. Proc Natl Acad Sci U S A. 2010;107:5018–23.
49. Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PloS ONE. 2010;5:e10054.
50. Tanaka A, Cleland MM, Xu S, Narendra DP, Suen D-F, Karbowski M, Youle RJ. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol. 2010;191:1367–80.
51. Kim NC, Tresse E, Kolaitis R-M, et al. VCP is essential for mitochondrial quality control by PINK1/Parkin and this function is impaired by VCP mutations. Neuron. 2013;78:65–80.
52. Lazarou M, Narendra DP, Jin SM, Tekle E, Banerjee S, Youle RJ. PINK1 drives Parkin self-association and HECT-like E3 activity upstream of mitochondrial binding. J Cell Biol. 2013;200:163–172.
53. Glauser L, Sonnay S, Stafa K, Moore DJ. Parkin promotes the ubiquitination and degradation of the mitochondrial fusion factor mitofusin 1. J Neurochem. 2011;118:636–45.
54. Germain D. Ubiquitin-dependent and independent mitochondrial protein quality controls: implications in ageing and neurodegenerative diseases. Mol Microbiol. 2008;70:1334–41.
55. Hamasaki M, Furuta N, Matsuda A, et al. Autophagosomes form at ER-mitochondria contact sites. Nature. 2013;495:389–93.
56. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;141:656–67.

57. Yang J-Y, Yang WY. Bit-by-bit autophagic removal of parkin-labelled mitochondria. Nat Commun. 2013;4:2428.
58. Chen Y, Dorn GW 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science. 2013;340:471–5.
59. Liu S, Sawada T, Lee S, et al. Parkinson's disease-associated kinase PINK1 regulates Miro protein level and axonal transport of mitochondria. PLoS Genet. 2012;8:e1002537.
60. Maday S, Wallace KE, Holzbaur ELF. Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. J Cell Biol. 2012;196:407–7.
61. Koyano F, Okatsu K, Ishigaki S, Fujioka Y, Kimura M, Sobue G, Tanaka K, Matsuda N. The principal PINK1 and Parkin cellular events triggered in response to dissipation of mitochondrial membrane potential occur in primary neurons. Genes Cells Devoted Mol Cell Mech. 2013;18:672–81.
62. Van Laar VS Arnold B Cassady SJ Chu CT Burton EA Berman SB. Bioenergetics of neurons inhibit the translocation response of Parkin following rapid mitochondrial depolarization. Hum Mol Genet. 2011;20:927–40.
63. Sterky FH, Lee S, Wibom R, Olson L, Larsson N-G. Impaired mitochondrial transport and Parkin-independent degeneration of respiratory chain-deficient dopamine neurons in vivo. Proc Natl Acad Sci U S A. 2011; 108:12937–42.
64. Liu L, Feng D, Chen G, et al. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. Nat Cell Biol. 2012;14:177–85.
65. Wu W, Tian W, Hu Z, et al. ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. EMBO Rep. 2014;15:566–75.
66. Chen G, Han Z, Feng D, et al. A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated mitophagy. Mol Cell. 2014. doi:10.1016/j.molcel.2014.02.034.
67. Ding W-X, Yin X-M. Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem. 2012. 393:547–64.
68. Zhu Y, Massen S, Terenzio M, Lang V, Chen-Lindner S, Eils R, Novak I, Dikic I, Hamacher-Brady A, Brady NR. Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. J Biol Chem. 2013;288:1099–113.
69. Rikka S, Quinsay MN, Thomas RL, Kubli DA, Zhang X, Murphy AN. Gustafsson ÅB. Bnip3 impairs mitochondrial bioenergetics and stimulates mitochondrial turnover. Cell Death Differ. 2011;18:721–31.
70. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. Nat Rev Mol Cell Biol. 2013;14:759–74.
71. Itakura E, Kishi-Itakura C, Koyama-Honda I, Mizushima N. Structures containing Atg9A and the ULK1 complex independently target depolarized mitochondria at initial stages of Parkin-mediated mitophagy. J Cell Sci. 2012;125:1488–99.
72. Michiorri S, Gelmetti V, Giarda E, et al. The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ. 2010;17:962–74.
73. Rabinowitz JD, White E. Autophagy and metabolism. Science. 2010;330:1344–8.
74. Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc Natl Acad Sci U S A. 2011;108:10190–5.
75. Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol. 2011;13:589–98.
76. Gautier CA, Kitada T, Shen J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc Natl Acad Sci U S A. 2008;105:11364–9.
77. Gegg ME, Cooper JM, Schapira AHV, Taanman J-W. Silencing of PINK1 expression affects mitochondrial DNA and oxidative phosphorylation in dopaminergic cells. PloS ONE. 2009;4:e4756.
78. Gusdon AM, Zhu J, Van Houten B, Chu CT. ATP13A2 regulates mitochondrial bioenergetics through macroautophagy. Neurobiol Dis. 2012;45:962–72.
79. Usenovic M, Knight AL, Ray A, Wong V, Brown KR, Caldwell GA, Caldwell KA, Stagljar I, Krainc D. Identification of novel ATP13A2 interactors and their role in a-synuclein misfolding and toxicity. Hum Mol Genet. 2012;21:3785–94.

80. Grünewald A, Arns B, Seibler P, Rakovic A, Münchau A, Ramirez A, Sue CM, Klein C. ATP13A2 mutations impair mitochondrial function in fibroblasts from patients with Kufor-Rakeb syndrome. Neurobiol Aging. 2012;33:1843.e1–7.
81. Sidransky E, Nalls MA, Aasly JO, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N Engl J Med. 2009;361:1651–61.
82. Siebert M, Sidransky E, Westbroek W. Glucocerebrosidase is shaking up the synucleinopathies. Brain J Neurol. 2014;37:1304–22.
83. Gegg ME, Burke D, Heales SJR, Cooper JM, Hardy J, Wood NW, Schapira AHV. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Ann Neurol. 2012;72:455–63.
84. Klein C, Westenberger A. Genetics of Parkinson's disease. Cold Spring Harb Perspect Med. 2012;2:a008888.
85. Suen D-F, Narendra DP, Tanaka A, Manfredi G, Youle RJ. Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. Proc Natl Acad Sci U S A. 2010;107:11835–40.
86. Hämäläinen RH, Manninen T, Koivumäki H, Kislin M, Otonkoski T, Suomalainen A. Tissue- and cell-type-specific manifestations of heteroplasmic mtDNA 3243A > G mutation in human induced pluripotent stem cell-derived disease model. Proc Natl Acad Sci U S A. 2013;110:3622–30.
87. Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. Lancet. 1989;1:1269.
88. Rana A, Rera M, Walker DW. Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan. Proc Natl Acad Sci U S A. 2013;110:8638–43.
89. De Pablo-Latorre R Saide A Polishhuck EV Nusco E Fraldi A Ballabio A. Impaired parkin-mediated mitochondrial targeting to autophagosomes differentially contributes to tissue pathology in lysosomal storage diseases. Hum Mol Genet. 2012;21:1770–81.
90. Osellame LD, Rahim AA, Hargreaves IP, Gegg ME, Richard-Londt A, Brandner S, Waddington SN, Schapira AHV, Duchen MR. Mitochondria and quality control defects in a mouse model of Gaucher disease–links to Parkinson's disease. Cell Metab. 2013;17:941–53.
91. Ordonez MP, Roberts EA, Kidwell CU, Yuan SH, Plaisted WC, Goldstein LSB. Disruption and therapeutic rescue of autophagy in a human neuronal model of Niemann Pick type C1. Hum Mol Genet. 2012;21:2651–62.
92. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. Lancet. 2008;372:1263–71.
93. Cleeter MWJ, Chau K-Y, Gluck C, Mehta A, Hughes DA, Duchen M, Wood NW, Hardy J, Mark Cooper J, Schapira AH. Glucocerebrosidase inhibition causes mitochondrial dysfunction and free radical damage. Neurochem Int. 2013;62:1–7.
94. Xu Y-H, Xu K, Sun Y, et al. Multiple pathogenic proteins implicated in neuronopathic Gaucher disease mice. Hum Mol Genet. 2014;doi:10.1093/hmg/ddu105.
95. Cao Y, Staropoli JF, Biswas S, Espinola JA, MacDonald ME, Lee J-M, Cotman SL. Distinct early molecular responses to mutations causing vLINCL and JNCL presage ATP synthase subunit C accumulation in cerebellar cells. PloS ONE. 2011;6:e17118.
96. Cao Y, Espinola JA, Fossale E, Massey AC, Cuervo AM, MacDonald ME, Cotman SL. Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis. J Biol Chem. 2006;281:20483–93.
97. Lee J-H, Yu WH, Kumar A, et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell. 2010;141:1146–58.
98. Coffey EE, Beckel JM, Laties AM, Mitchell CH. Lysosomal alkalization and dysfunction in human fibroblasts with the Alzheimer's disease-linked presenilin 1A246E mutation can be reversed with cAMP. Neuroscience. 2014;263:111–24.
99. Nalls MA, Duran R, Lopez G, et al. A multicenter study of glucocerebrosidase mutations in dementia with Lewy bodies. JAMA Neurol. 2013;70:727–35.

100. Tresse E, Salomons FA, Vesa J, Bott LC, Kimonis V, Yao T-P, Dantuma NP, Taylor JP. VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes and this function is impaired by mutations that cause IBMPFD. Autophagy. 2010;6:217–27.
101. Bartolome F, Wu H-C, Burchell VS, et al. Pathogenic VCP mutations induce mitochondrial uncoupling and reduced ATP levels. Neuron. 2013;78:57–64.

Chapter 4
Dual Role of Autophagy in Neurodegenerative Diseases: The Case of Amyotrophic Lateral Sclerosis

Leslie Bargsted, René L. Vidal, Claudio Hetz and Soledad Matus

Abstract Amyotrophic lateral sclerosis (ALS) is an adult onset and fatal neurodegenerative disease. A major histophatological hallmark of the disease patient-derived tissue and ALS mouse models is the presence of protein aggregates containing misfolded specific proteins. Strategies to clear out these abnormal protein species are proposed as a possible target for disease intervention. Autophagy, a catabolic route that selective degradates abnormally-folded proteins by the lysosomal pathway, has emerged as an attractive target to treat neurodegenerative diseases, like ALS. Accumulating evidence indicates that autophagy impairment also occurs in ALS and can contribute to the neurodegenerative condition. In this article we discuss the evidence involving autophagy alteration associated to ALS phenotype and the evidence that place autophagy as a therapeutic opportunity to treat this neurodegenerative disease.

Keywords Rapamycin · MTOR · Autophagy · ALS · Beclin 1 · Aggregation · Neurodegenerative disease

Introduction

The most common human neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Huntington's disease (HD), share a common neuropathology characterized by the presence

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of protein inclusions in neuronal subcellular compartments. Protein misfolding and its propagation through a seeding mechanism have been associated to neuronal dysfunction in most neurodegenerative diseases, triggering cellular stress reactions [1–3]. For this reason these pathologies are classified as protein misfolding disorders (PMDs). The abnormal aggregation processes in neurodegenerative context comprises also the generation of highly diffusible small oligomers, fibrillar aggregates and large aggregates that are visualized as protein inclusions with amyloid properties. Thus, the maintenance of protein homeostasis (referred to as proteostasis) is crucial to sustain neuronal function in stress conditions that are frequent in neuronal disorders and also during aging. Thus, strategies to remove the load of protein aggregates and oligomers are becoming a relevant target for therapeutic intervention in PMDs (reviewed in [4]).

Macroautophagy (here termed as autophagy) is a selective and efficient catabolic route that degrades misfolded mutant proteins associated to neurodegeneration and damaged organelles. Hence, this cellular pathway is attractive for searching new targets in order to enhance degradation of misfolded proteins through pharmacological and genetic strategies. Regulation of activation of autophagy is commanded by complex signaling mechanisms, which have been classified as mTOR-dependent and—independent pathways. Rapamycin is an mTOR inhibitor and it is the most widely used compound to evaluate the effects of enhancing autophagy in many neurodegeneration models [5, 6]. Although rapamycin treatments have proven neuroprotective in certain in vivo disease models, in ALS the consequences are contradictory and difficult to interpret [7–9]. On the other hand, drugs identified to enhance autophagy through mTOR-independent pathway provided neuroprotection in several models of PMDs [6]. In the case of ALS, trehalose, an mTOR-independent autophagy inducer, generated beneficial effects in SOD1 mutant transgenic mouse models [10, 11]. Also, it is very important to mention that recently new compounds have been tested in cellular and animal models of ALS with TAR DNA-binding protein 43 (TDP43) pathology (see below), and very promising results were obtained in terms of neuroprotection [12].

Despite the promising results reported indicating positive effects of modulating autophagy in several neurodegenerative pathologies, accumulating evidence also indicates that alteration of different components of autophagy pathway may participate in disease progression as part of the etiology off the disease, involving the global dysfunction of the degradative functions of the cells [13]. Furthermore, the possible effects of intervening key players of the autophagy pathway to enhance the process are not easy to predict. In the case of ALS, for example, we described that enhancing autophagy through targeting the proteostasis network in vivo triggers neuroprotection [14]. However, direct targeting of the autophagy regulator Beclin 1, augmented the life span of an ALS mouse model [15]. In this chapter, we discuss the link between ALS pathology with the autophagy process, and the possible avenues for future therapeutic interventions.

Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive, lethal and degenerative disorder that is characterized by the selective death of motoneurons present in spinal cord and frontal cortex. Clinically, this disease involves the progressive paralysis due an atrophy of the muscles, as a lower motor manifestation; and also an increased tone and exaltation of tendon reflexes as upper motor manifestations [16, 17]. In the majority of cases, this motor disorder has a mortal prognosis because of respiratory failures 2–3 years after the onset of symptoms [18]. A small fraction of total documented cases (10 %) are associated with genetic mutations and the disease is inherited in a dominant manner, providing them a familial category. In familial forms of ALS (fALS) the mutation given by an abnormal hexanucleotide repeat expansion in the gene *C9ORF72*, constitutes the highest incidence factor, representing 40 % of familial cases [19, 20] and is follow by point mutations in the gene encoding superoxide dismutase (*SOD1*), accounting 20 % of fALS cases [21]. The remaining 90 % of the cases are not been associated with clear genetic dominant elements and are classified as sporadic cases (sALS), because of the lack of familial history of the disease. In most sporadic cases it has been described the presence of aggregates and abnormal modification of TDP43, a transcriptional regulator that is directing involved in DNA/RNA metabolism [22]. TDP43 is also the main component of inclusions of patients of frontotemporal lobar degeneration with ubiquitinated inclusion (FTLD-U), a type of frontotemporal dementia (FTD) [22].

The phenomenon of protein misfolding is a hallmark of ALS and other brain diseases. At the celular level it is represented by the accumulation of phosphorylated neurofilament, Bunina bodies, Lewy body-like inclusions, and on the other hand by inclusions and accumulation of ubiquitinated material at axons motoneurons [23]. It is also very common in ALS activation and proliferation of astrocytes and microglia in patients and animal models of the disease [24–28]. Unfortunately, currently there is no primary therapy for this disorder and Riluzole is the only drug approved for the treatment, which improves symptoms only in a slight manner [29]. Therefore, the search of new pharmacological strategies and the development of alternative therapeutic tools are still of great importance, and the enhancement of autophagy may in theory provide beneficial effects to alleviate neurodegeneration.

Autophagy Degradation System and its Key Components

Autophagy is a cellular mechanism comprising the degradation of different components of the cytoplasm within lysosomes [30, 31]. This process is involved in many important cellular functions, such as metabolism, development, infection, aging, immunity, cell survival and cell death [32]. The autophagy pathway are regulated by several Autophagy-related (ATG) genes, and it is mediated by different regulatory

steps, including autophagy induction, nucleation/autophagosome formation, vesicle expansion, cargo recognition, crosstalk between endocytosis and autophagy, and autophagosome clearance [33, 34]. The autophagy process initiates, in part, by a protein kinase complex that responds to upstream signals (atg1 and atg13 in yeast). The serine/threonine protein kinase mTOR (mammalian target of rapamycin) plays a role as a regulator of autophagy through the suppression of the pathway under nutrient-rich conditions, and it is a component of the mTOR complex 1 (mTORC1). The nucleation/autophagosome formation complex is regulated by protein complexes involving in the generation of phosphatidylinositol-3-phosphate (PI_3P) as the class III phosphatidylinositol-3-kinase (PI_3K), which control the localization of other autophagy proteins in the pre-autophagosomal structure and it is part of the process of nucleation of the autophagosome [34]. The nucleation complex is comprised by Beclin 1 (BCL-2 interacting protein) and other essential components, including VPS34 [35]. The anti-apoptotic proteins BCL-2 and BCL-X_L negatively regulate Beclin 1 at the endoplasmic reticulum (ER) membrane [36]. Vesicle expansion is executed by two ubiquitin-like conjugation systems. One of them is carried out by the covalent conjugation of ATG12 to ATG5 that, in turn, forms a protein complex associated to ATG16, translocating to the membrane of early autophagosomes. The other signaling pathway promote the conjugation of microtubule-associated protein light chain 3 (LC3) to phosphatidylethanolamine (PE) [37]. After conjugation, the soluble LC3-I translocates to the autophagosome membrane and is called LC3-II. Finally, autophagosomes acquire hydrolytic activity fusing with acidic lysosomes and forming, in this manner, the autophagolysosome where cargo is degraded [38].

The mTOR-dependent autophagy activation is the most studied pathway and it is classically related to conditions of nutrient starvation. However, autophagy is also regulated by mTOR-independent mechanisms, which were discovered through pharmacological screenings aimed to determine novel molecular targets to enhance autophagy [39–41]. Currently, the molecular components of mTOR-independent pathways are poorly described, but some of them have been identified involving fluctuations in intracellular calcium levels and the turnover of phosphatidyl-inositol (inositol phosphates). mTOR-independent pathways are induced by agents that decrease inositol-3-phosphate (IP3) levels like lithium, valproate and carbamazepine [6]. Also calcium could activate calpain to induce mTOR-independent autophagy through a mechanism in which cyclic AMP (cAMP) levels regulate IP_3 production [41].

Furthermore, autophagy has been engaged to cellular stress conditions [42]. Within them, it has been described that the occurence of ER stress by protein folding impairment induces autophagy through the activation of the unfolded protein response (UPR) sensor IRE1 and the downstream activation of c-Jun N-terminal kinase (JNK) [43, 44], which would participate in the dissociation of the Beclin 1 and BCL-2 complex [45]. ER stress has been related to the induction of autophagy through several UPR transcription factors and also FOXO1, a critical regulator of autophagy in neurons [46]. In this manner, there are multiple mechanisms that regulate autophagy levels depending of the cellular context in order to maintain the protein and organelle homeostasis.

Role of Autophagy in ALS

Autophagy is a relevant cellular process in the nervous system. This idea was demonstrated by the genetic inactivation of autophagy in mice deficient for *atg5* or *atg7* (essential regulatory genes) in the nervous system that caused spontaneous neurodegeneration, and accumulation of protein aggregates, massive neuronal loss, and premature death of the animal [47, 48].

In most common proteins linked to PMDs abnormal interactions of disease-related proteins have been reported with specific components of the autophagy machinery [13, 49–51]. In ALS Beclin 1 was shown to have an important role in the development of the disease. We described that in pathological context of ALS, Beclin 1 is upregulated in familial and sporadic patients [14]. This hallmark has also been observed in models of brain ischemia [52], HD [53], traumatic brain injury [54], PD [55], and lipid storage disorders [56, 57]. On the other hand, when Beclin 1 levels are reduced in an animal model of ALS (mutant SOD1 transgenic mice), significant neuroprotection was generated and the lifespan of the animal prolonged [15]. These unexpected results were associated with an accumulation of monomeric and high molecular weight species of mutant SOD1 and a reduction of oligomeric forms of the protein. This is the first direct evidence linking autophagy with ALS in vivo, since previous studies were either correlative or based on the use of drugs that do not specifically target the autophagy pathway. According with this observation, recent reports suggest that the presence of large SOD1 protein inclusions actually have neuroprotective effects on ALS, and also in HD, by decreasing specific concentrations of misolded proteins, because they could trap highly diffusible neurotoxic oligomeric species [58, 59]. Also, in this report we show that mutant SOD1 associates with the Beclin-1/BCL-X_L complex, destabilizing this inhibitory interaction and thereby altering Beclin-1 function in the autophagy process due abnormal protein-protein interactions [15], as reported in other diseases [4] (Fig. 4.1). Thus, the genetic strategy to decrease the expression levels of Beclin 1 using heterozygous mice may bypass this specific molecular alteration on the pathway by reducing the rate of overactivation of the autophagic processes. In agreement with this concept, another report showed that mutant huntingtin inclusions sequester Beclin 1, which could impair its function [60]. Thus, Beclin 1 may have a dual participation in ALS, a downstream effect given by the promotion of the degradation of misfolded proteins and damaged organelles; and an upstream alteration related with the modulation of the threshold for autophagy activation due an abnormal interaction with mutant SOD1 [15].

Genetic Alterations to the Autophagy Pathway in ALS

Genetic evidence also suggests that autophagy may be part of the etiology of the disease. Several mutations have been identified in different genes related with the regulation of autophagy; for example mutations in the genes *UBQLN2* (coding for

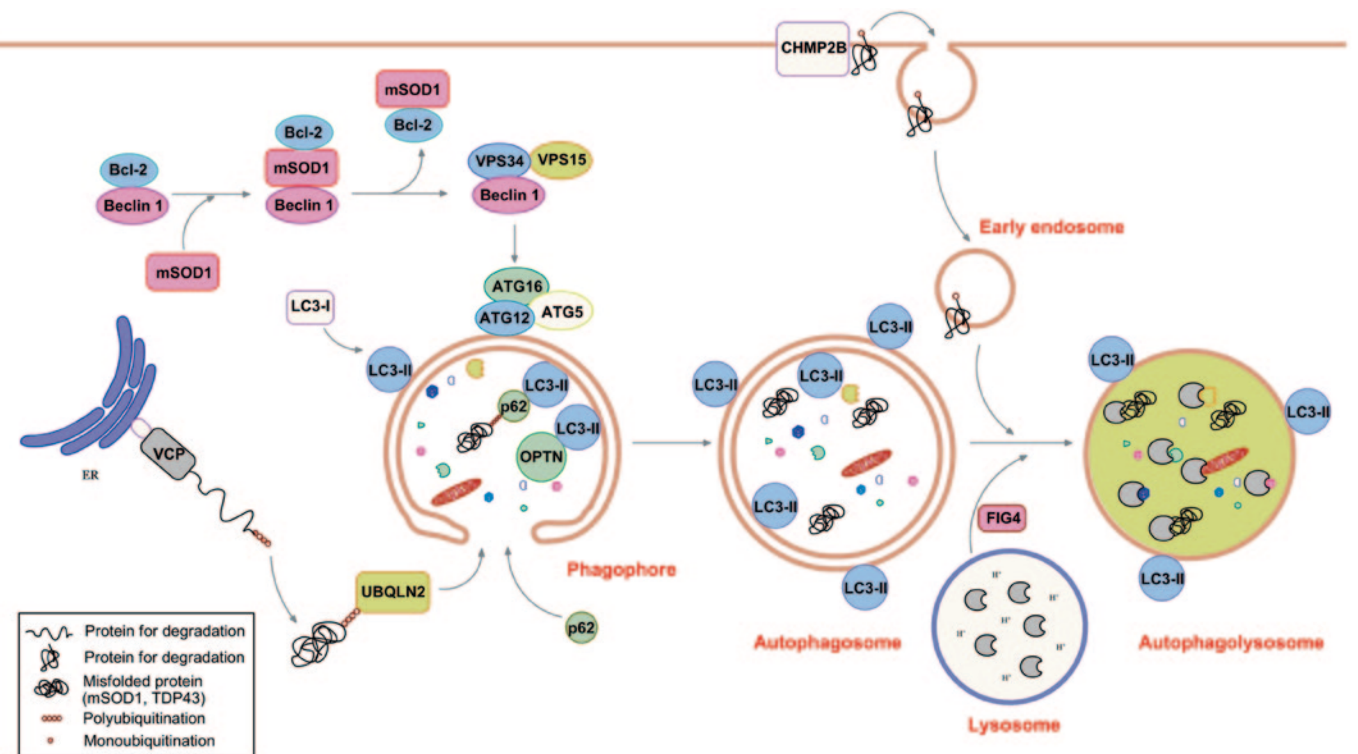

Fig. 4.1 Autophagy impairment in amyotrophic lateral sclerosis. Beclin 1 is part of the nucleation complex together with VPS34 and VPS15, which is fundamental for early autophagosome formation and autophagy initiation. The interaction between Beclin 1 and BCL-2 and BCL-XL inhibits nucleation process and consequently autophagy. In an ALS context, mutant SOD1 (*mSOD1*) and TDP43 aggregates can be degraded by the autophagy pathway. Mutant SOD1 can abnormally associate with the Beclin 1/BCL-XL complex, destabilizing this interaction, over-activating autophagy. Thus, in fALS, mutant SOD1 monomers and oligomers would trigger phagophore formation in a deregulated manner and also generate an abnormal autophagy process. Alterations in autophagic genes UBQLN2, p62/SQSTM1, OPTN, VCP, CHMP2B, and FIG4 proteins is also linked with ALS, and in some cases frontotemporal dementia (*FTD*). These

ubiquilin protein) [61–63], *SQSTM1* (coding for p62/SQSTM1 protein) [64–66], *OPTN* (coding for optineurin protein, OPTN) [67–70] and *VCP* (coding Valosin-containing protein, VCP) [71] that are directly involved in protein degradation, and in *CHMP2B* (coding for charged multivesicular body protein 2B, or chromatin-modifying 2B, CHMP2B) [72, 73] and *FIG4* (coding for FIG4 protein) [74] that are required for autophagosome maturation.

UBQLN2 is a protein that transports polyubiquinated proteins to the degradation processes by proteasome and autophagy; and ALS-linked mutations in this gene may impair overall protein degradation [61]. UBQLN2 alterations have been reported in sporadic ALS and familial ALS cases [61]. Further, UBQLN2 and ubiquitin co-localize in pathological inclusions and they also contain TDP43, FUS/TLS or OPTN [61, 63], suggesting that altered proteostasis is involved as a pathogenic mechanism in ALS. On the other hand, p62/SQSTM1 is a well-described autophagy substrate that acts as an adaptor protein that bridges aggregates and autophagy clearance, through the binding with LC3 [64]. This is consistent with the observation that p62/SQSTM1 promotes the autophagy-dependent degradation of mutant SOD1 [75] and TDP43 [76]. OPTN is a multifunctional protein that binds both polyubiquinated proteins and LC3, and has been proposed as a receptor of autophagy [77]. In patients with OPTN mutations there is the presence of OPTN-positive inclusions, but also the protein OPTN was found as a component of TDP43 inclusions in sporadic patients and being part of SOD1-positive inclusions in patients with SOD1 mutations [69]. VCP is a protein that interacts with ubiquitinated proteins to allow degradation or recycling processes, participating in several protein clearance pathways including the removing of misfolded proteins from the ER and sorting of endosomal proteins during their trafficking [78]. Depletion of VCP generates accumulation of immature autophagosomes, thus suggesting that VCP is required for a proper autophagy process [79]. Mutations VCP were identified in inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBM-FTD) [80] and subsequently in ALS [71]. Interestingly, TDP43 has been found mislocalized to the cytosol in the context of VCP-mediated autophagic dysfunction.

CHMP2B is a crucial component of endosomal sorting complexes [81]. Many reports support the disruption of normal morphology and function of endosomes and lysosomes with mutations in CHMP2B [82, 83], realizing the important role of CHMP2B in autophagy. Mutations in CHMP2B were primarily identified in FTD

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genetic alterations may also contribute to alterations in the autophagy process in ALS. UBQLN2 transports polyubiquitinated proteins for autophagy degradation. p62/SQSTM1 binds ubiquitinated aggregates to target them to autophagy-mediated clearance through interaction with LC3. Similarly, OPTN acts as an adaptor protein for polyubiquinated proteins and as an autophagy receptor. VCP participates in the extraction of misfolded proteins from the ER and in the targeting for autophagy degradation. CHMP2B is a key component of endosomal sorting complexes, which are crucial for autophagosome maturation. Finally, FIG4 is a phosphatase that is important for the regulation of endosome-lysosome trafficking

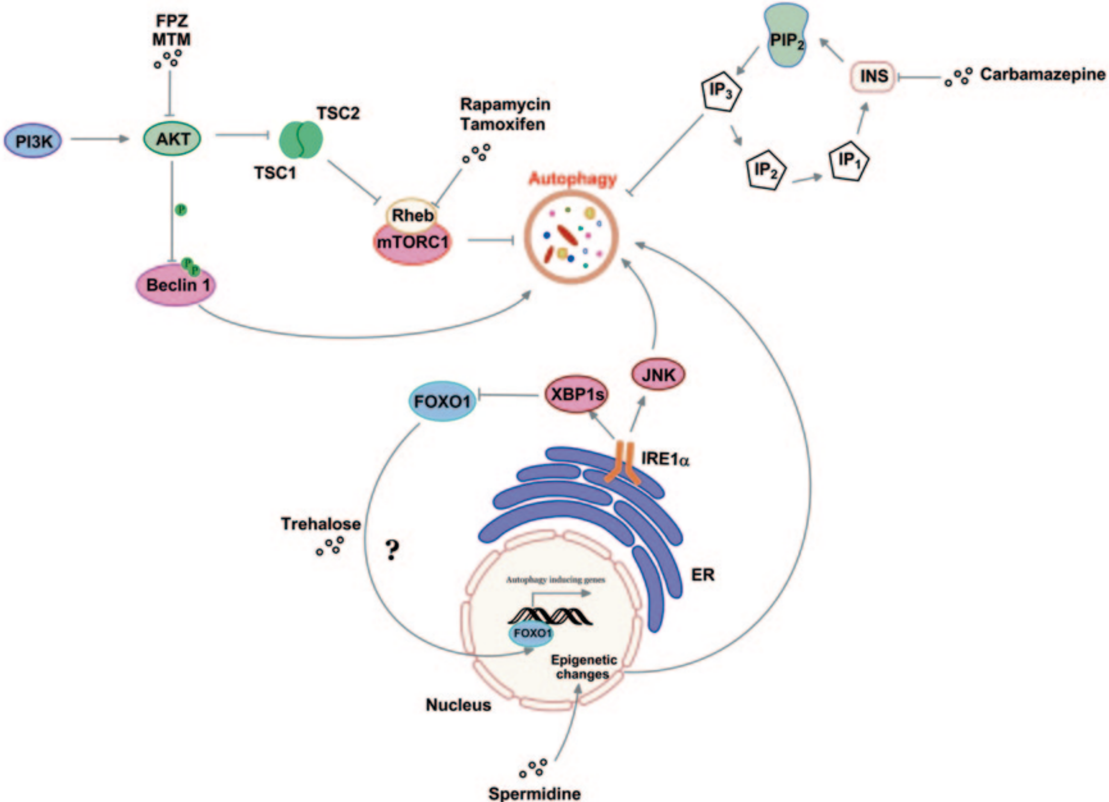

Fig. 4.2 Pharmacological targeting of the autophagy pathway in ALS models. Under conditions of nutrient starvation autophagy is activated through the inhibition of mTORC1 by tuberus sclerosis complex (*TSC1/2*). Fluphenazine (*FPZ*) and methotrimeprazine (*MTM*) are derivatives of 10-(4`-(N-diethylamino)butyl)-2-chlorophenoxazine (*NCP*), molecule that induce autophagy by inhibiting AKT. It is not well known if the action of these molecules depends on mTOR since

[83, 84] and then in ALS [72, 73]. Transgenic mice expressing the intron 5-retention mutant of *CHMP2B*, develop a progressive neurological impairment characterized by axonal disorder and early mortality [85]. Instead, loss of CHMP2B function in mice after gene disruption does not produce any phenotype [85]. Finally, FIG4 is a phosphatase that regulates endosomal-lysosomal vesicle trafficking through the control of amounts of phosphatidyl-inositol-3,5-biphosphate (PI(3,5)P_2) [86]. Heterozygote mutations in *FIG4*, i.e. the combination of a missense allele with a null allele, have been described in cases of Charcot-Marie-Tooth disease type 4 (CMT4J) [87] and ALS [74]. In mice, recessive mutation in FIG4 induces a pathological phenotype characterized by abnormal gait, severe tremor, degeneration of motor and sensory neurons and diluted pigmentation [87, 88]. Mice missing for FIG4 show altered levels of PI (3, 5)P_2 and autophagy pathway is impaired due a dysfunction of the late endosome-lysosome axis [87]. Thus, independent reports have identified distinct rare mutations in several genes involved in autophagy regulation in ALS cases, suggesting that alterations in this catabolic process are detrimental to motoneuron function (Fig. 4.1).

Pharmacological Targeting of Autophagy in ALS

A large group of compounds have been identified enhancing autophagy in high-throughput screenings with proven efficacy in several preclinical disease models [4, 89, 90]. The brain poorly responds to global nutrient deprivation in comparison with other tissues; however autophagy induction through the mTOR inhibitor rapamycin provides protective effects in some experimental models of neurodegeneration. For example, rapamycin treatment delays experimental HD [91], it reduces cognitive anomalies in an animal model of AD (Majumder et al. 2011; Caccamo et al. 2010, 2011; Spilman et al. 2010), it delays spinocerebellar ataxia [91], also PD (Dehay et al. 2010; Malagelada et al. 2010). In contrast to all this reports, the scenario in ALS is much more complex to interpret. In sharp contrast, rapamycin administration to mutant SOD1 mice did not generate any obvious beneficial effects [92, 93] or even have damaging results in terms of disease progression [9]. In VCP transgenic mice, treatment with rapamicyn also induced drastic detrimental effects, exacerbating histopathological alterations [7]. However, administration of rapamycin in a

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AKT they can turn off autophagy by inhibiting TSC1/2 and inactivating Beclin 1 by phosphorylation. Rapamicyn and tamoxifen induces autophagy by inhibiting kinase activity of mTOR through the stabilization of the complex. Carbamazepine triggers autophagy by inhibiting inositol (*Ins*) synthesis. Spermidine induces epigenetic changes that lead to an activation of autophagy. Carbamazepine and spermidine are examples of mTOR-independent autophagy inducers. Stress signals from the endoplasmic reticulum (*ER*) can also play a role in modulation of autophagy, through the unfolded protein response (*UPR*) transcription factor XBP1s that could inhibit FOXO1. The UPR stress sensor IRE1 also enhances autophagy through the JNK pathway. Finally, trehalose induces mTOR-independent autophagy in part by the expression of a cluster of ATG genes and activation of FOXO1, a key transcription factor in autophagy process in neurons

transgenic murine model that overexpress a WT form of TDP43 in forebrain delayed disease progression, improved life span and reduced neuronal loss and neuromuscular alterations [8].

Trehalose is a non-reducing disaccharide naturally produced by some non-mammals organisms under stress conditions that efficiently triggers mTOR-independent autophagy even through oral administration. This disaccharide is widely used in the food industry as a preserving agent and it is approved by the FDA. Trehalose was initially described as a chemical chaperone that could prevent protein aggregation through the stabilization of protein conformations. Treatment with trehalose induces the degradation of diverse aggregation-prone proteins through autophagy enhancement in cell culture models [40]. Also, studies with trehalose in vivo have shown remarkable positive effects in experimental models of HD [94], PD [95], AD [96], oculopharyngeal muscular dystrophy [97], and tauopathies models [98]. Thus, the translational potential of trehalose is remarkable since this compound is able to decrease protein aggregation rate, promote neuronal survival and improve pathological phenotypes through oral treatments in animals. However, it is important to emphasize that only some few recent reports have actually related the neuroprotective effects of trehalose with autophagy induction in vivo [98]. In the context of ALS, we recently demonstrated the beneficial effects of trehalose administration in a transgenic mouse of mutant SOD1, which correlated with the induction of mTOR-independent autophagy [10]. We also demonstrated the induction of autophagy fluxes in vivo similar to rapamycin using a novel tool to monitor autophagy in the nervous system [99], which has important implications for future drug testing [100]. Trehalose treatment led to the increase of animal survival and reduction of disease severity, effects that were accompanied with enhanced mutant SOD1 degradation [10]. Importantly, virtually identical results were recently reported in another transgenic mouse model of SOD1 mutant, where trehalose administration significantly delayed disease onset and decreased neuronal loss [11]. One possible mechanism of action of trehalose is the upregulation of a cluster of *atg* genes [10] and the activation of FOXO1, a transcription factor that play key roles during aging and in autophagy regulation [46] (Fig. 4.2). In summary so far rapamicyn and trehalose are the most studies compounds used to test the possible contribution of autophagy to neurodegeneration, and they are widely tested in preclinical models. However, in ALS models the impacts of both pharmacological agents are opposite depending on the model used. Furthermore, other small molecules have been employed to target autophagy in ALS, including carbamazepine, spermidine and tamoxifen, which rescued motor anomalies of mutant TDP43 transgenic mice [8] (Fig. 4.2). These therapeutic effects were correlated with an induction of autophagy markers in vivo.

Recently, an interesting study showed the testing of autophagy-inducing new compounds in cellular models of ALS that overexpress TDP43 through a screening of an *in silico* library of over 1 million compounds [12]. This work showed that treatment with Fluphenazine (FPZ) and methotrimeprazine (MTM) in transfected cultured cortical mouse neurons decrease TDP43 levels and its half-life. FPZ and MTM act by inducing autophagy and their action reduced the proportion of neurons with cytoplasmatic mutant TDP43 (Fig. 4.2). Finally, both pharmacological

compounds diminished the risk of death of human stem cell–based ALS models, which consisted of motoneurons and astrocytes derived from human induced pluripotent stem cells (iPSCs) with a pathogenic TDP43 mutation [12]. These findings are quite relevant for the investigation field focused in searching new drugs that could be effective in reestablish the homeostasis of the cell in an ALS context. In this case, these results indicate that levels and localization of TDP43 determine neurotoxicity and the induction of autophagy directly affects on TDP43 clearance and also mitigates neurodegeneration.

Autophagy is not an isolated degradation process in the cell. There is a complex proteostasis network that comprises a dynamic interconnection between different stress pathways besides autophagy like UPR, the heat shock response, proteasome system, quality control mechanisms, and other cellular processes [101]. According to this, we demonstrated that genetic manipulation of XBP1, a crucial transcription factor of the UPR pathway, induces autophagy and generates neuroprotective effects in mouse models of ALS [14], and also in HD [102]. These observations were accompanied with a decrease in the amount of protein aggregates, an improvement of neuronal survival and a prolongation of mice lifespan [14, 102]. Thereby, it is possible modulate autophagic processes targeting elements of another pathways that configure the proteostasis network, and this would be interesting to explore in a disease context in the future.

Concluding Remarks

The autophagy process is becoming an interesting topic to develop therapeutic strategies to delay ALS. However, we caution that modulation of this cellular pathway could have detrimental effects. Therefore, it is extremely important to understand in detail the mechanisms that could trigger autophagy impairment in pathological models of neurodegenerative disorders. This is crucial to generate strategies to bypass or revert possible autophagy defects. Autophagy enhancement should be induced on a physiological level, so the modulations of the pathway have to be under a homeostatic limit to avoid over-degradation of cellular components. The identification of different chemical agents that target crucial components of autophagy is configuring an emerging field focused in the development of therapeutic interventions in ALS. However, we predict that a challenging issue for future clinical trial is the calibration of drug concentrations and regimens for chronic use of these compounds in order to avoid secondary effects. The use of novel iPS-derived models of ALS will be of great contribution to the search of compounds that are effective in the context of sALS [103]. Since there is a large amount of available drugs that modulates biological processes beyond autophagy it is necessary to complement this search with more sophisticated high-throughput screenings that identified compound that selectively engage the pathway. Gene therapy may also emerge as an attractive strategy to enhance autophagy only in motoneurons as reported in other brain diseases [4]. Uncovering the possible side consequences of targeting

autophagy in the long term at systemic levels is an urgent need for the validation of possible drugs that may move into trials in the future.

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References

1. Hetz C, Mollereau B. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. Nat Rev Neurosci. 2014;15(4):233–49.
2. Matus S, Glimcher LH, Hetz C. Protein folding stress in neurodegenerative diseases: a glimpse into the ER. Curr Opin Cell Biol. 2011;23(2):239–52.
3. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci. 2003;4(1):49–60.
4. Vidal R, Matus S, Bargsted L, Hetz C. Targeting autophagy in neurodegenerative diseases. Trends Pharmacol Sci. 2014;35:583–91.
5. Sarkar S. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. Biochem Soc Trans. 2013;41(5):1103–30.
6. Sarkar S, Ravikumar B, Floto RA, Rubinsztein DC. Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies. Cell Death Differ. 2009;16(1):46–56.
7. Ching JK, Elizabeth SV, Ju JS, Lusk C, Pittman SK, Weihl CC. mTOR dysfunction contributes to vacuolar pathology and weakness in valosin-containing protein associated inclusion body myopathy. Hum Mol Genet. 2013;22(6):1167–79.
8. Wang IF, Guo BS, Liu YC, Wu CC, Yang CH, Tsai KJ, et al. Autophagy activators rescue and alleviate pathogenesis of a mouse model with proteinopathies of the TAR DNA-binding protein 43. Proc Natl Acad Sci U S A. 2012;109(37):15024–9.
9. Zhang X, Li L, Chen S, Yang D, Wang Y, Wang Z, et al. Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis. Autophagy. 2011;7(4):412–25.
10. Castillo K, Nassif M, Valenzuela V, Rojas F, Matus S, Mercado G, et al. Trehalose delays the progression of amyotrophic lateral sclerosis by enhancing autophagy in motoneurons. Autophagy. 2013;9(9):1308–20.
11. Zhang X, Chen S, Song L, Tang Y, Shen Y, Jia L, et al. MTOR-independent, autophagic enhancer trehalose prolongs motor neuron survival and ameliorates the autophagic flux defect in a mouse model of amyotrophic lateral sclerosis. Autophagy. 2014;10(4):588–602.
12. Barmada SJ, Serio A, Arjun A, Bilican B, Daub A, Ando DM, et al. Autophagy induction enhances TDP43 turnover and survival in neuronal ALS models. Nat Chem Biol. 2014;10(8):677–85.
13. Marino G, Madeo F, Kroemer G. Autophagy for tissue homeostasis and neuroprotection. Curr Opin Cell Biol. 2011;23(2):198–206.
14. Hetz C, Thielen P, Matus S, Nassif M, Court F, Kiffin R, et al. XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy. Genes Dev. 2009;23(19):2294–306.

15. Nassif M, Valenzuela V, Rojas-Rivera D, Vidal R, Matus S, Castillo K, et al. Pathogenic role of BECN1/Beclin 1 in the development of amyotrophic lateral sclerosis. Autophagy. 2014;10(7):1256–71.
16. Mulder DW. Clinical limits of amyotrophic lateral sclerosis. Adv Neurol. 1982;36:15–22.
17. Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci. 2006;7(9):710–23.
18. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. N Engl J Med. 2001;344(22): 1688–700.
19. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron. 2011;72(2):245–56.
20. Renton AE, Majounie E, Waite A, Simon-Sanchez J, Rollinson S, Gibbs JR, et al. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron. 2011;72(2):257–68.
21. Andersen PM, Al-Chalabi A. Clinical genetics of amyotrophic lateral sclerosis: what do we really know? Nat Rev Neurol. 2011;7(11):603–15.
22. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. 2006;314(5796):130–3.
23. Ince PG. Amyotrophic lateral sclerosis. In: Brown RHJ, Meininger K, Swash M, Editors. Neuropathology. London: Martin Dunitz; 2000. pp. 83–112.
24. Kushner PD, Stephenson DT, Wright S. Reactive astrogliosis is widespread in the subcortical white matter of amyotrophic lateral sclerosis brain. J Neuropathol Exp Neurol. 1991;50(3):263–77.
25. Murayama S, Inoue K, Kawakami H, Bouldin TW, Suzuki K. A unique pattern of astrocytosis in the primary motor area in amyotrophic lateral sclerosis. Acta Neuropathol. 1991;82(6):456–61.
26. Nagy D, Kato T, Kushner PD. Reactive astrocytes are widespread in the cortical gray matter of amyotrophic lateral sclerosis. J Neurosci Res. 1994;38(3):336–47.
27. Tsao W, Jeong YH, Lin S, Ling J, Price DL, Chiang PM, et al. Rodent models of TDP-43: recent advances. Brain Res. 2012;1462:26–39.
28. Turner BJ, Talbot K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Prog Neurobiol. 2008;85(1):94–134.
29. Bensimon G, Lacomblez L, Meininger V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. N Engl J Med. 1994;330(9):585–91.
30. Cuervo AM. Autophagy: in sickness and in health. Trends Cell Biol. 2004;14(2):70–7.
31. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. J Cell Sci. 2005;118(Pt 1):7–18.
32. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069–75.
33. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67–93.
34. He C, Levine B. The Beclin 1 interactome. Curr Opin Cell Biol. 2010;22(2):140–9.
35. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep. 2001;2(4):330–5.
36. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122(6):927–39.
37. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741–52.
38. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012;8(4):445–544.
39. Sarkar S. Chemical screening platforms for autophagy drug discovery to identify therapeutic candidates for Huntington's disease and other neurodegenerative disorders. Drug Discover Today Technol. 2013;10(1):e137–44.

40. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. 2007;282(8):5641–52.
41. Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol. 2008;4(5):295–305.
42. Kroemer G, Marino G, Levine B. Autophagy and the integrated stress response. Mol Cell. 2010;40(2):280–93.
43. Criollo A, Vicencio JM, Tasdemir E, Maiuri MC, Lavandero S, Kroemer G. The inositol trisphosphate receptor in the control of autophagy. Autophagy. 2007;3(4):350–3.
44. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006;26(24):9220–31.
45. Castillo K, Rojas-Rivera D, Lisbona F, Caballero B, Nassif M, Court FA, et al. BAX inhibitor-1 regulates autophagy by controlling the IRE1alpha branch of the unfolded protein response. EMBO J. 2011;30(21):4465–78.
46. Vidal RL, Hetz C. Unspliced XBP1 controls autophagy through FoxO1. Cell Res. 2013;23(4):463–4.
47. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441(7095):885–9.
48. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880–4.
49. Nassif M, Hetz C. Targeting autophagy in ALS: a complex mission. Autophagy. 2011;7(4):450–3.
50. Sridhar S, Botbol Y, Macian F, Cuervo AM. Autophagy and disease: always two sides to a problem. J Pathol. 2012;226(2):255–73.
51. Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci. 2010;13(7):805–11.
52. Rami A, Langhagen A, Steiger S. Focal cerebral ischemia induces upregulation of Beclin 1 and autophagy-like cell death. Neurobiol Dis. 2008;29(1):132–41.
53. Wu JC, Qi L, Wang Y, Kegel KB, Yoder J, Difiglia M, et al. The regulation of N-terminal Huntingtin (Htt552) accumulation by Beclin1. Acta Pharmacol Sin. 2012;33(6):743–51.
54. Diskin T, Tal-Or P, Erlich S, Mizrachy L, Alexandrovich A, Shohami E, et al. Closed head injury induces upregulation of Beclin 1 at the cortical site of injury. J Neurotrauma. 2005;22(7):750–62.
55. Wills J, Credle J, Oaks AW, Duka V, Lee JH, Jones J, et al. Paraquat, but not maneb, induces synucleinopathy and tauopathy in striata of mice through inhibition of proteasomal and autophagic pathways. PLoS ONE. 2012;7(1):e30745.
56. Pacheco CD, Lieberman AP. Lipid trafficking defects increase Beclin-1 and activate autophagy in Niemann-Pick type C disease. Autophagy. 2007;3(5):487–9.
57. Takamura A, Higaki K, Kajimaki K, Otsuka S, Ninomiya H, Matsuda J, et al. Enhanced autophagy and mitochondrial aberrations in murine G(M1)-gangliosidosis. Biochem Biophys Res Commun. 2008;367(3):616–22.
58. Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature. 2004;431(7010):805–10.
59. Brotherton TE, Li Y, Glass JD. Cellular toxicity of mutant SOD1 protein is linked to an easily soluble, non-aggregated form in vitro. Neurobiol Dis. 2013;49:c49–56.
60. Shibata M, Lu T, Furuya T, Degterev A, Mizushima N, Yoshimori T, et al. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J Biol Chem. 2006;281(20):14474–85.
61. Deng HX, Chen W, Hong ST, Boycott KM, Gorrie GH, Siddique N, et al. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. Nature. 2011;477(7363):211–5.
62. Gellera C, Tiloca C, Del Bo R, Corrado L, Pensato V, Agostini J, et al. Ubiquilin 2 mutations in Italian patients with amyotrophic lateral sclerosis and frontotemporal dementia. J Neurol Neurosurg Psychiatry. 2013;84(2):183–7.

63. Williams KL, Warraich ST, Yang S, Solski JA, Fernando R, Rouleau GA, et al. UBQLN2/ubiquilin 2 mutation and pathology in familial amyotrophic lateral sclerosis. Neurobiol Aging. 2012;33(10):2527 e3–10.
64. Fecto F, Yan J, Vemula SP, Liu E, Yang Y, Chen W, et al. SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. Arch Neurol. 2011;68(11):1440–6.
65. Rubino E, Rainero I, Chio A, Rogaeva E, Galimberti D, Fenoglio P, et al. SQSTM1 mutations in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Neurology. 2012;79(15):1556–62.
66. Teyssou E, Takeda T, Lebon V, Boillee S, Doukoure B, Bataillon G, et al. Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: genetics and neuropathology. Acta Neuropathol. 2013;125(4):511–22.
67. Del Bo R, Tiloca C, Pensato V, Corrado L, Ratti A, Ticozzi N, et al. Novel optineurin mutations in patients with familial and sporadic amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry. 2011;82(11):1239–43.
68. Iida A, Hosono N, Sano M, Kamei T, Oshima S, Tokuda T, et al. Optineurin mutations in Japanese amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry. 2012;83(2):233–5.
69. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, et al. Mutations of optineurin in amyotrophic lateral sclerosis. Nature. 2010;465(7295):223–6.
70. van Blitterswijk M van Vught PW, van Es MA, Schelhaas HJ, van der Kooi AJ, de Visser M, et al. Novel optineurin mutations in sporadic amyotrophic lateral sclerosis patients. Neurobiol Aging. 2012;33(5):1016 e1–7.
71. Johnson JO, Mandrioli J, Benatar M, Abramzon Y, Van Deerlin VM, Trojanowski JQ, et al. Exome sequencing reveals VCP mutations as a cause of familial ALS. Neuron. 2010;68(5):857–64.
72. Cox LE, Ferraiuolo L, Goodall EF, Heath PR, Higginbottom A, Mortiboys H, et al. Mutations in CHMP2B in lower motor neuron predominant amyotrophic lateral sclerosis (ALS). PLoS ONE. 2010;5(3):e9872.
73. Parkinson N, Ince PG, Smith MO, Highley R, Skibinski G, Andersen PM, et al. ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). Neurology. 2006;67(6):1074–7.
74. Chow CY, Landers JE, Bergren SK, Sapp PC, Grant AE, Jones JM, et al. Deleterious variants of FIG4, a phosphoinositide phosphatase, in patients with ALS. Am J Hum Genet. 2009;84(1):85–8.
75. Gal J, Strom AL, Kwinter DM, Kilty R, Zhang J, Shi P, et al. Sequestosome 1/p62 links familial ALS mutant SOD1 to LC3 via an ubiquitin-independent mechanism. J Neurochem. 2009;111(4):1062–73.
76. Brady OA, Meng P, Zheng Y, Mao Y, Hu F. Regulation of TDP-43 aggregation by phosphorylation and p62/SQSTM1. J Neurochem. 2011;116(2):248–59.
77. Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, Brady NR, et al. Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science. 2011;333(6039):228–33.
78. Meyer H, Weihl CC. The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. J Cell Sci. 2014;127:3877–83.
79. Ju JS, Fuentealba RA, Miller SE, Jackson E, Piwnica-Worms D, Baloh RH, et al. Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. J Cell Biol. 2009;187(6):875–88.
80. Watts GD, Wymer J, Kovach MJ, Mehta SG, Mumm S, Darvish D, et al. Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat Genet. 2004;36(4):377–81.
81. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature. 2009;458(7237):445–52.
82. Urwin H, Authier A, Nielsen JE, Metcalf D, Powell C, Froud K, et al. Disruption of endocytic trafficking in frontotemporal dementia with CHMP2B mutations. Hum Mol Genet. 2010;19(11):2228–38.
83. van der Zee J, Urwin H, Engelborghs S, Bruyland M, Vandenberghe R, Dermaut B, et al. CHMP2B C-truncating mutations in frontotemporal lobar degeneration are associated with an aberrant endosomal phenotype in vitro. Hum Mol Genet. 2008;17(2):313–22.

84. Momeni P, Rogaeva E, Van Deerlin V, Yuan W, Grafman J, Tierney M, et al. Genetic variability in CHMP2B and frontotemporal dementia. Neurodegener Dis. 2006;3(3):129–33.
85. Ghazi-Noori S, Froud KE, Mizielinska S, Powell C, Smidak M, Fernandez de Marco M, et al. Progressive neuronal inclusion formation and axonal degeneration in CHMP2B mutant transgenic mice. Brain. 2012;135(Pt 3):819–32.
86. Kon T, Mori F, Tanji K, Miki Y, Toyoshima Y, Yoshida M, et al. ALS-associated protein FIG4 is localized in Pick and Lewy bodies, and also neuronal nuclear inclusions, in polyglutamine and intranuclear inclusion body diseases. Neuropathology. 2014;34(1):19–26.
87. Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, et al. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. Nature. 2007;448(7149):68–72.
88. Zhang X, Chow CY, Sahenk Z, Shy ME, Meisler MH, Li J. Mutation of FIG4 causes a rapidly progressive, asymmetric neuronal degeneration. Brain. 2008;131(Pt 8):1990–2001.
89. Fleming A, Noda T, Yoshimori T, Rubinsztein DC. Chemical modulators of autophagy as biological probes and potential therapeutics. Nat Chem Biol. 2011;7(1):9–17.
90. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov. 2012;11(9):709–30.
91. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004;36(6):585–95.
92. Bhattacharya A, Bokov A, Muller FL, Jernigan AL, Maslin K, Diaz V, et al. Dietary restriction but not rapamycin extends disease onset and survival of the H46R/H48Q mouse model of ALS. Neurobiol Aging. 2012;33(8):1829–32.
93. Staats KA, Hernandez S, Schonefeldt S, Bento-Abreu A, Dooley J, Van Damme P, et al. Rapamycin increases survival in ALS mice lacking mature lymphocytes. Mol Neurodegener. 2013;8:31.
94. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, et al. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. Nat Med. 2004;10(2):148–54.
95. Perucho J, Casarejos MJ, Gomez A, Solano RM, de Yebenes JG, Mena MA. Trehalose protects from aggravation of amyloid pathology induced by isoflurane anesthesia in APP(swe) mutant mice. Curr Alzheimer Res. 2012;9(3):334–43.
96. Rodriguez-Navarro JA, Rodriguez L, Casarejos MJ, Solano RM, Gomez A, Perucho J, et al. Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. Neurobiol Dis. 2010;39(3):423–38.
97. Davies JE, Sarkar S, Rubinsztein DC. Trehalose reduces aggregate formation and delays pathology in a transgenic mouse model of oculopharyngeal muscular dystrophy. Hum Mol Genet. 2006;15(1):23–31.
98. Schaeffer V, Lavenir I, Ozcelik S, Tolnay M, Winkler DT, Goedert M. Stimulation of autophagy reduces neurodegeneration in a mouse model of human tauopathy. Brain. 2012;135(Pt 7):2169–77.
99. Castillo K, Valenzuela V, Matus S, Nassif M, Onate M, Fuentealba Y, et al. Measurement of autophagy flux in the nervous system in vivo. Cell Death Dis. 2013;4:e917.
100. Matus S, Valenzuela V, Hetz C. A new method to measure autophagy flux in the nervous system. Autophagy. 2014;10(4):710–4.
101. Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. Science. 2008;319(5865):916–9.
102. Vidal RL, Figueroa A, Court FA, Thielen P, Molina C, Wirth C, et al. Targeting the UPR transcription factor XBP1 protects against Huntington's disease through the regulation of FoxO1 and autophagy. Hum Mol Genet. 2012;21(10):2245–62.
103. Matus S, Medinas DB, Hetz C. Common ground: stem cell approaches find shared pathways underlying ALS. Cell Stem Cell. 2014;14(6):697–9.

Chapter 5
Autophagy Pathways in Huntington's Disease

María Ángeles Mena, Juan Perucho, Marian Fernandez-Estevez and Justo García de Yébenes

Abstract Huntington's disease (HD) is a hereditary neurodegenerative disorder characterized by motor, cognitive and behavioural abnormalities. HD is caused by a mutation in the huntingtin gene which produces an enlarged chain of CAG triplets in this gene and an expanded chain of poliglutamines in the N terminal portion of the protein. HD is characterized by neuronal loss and atrophy of several brain nuclei, preferentially in the striatum.

The pathogenic mechanisms responsible for HD are partially unknown. Mutant huntingtin aggregates in insoluble filaments, changes its localization from the cytoplasm to the nucleus and changes the transcription of genes, inhibits mitochondrial function, activates caspases, block microtubules, interacts with Ca2+channels and excitatory receptors and inhibits the production of neurotrophic factors.

There are abnormalities of the ubiquitin proteasomal system (UPS) in HD. In samples of human brain from patients with HD, it has been observed that there are intranuclear inclusions of huntingtin fragments which stain with antibodies against ubiquitine. These inclusions are present even before the presence of clinical deficits and their severity correlates with the size of the expansion. Proteasomal function, however, is preserved suggesting that the polyglutamine chains block the

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ubiquitylation pathway. In human fibroblasts from patients with HD, activation of autophagy compensates the deficits of the UPS.

Similarly, in experimental models of HD there are intraneuronal inclusions which appear before the clinical deficits and stimulation of the autophagy reduces the number of inclusions. Autophagy could compensate deficits related with blockade of the UPS in HD.

Keywords Huntington's disease · Huntingtin · Autophagy · Aggregation · Polyglutamines

Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder, characterized by motor, cognitive and behavioral deficits [1–3], produced by the expansion of a CAG trinucleotide repeat in the huntingtin gene, which causes an enlarged poliglutamine chain at the N-terminal region of huntingtin [4, 5]. The expanded huntingtin mutant protein, which is ubiquitously expressed in the body and the brain, causes preferential neurodegeneration in distinct brain regions with a pattern that varies according to the size of the expansion. This is done in such a way that subjects with relatively small expansions show neuronal loss almost limited to the striatum while those with large expansion show widespread degeneration throughout the brain (striatum > thalamus > cerebral cortex > diencephalon > brain stem > cerebellum) and even with very large expansions into the muscle. In the striatum, the huntingtin protein accumulates and causes striatal atrophy [6] and the death of the medium and small size projecting spiny neurons [7, 8].

At the cellular levels, the most relevant finding observed in the neurons, which remain in different brain nuclei, is the presence of intranuclear inclusions, present in most neurons, that stain with antibodies anti-ubiquitin and anti-huntingtin. Since normal huntingtin is a cytoplasmic protein, the presence of immunoreactive intranuclear inclusion suggests a translocation of the mutant protein from cytoplasm to nucleus and a change of protein function.

Pathogenic Mechanisms The mechanism of the disease is unknown though a number of molecular events are related to the abnormal protein (Fig. 5.1).

The most important mechanisms involved in the neurodegeneration of neurons in HD are the following:

a. Aggregation of huntingtin in insoluble protofilaments
b. Translocation to the nucleus and changes in transcription
c. Inhibition of mitochondrial function
d. Activation of caspases and interaction with other proteins
e. Blockade of microtubules
f. Interaction with neuronal excitability and regulation of Ca2+
g. Deficits of neurotrophins and excessive inflammation

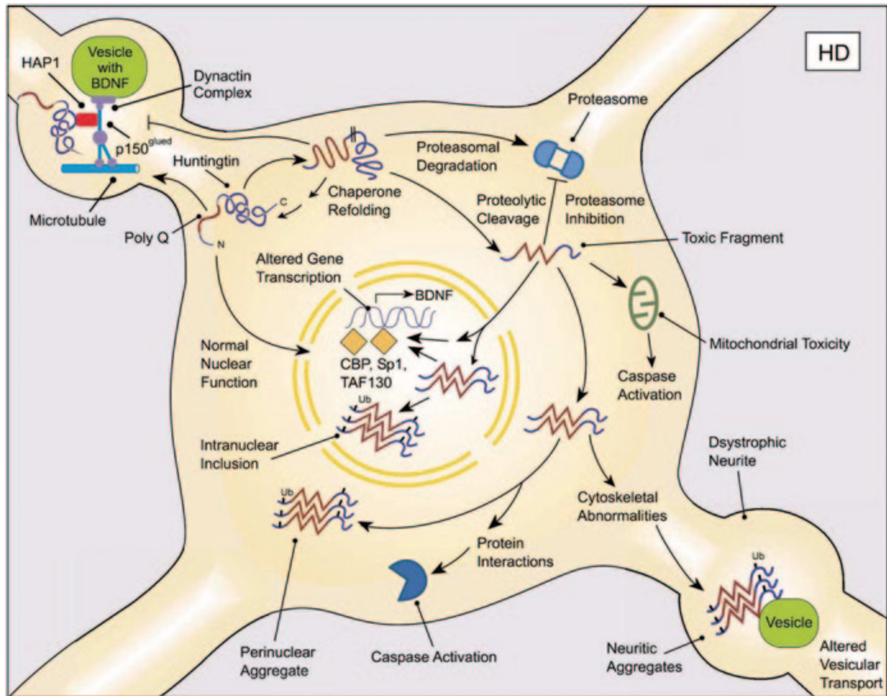

Fig. 5.1 Mechanism of toxicity of mutant huntingtin [9]. Normal huntingtin is localized in the cytoplasm and regulates vesicle transport, including that of BDNF. It also regulates gene transcription. The mutation produces residues resistant to degradation, which are hyperphosphorylated and interfere with BDNF receptor signaling. Insoluble aggregates, activate caspases directly or via mitochondrial effects

Most of these mechanisms are interrelated and linked in multiple circular pathways. It is difficult at this time to pinpoint which of these pathogenic mechanisms is primary and which are secondary. Without this knowledge it is difficult to design neuroprotective studies based on rational backgrounds. In any event, abnormal processing of the mutant huntingtin or poliglutamine fragments derived from it could play a role in other cellular and molecular abnormalities observed en patients with Huntington's disease. In order to discuss the data already available regarding the processing of abnormal proteins or protein segments in HD we shall divide our discussion in two parts, (a) data obtained in patients with HD and (b) data obtained in experimental models (Fig. 5.2).

Protein Processing in Patients with HD

Shortly after it was discovered that the gene defect responsible for HD was an expansion of the variable sequence of CAG in the 5′-terminal region of the huntingtin gene [4], which translates into an enlarged polyglutamine chain in the N-terminal

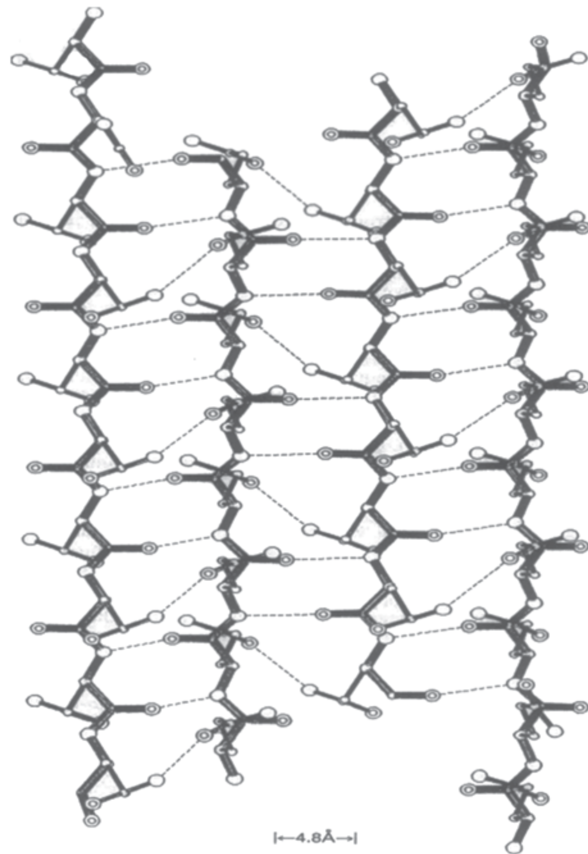

Fig. 5.2 Polar zippers of poliglutamines [10]

region of the huntingtin protein, it was found that in models of four diseases characterized by polyglutamine expansions these polyglutamines form β-sheets strongly held together by hydrogen bonds [10]. It was also suggested that "glutamine repeats may function as polar zippers, for example, by joining specific transcription factors bound to separate DNA segments. Their extension may cause disease either by increased, nonspecific affinity between such factors or by gradual precipitation of the affected proteins in neurons" [10].

Neuropathological studies performed in brains of patients with HD showed neuronal intranuclear inclusions and dystrophic neurites containing fragments of polyglutamine derived from the N-terminal region of the huntingtin protein [11]. The extent of huntingtin accumulation increased with the size of the polyglutamine expansions. The neuronal intranuclear inclusions and the dystrophic neurites contained ubiquitin, which suggest that mutant huntingtin was labeled for protesomal processing but resistant to digestion [11]. The authors considered that abnormal aggregation of huntingtin was one of the pathogenic mechanisms of the disease.

The types, location, numbers, forms, and composition of microscopic huntingtin aggregates in human brain have been studied with a fusion protein antibody against the first 256 amino acids which preferentially recognizes aggregated huntingtin [12]. Neuropil aggregates are much more common than nuclear aggregates and can be present in large numbers in presymptomatic mutation carriers before the onset of clinical symptoms. There are also many more aggregates in cortex than in striatum, where they are actually uncommon. Although the striatum is the most affected region in HD, only 1–4% of striatal neurons in all grades of HD have nuclear aggregates. Neuropil aggregates, which have been identified by electron microscopy to occur in dendrites and dendritic spines, could play a role in the known dendritic pathology that occurs in HD. Aggregates increase in size in advanced grades, suggesting that they may persist in neurons that are more likely to survive. Ubiquitination is apparent in only a subset of aggregates, suggesting that ubiquitin-mediated proteolysis of aggregates may be late or variable [12]. The preferential localization of the intranuclear inclusions in cortical neurons in spite of a more severe cell loss in the striatum could be interpreted in different ways as (a) there are fewer inclusions in striatal neurons because there are fewer surviving striatal neurons and (b) there are more intranuclear inclusions in surviving cortical neurons because ubiquitination is, somehow, a way of neutralization of the toxic properties of huntingtin.

Cellular clearing of abnormal proteins could take place through the ubiquitine-proteasome pathway or through autophagy, a process which could be divided in three categories, macro-autophagy, micro-autophagy and chaperone mediated autophagy. In general, both pathways, ubiquitination + proteolysis and autophagy, are inter-related and when one of these processes is blocked there is a compensatory activation of the other in healthy cells [13]. Therefore, if ubiquitin related proteolysis is depressed in patients with HD, one could expect that autophagy is an important compensatory pathway for protein processing and that stimulation of autophagy could be a possible disease modifying therapeutic approach.

The role of autophagy in HD has been unveiled by recent studies which show that the age at onset of the disease could be modified by the V471A polymorphism of the ATG7 gene, a key factor in autophagy [14, 15]. We have further investigated the possible effects of manipulation of ubiquitination and autophagy in fibroblasts from patients with HD. These fibroblasts have increased levels of ubiquitilated proteins and higher levels of reactive oxygen species (ROS), huntingtin and the autophagy marker LAMP2A. Baseline replication rates were higher in HD than in control fibroblasts but that was reverted after 12 passages. Epoxomicin, an inhibitor of the ubiquitin pathway, increases the activated caspase-3, HSP70, huntingtin, ubiquitinated proteins and ROS levels in fibroblasts from both HD and controls. Treatment with trehalose, an activator of autophagy, counteracts the effects of epoxomicin such as an increase in ROS, ubiquitinated proteins, huntingtin and activated caspase-3 levels and also increases the autophagy marker LC3 levels more in HD fibroblast than controls. These results suggest that activation of autophagy by trehalose could revert protein processing abnormalities observed in patients with HD (Fig. 5.3).

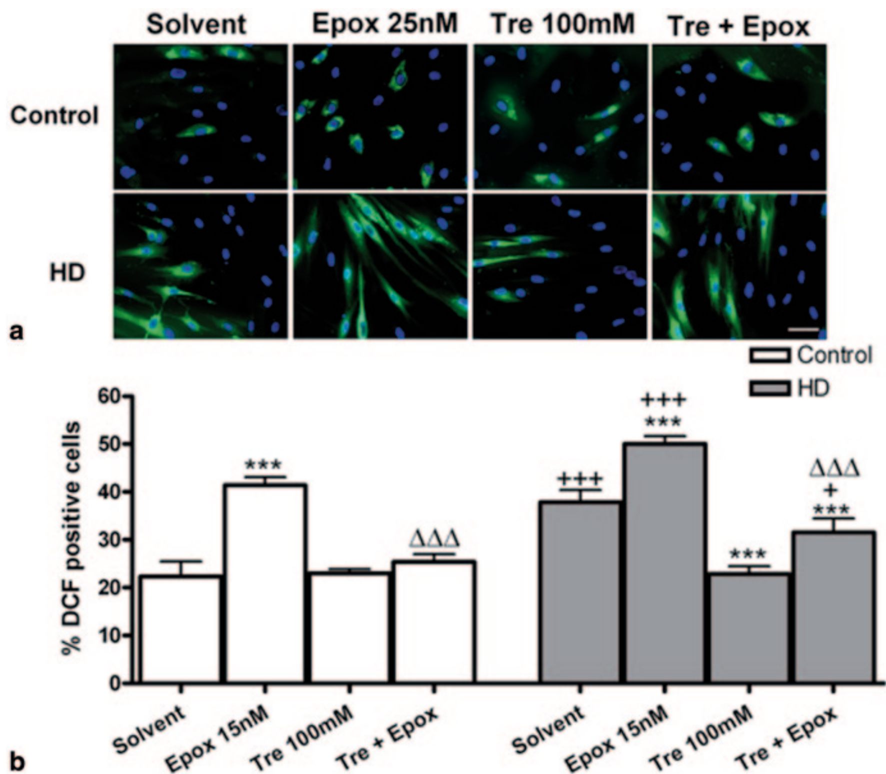

Fig. 5.3 Epoxomicin increases ROS levels, which are reduced by trehalose in HD firboblasts. **a** 29, 79–dichlorofluorescin (DCF) immunocytochemistry (*green*) and total nuclei stained with bis-benzimide (*blue*) in control and HD fibroblasts and **b** percentage of DCF positive cells respect to the total number. (Scale bar = 20 mm). Values are expressed as the mean ± SD, $n = 4$ patients. Control cell number (mean per field), $n = 4$. HD cell number (mean per field), $n = 4$. The data of each patient was obtained using 4 replicates. Statistical analysis was performed by one-way ANOVA with repeated measures followed by Bonferroni multiple comparison test: *** = $p < 0.001$ vs solvent; + = $p < 0.05$, +++ = p Σψμβολ 0.001 HD vs controls; ΔΔΔ = $p < 0.001$ trehalose + epoxomicin vs epoxomicin [16]

Ubiquitination and Autophagy in Models of HD

Shortly after the discovery of the genetic mutation that causes HD several animal models of HD have been created in experimental animals from nematodes to mammals, most commonly mice and more recently pigs and sheep. These experimental models essentially confirm the same abnormalities of protein processing observed in patients with HD, and most importantly, the presence of neuronal intranuclaer inclusions in different brain areas, a correlation between the size of the expansion and density of inclusions, normal proteasomal function in vitro and alternative

compensatory autophagic function. Both the UPS and autophagy pathway are important for processing of mutant huntingtin or its polyglutamine fragments since the inhibition of the two systems increases the the levels of huntingtin in PC12 cells [17].

Autophagic activity can be enhanced in Akt- and m-TOR-independent mechanisms by a number of small molecules including phenoxamine and its derivatives [18–20]. Autophagic enhancement in experimental models of HD reduces the number of inclusions [19].

Very interesting experiments have been published recently regarding the selective impairment of the striatum and its possible relation with autophagy [21]. These authors have shown that the striatal specific protein Rhes binds huntingtin and enhances its cytotoxicity, since Rhes deleted mice are protected in HD. In PC12 cells deletion of Rhes decreases autophagy and Rhes overexpression increases autophagy. These effects are independent of mTOR and opposite to the direction predicted by the known activation of mTOR by Rhes. Rhes robustly binds the autophagy regulator Beclin-1, decreasing its inhibitory interaction with Bcl-2, independent of JNK-1 signaling. Finally, co-expression of mutant huntingtin blocks Rhes-induced autophagy activation. Thus, the preferential striatal pathology and delayed onset of HD may reflect the striatal-selective expression and changes in autophagic activity of Rhes [21].

In mice and fly models of HD mTOR is bound to huntingtin aggregates [22]. Treatment with rapamycin, which inhibits mTOR related kinase activity and increases autophagy, reduces the number of huntingtin aggregates [22].

Another important factor in the neuropathology is the cell-interactions during the disease and in the brain up to 90% are glial cells supporting the survival and function of the neuronal cells. Glial pathology has also been found in HD models [23, 24] and in brains of patients with HD, analyzed post-mortem [25–27]. Mutant huntingtin is expressed also in glial cells and directly affects neuron pathology in HD [28], but comparing the amount of huntingtin inclusions, neurons accumulate much more than glial cells in mouse brains [29, 30]. Glial cells may be able to clear truncated proteins more efficiently than neurons, and astrocytes also produce neurotrophic factors and cytokines to regulate the forms and functions of neurons. Recent studies have shown that R6/1 mice implanted with a pump which delivers glial conditioned medium in one striatum have fewer intranuclear inclusions than littermates infused with artificial cerebro spinal fluid (Fig. 5.4) [31].

This protective effect of glial conditioned medium on huntingtin pathology correlates with enhancement of autophagy [31]. The relative sparing of glia from huntingtin related pathology is, therefore, probably related to the production by glia of soluble factors, which diffuse into the glia conditioned medium and enhance authophagy and clearance of mutant huntingtin (Fig. 5.5).

In summary, there is evidence of impairment of the UPS by mutant huntingtin. It is also clear that cells of patients with HD try to compensate the UPS dysfunction by enhancing autophagy. This strategy could be used therapeutically.

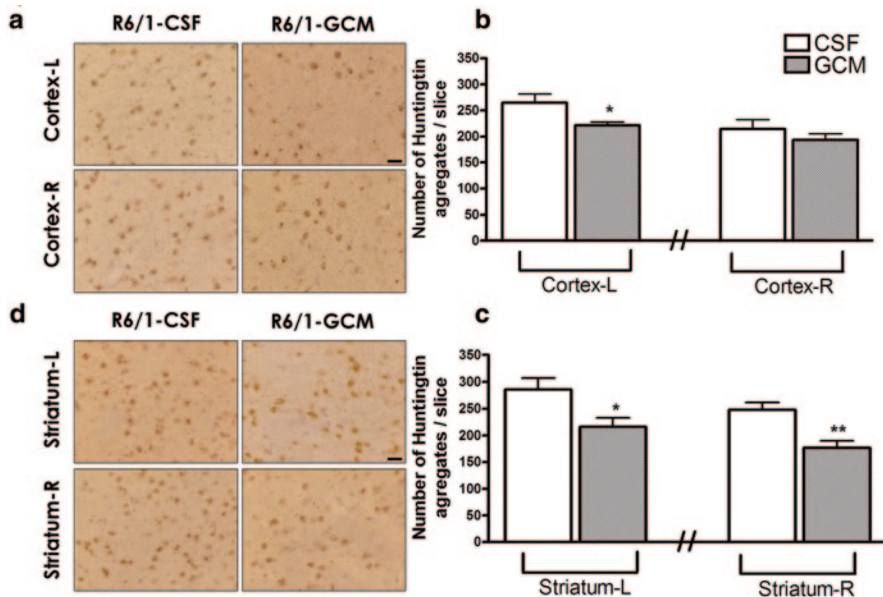

Fig. 5.4 GCM treatment (30 days) diminishes the number of huntingtin inclusions in cortex and striatum. **a** Microphotographs of the right and left cortex with the specific antibody anti-HTT inclusions and **b** quantification of both cortex, left and right. **c** Microphotographs of the right and left striatum with the specific antibody anti-HTT inclusions and **d** quantification of both striatum, left and right. The values are expressed as the mean ±SEM (n=6 mice in each experimental group). The statistical analysis was performed by student's t test. *=$p<0.05$, **=$p<0.01$ GCM vs control CSF groups [31]

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Fig. 5.5 GCM treatment for 15 days activates autophagy pathways. **a** Representative bands and western blot quantification of LC3 II/LC3 I ratio. The presence of LC3 in autophagosomes and the conversion of LC3 to the lower migrating form LC3-II are used as an indicator of autophagy. **b** Representative bands and quantification of Beclin-1 antibody, direct marker of the autophagosome formation. **c** Western blots representative bands and quantification of p-62 specific antibody, as a substrate of autophagy and **d** LAMP-2 quantification, corrected by b-actin. Values are from the hemibrains, expressed as mean ± SEM (n=6 in each group). Statistical analysis performed by t student. *p,0.05 GCM vs control CSF group and + p,0.05 right vs left hemispheres. **e** Representative

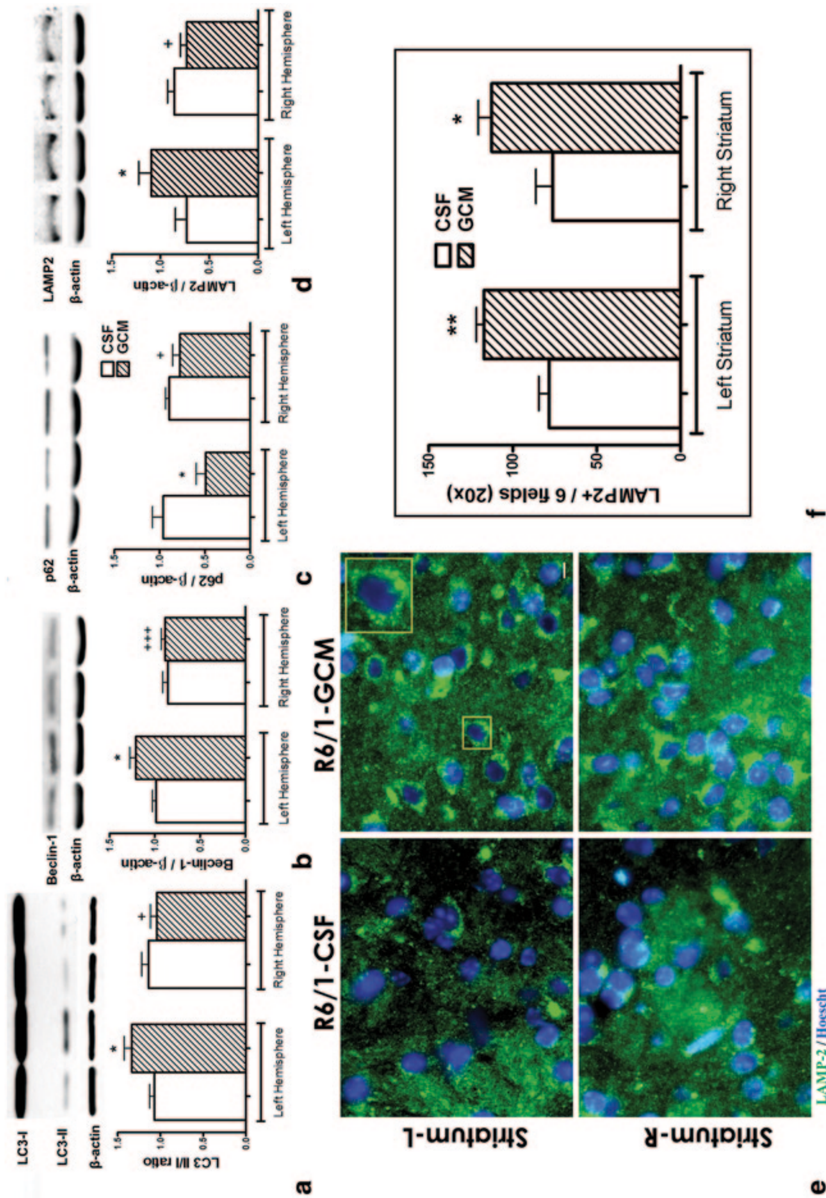

Fig. 5.5 (continued) microphotographs of Lysosomal-associated membrane protein, LAMP-2 A antibody, present in lysosomes and endosomes, which implies autophagy activation. LAMP-2A staining (green) and nuclei (Hoescht, blue) immunofluorescence in striatum of R6/1 mice, with CSF and GCM infusion. (406 magnification, scale bar = 10 mm). f Quantification of number of cells with LAMP-2A positive vesicle distribution in the perinuclear region, around the nucleus cell. Numbers of cells counted in 6 random fields of the striatum at 206 magnification. Mean ± SEM (n = 6 in each group). Statistical analysis performed by student's t test. *p,0.05, **p,0.01 GCM vs control CSF group; +p,0.05, +++p,0.001 right vs left hemispheres [31]

References

1. Bruyn GW, Went LN. Huntington's chorea. In: Vinken BG, Vinken PJ, Klawans HL, editors. Handbook of clinical neurology. Amsterdam: Elsevier; 1986. pp. 267–97.
2. Huntington G. On Chorea. Med Surg Report Wkly J. April 13, 1872;26(15):317–321. (Philadelphia: S.W. Butler).
3. Ruiz PJ, et al. Huntington's disease: a multidisciplinary study. Eur J Neurol. 1995;2(3):185–91.
4. Gusella JF, MacDonald ME. Hunting for Huntington's disease. Mol Genet Med J Neurol. 1993;3:139–58.
5. Gusella JF, et al. A polymorphic DNA marker genetically linked to Huntington's disease. Nature. 1983;306(5940):234–8.
6. Vonsattel JP, Keller C, Cortes Ramirez EP. Huntington's disease—neuropathology. Handb Clin Neurol. 2011;100:83–100.
7. Roze E, et al. Pathophysiology of Huntington's disease: an update. Rev Neurol (Paris). 2008;164(12):977–94.
8. Vonsattel JP, DiFiglia M. Huntington disease. J Neuropathol Exp Neurol. 1998;57(5):369–84.
9. Ross CA. Huntington's disease: new paths to pathogenesis. Cell. 2004;118(1):4–7.
10. Perutz M. Polar zippers: their role in human disease. Protein Sci. 1994;3(10):1629–37.
11. DiFiglia M, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science. 1997;277(5334):1990–3.
12. Gutekunst CA, et al. Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. J Neurosci. 1999;19(7):2522–34.
13. Li X, et al. Inhibiting the ubiquitin-proteasome system leads to preferential accumulation of toxic N-terminal mutant huntingtin fragments. Hum Mol Genet. 2010;19(12):2445–55.
14. Metzger S, et al. Age at onset in Huntington's disease is modified by the autophagy pathway: implication of the V471A polymorphism in Atg7. Hum Genet. 2010;128(4):453–9.
15. Metzger S, et al. The V471A polymorphism in autophagy-related gene ATG7 modifies age at onset specifically in Italian Huntington disease patients. PLoS One. 2013;8(7):e68951.
16. Fernandez-Estevez MA, et al. Trehalose reverses cell malfunction in fibroblasts from normal and Huntington's disease patients caused by proteosome inhibition. PLoS One. 2014;9(2):e90202.
17. Wu JC, et al. The regulation of N-terminal Huntingtin (Htt552) accumulation by Beclin1. Acta Pharmacol Sin. 2012;33(6):743–51.
18. Sarkar S, et al. Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat Chem Biol. 2007;3(6):331–8.
19. Tsvetkov AS, et al. A small-molecule scaffold induces autophagy in primary neurons and protects against toxicity in a Huntington disease model. Proc Natl Acad Sci U S A. 2010;107(39):16982–7.
20. Williams A, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol. 2008;4(5):295–305.
21. Mealer RG, et al. Rhes, a striatal-selective protein implicated in Huntington disease, binds beclin-1 and activates autophagy. J Biol Chem. 2014;289(6):3547–54.
22. Ravikumar B, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004;36(6):585–95.
23. Reddy PH, et al. Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat Genet. 1998;20(2):198–202.
24. Yu ZX, et al. Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. J Neurosci. 2003;23(6):2193–202.
25. Myers RH, et al. False-negative results with levodopa for early detection of Huntington's disease. N Engl J Med. 1982;307(9):561–2.
26. Myers RH, et al. Homozygote for Huntington disease. Am J Hum Genet. 1989;45(4):615–8.

27. Sapp E, et al. Early and progressive accumulation of reactive microglia in the Huntington disease brain. J Neuropathol Exp Neurol. 2001;60(2):161–72.
28. Bradford J, et al. Mutant huntingtin in glial cells exacerbates neurological symptoms of Huntington disease mice. J Biol Chem. 2010;285(14):10653–61.
29. Shin JY, et al. Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. J Cell Biol. 2005;171(6):1001–12.
30. Tydlacka S, et al. Differential activities of the ubiquitin-proteasome system in neurons versus glia may account for the preferential accumulation of misfolded proteins in neurons. J Neurosci. 2008;28(49):13285–95.
31. Perucho J, et al. Striatal infusion of glial conditioned medium diminishes huntingtin pathology in r6/1 mice. PLoS One. 2013;8(9):e73120.

Chapter 6
Control of Autophagy in Parkinson's Disease

Rosa A. González-Polo, Rubén Gómez-Sánchez, Elisa Pizarro-Estrella, Sokhna M. S. Yakhine-Diop, Mario Rodríguez-Arribas and José M. Fuentes

Abstract Parkinson's disease (PD) is a neurodegenerative disorder characterized by a dysregulation of cellular degradation system. An accumulation of misfolded proteins has been founded in the brains of parkinsonian patients, causing neuroinflammation and oxidative stress, and leading to a progressive neurodegeneration. Autophagy plays an important role in the progression of PD. In this chapter, we analyze the relationship of different types of autophagy (microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy) with the oxidative stress and with several proteins involved in PD, showing deregulation of these degradative processes when these proteins are mutated. Also, we show a possible therapeutic alternative based on autophagy inducers that might be a potential drug for PD treatment.

Keywords Autophagy · Neurotoxicity · Parkinson's disease · PARK genes · Pesticides

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Abbreviations

ARE	Antioxidant response element
Atgs	Autophagy-related genes
AV	Autophagic vacuole
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
$\Delta\psi m$	Mitochondrial membrane potential
ΔN-PINK1	Cleaved PINK1
CMA	Chaperone mediated autophagy
CNS	Central nervous system
COR	C-terminal of ROC
DA	Dopamine
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Fbxo7	F-box protein 7
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FL-PINK1	Full-length PINK1
GSH	glutathione
Hsc70	Heat-shock cognate 70
Hsp90	Heat shock proteins 90
JDP2	Jun dimerization protein 2
Keap1	Kelch-like ECH-associated protein 1
L-DOPA	L-3,4-dihydroxyphenylalanine
LAMP2A	Lysosomal associated membrane protein-2A
LB	Lewy body
LC3	Microtubule-associated protein 1 light chain 3
LRR	Leucine-rich repeat
LRRK2	Leucine rich-repeat-kinase 2
MAO-B	monoamine oxidase B
MAPK	Mitogen-activated protein kinase
MafK	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog K
Mfn	Mitofusin
MPP^{+}	1-methyl-7-phenylpyridine
MPTP	1-methyl–4-phenyl-1,2,3,6-tetrahidropyridine
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NRF-1	Nuclear respiratory factor 1
Nrf2	Nuclear factor (erythroid-derived)-like 2
6-OHDA	6-hidroxydopamine
PARL	Presenilin-associated rhomboid-like
PARIS	Parkin interacting substrate
PARL	Presenelin-associated rhomboid-like
PBR	Ring-in-between-ring
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor gamma
PI3K	Phosphoinositide-3-kinase
PINK1	Phosphatase and tensin homolog (PTEN)-induced kinase 1

PP2A	Protein phosphatase type 2A
PQ	Paraquat
RING	Really interesting new gene domain
ROC	Ras of complex proteins
ROS	Reactive oxygen species
SMAF	Small Maf proteins
SMERs	Small molecule enhancers of rapamycin
α-syn	α-synuclein
TFAM	Mitochondrial transcription factor A
ULK1	Unc-51-like kinase
UPS	Ubiquitin proteasome system
UCH-L1	Ubiquitin C-terminal hydrolase L1
VDAC	Voltage-dependent anion channel
WT	Wild type

Introduction

Parkinson's Disease

Definition and Epidemiology

Neurodegenerative diseases are chronic and progressive processes characterized by loss of neurons in different regions of the nervous system. The most prevalent neurodegenerative diseases are Alzheimer's disease (AD) and Parkinson's disease (PD), although there are other important diseases such as Huntington 's disease, ataxia or amyotrophic lateral sclerosis [1].

Therefore, as just indicated, PD is one of the most common neurodegenerative disorders in the world population, affecting 2 % of the population over 65 years with the diagnosis 5–24 cases per 100,000 population [2].

The first symptoms of the disease usually appear about 60 years, to a major in the course of 5 or 15 years with incapacitation [3]. Its incidence is increased with age, although in the past 40 years, the age of onset has been anticipating, being currently more common in younger people. The appearance of the disease in less than 20 years age is very low and is called juvenile PD, while the appearance between 20 and 40 years affects less than 5 % of all cases and is called early-onset PD**.**

Origin

The source of this disorder is a loss of, at least, 50 % of the neurons in the midbrain area known as *substantia nigra*. These neurons have a characteristic dark pigmentation due to the presence of neuromelanin. Under normal physiological conditions, these neurons are responsible of dopamine (DA) production, a messenger

sent by which inhibitory signals to the striatum, responsible for performing smooth, purposeful movement. In a PD patient, the death of neurons in the *substantia nigra* in the striatum causes a depletion of DA which produces akinesia [4, 5]. There are also other injuries, such as degeneration of dopaminergic ventral tegmental area [6]. This nonnigral lesion produces cognitive and psychological disorders, such as dementia, which mainly appears in 30% of cases of the disease, usually in the later case [7].

Symptoms and Treatments

Idiopathic PD is clinically defined by a serie of symptoms usually characterized by loss of movement coordination. They can be differentiated between motor and non-motor symptoms [8].

Motor symptoms that may present in an individual with PD are:

- Tremor: the most common and easily recognizable feature of PD. They are usually unilateral, with a frequency of 4.6 Hz (slow) and characteristics of supination-pronation are tremors.
- Micrograph: slow, small strokes with scripture.
- Bradykinesia: slowness of movement that hinder the performance of everyday actions.
- Rigidity: is characterized by an increased resistance and is often accompanied by pain.
- Akinesia: muscular paralysis.
- Postural inestability: it is due to the loss of postural reflexes and is usually a manifestation of the later stages of PD.
- Hypomimia: reduced facial expression (this is related to bradykinesia).
- Dysarthria: speech problem. Monotony, decreased volume (dysprosody), reduced stress and loose coupling.
- Dysphagia: difficulty eating, accompanied by hypersalivation (excessive production of saliva).
- Dystonia: loss of muscle tone.
- Difficult march, shuffling.
- Rotary movements in bed.
- Scoliosis deviation of the spine.
- Camptocormia: abnormal pronounced trunk flexion.

However, individuals affected by PD may also have a high number of non-motor symptoms such as:

- Cognitive impairment: loss of mental functions (memory, orientation, or language), and considered prior to dementia.
- Bradyphrenia: slowed thinking.
- Depression and apathy.
- Anhedonia: inability to experience pleasure.
- Fatigue, back and shoulder pain.

- Anosmia: loss or reduction of smell.
- Orthostatic hypotension: excessive reduction of blood pressure when standing.
- Constipation, urinary and sexual dysfunction.
- Excessive sweating and seborrhea.
- Weight loss.
- Sleep disorders.

Current treatments are able to reduce the symptoms caused in part by the PD, however, the disease has not any effective treatment able to cure it.

Pharmacologically, L-3,4-dihydroxyphenylalanine (L-DOPA), DA agonists and inhibitors of monoamino oxidase B (MAO-B) are used. The use of either treatment is considered in relation to the individual response of each patient to such treatment and the phase in which the patient is located.

Etiology

Over time, various studies have suggested that PD has a multifactorial etiology, in which both genetic and environmental factors are included [9]. In the late 19th century, Gowers reported on the PD *"… in any occasions the influence of the inheritance… could be important"*. This was the general opinion among researchers until 1980, when the discovery of the toxin 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) [10], which produces a selective death of neurons of the *substantia nigra* in human and experimental models [11], and induces symptoms similar to those seen in the PD engines. This discovery opened the door to a more thorough investigation of possible environmental factors that could be related to the disease. Today a large number of pesticides, herbicides and industrial chemicals involved in the development of PD are known. Among the most studied compounds found rotenone, a substance of plant origin used as an insecticide and acts as a potent inhibitor of complex I of the mitochondrial respiratory chain, 6-hydroxydopamine (6-OHDA), oxidative stress-inducing neurotoxin [12], or bipiridinium compounds such as the 1-methyl-4-phenyl-pyridine ion (MPP^+) or the herbicide paraquat (PQ), which involvement in neurodegenerative processes has been extensively studied [12–18]. Thus, the knowledge of the genetic factors involved in this disease is paramount when it comes to elucidate the possible causes and mechanisms that occur during the development of the disease.

Nowadays, thanks to epidemiological studies, it is known that most cases of individuals with PD are sporadic and only 5–10 % has a familiar pattern of inheritance, highlighting the importance of environmental factors in the origin of this disease. Therefore, it has been postulated that the cause of PD may be the interaction between heredity and environment, where the gene acts as a predisposing factor but does not determine the development of the disease. There are studies supporting this interaction, an example can be given in studies that show how individuals with mutations in *PARK9* gene have an increased susceptibility to excess manganese poisoning, and subsequently neuronal death, so if these individuals are found in environments with high concentrations of the metal, present a predisposition to PD [19].

Environmental Factors

Some years ago, environmental factors are suspected to be the main cause or at least the major in PD. This theory gained strength after the influenza pandemic of 1918, as a large number of individuals (around five million people) developed postencephalitic parkinsonism attributed to infectious agents in the environment [20, 21]. The discovery and identification of the MPTP toxin in early 1980, drug used intravenously for young people with symptoms compatible with PD, further strengthened environmental theory [10]. More thorough studies revealed that MPTP is capable of crossing the blood-brain barrier and is converted by MAO-B in a reactive molecule, the MPP^{+}ion, which accumulates in dopaminergic cells and produces cell degeneration as a result of mitochondrial complex I inhibition, increased oxidative stress levels and redistribution of DA from the vesicles into the cytosol [22, 23]. Chronic exposure to the toxin in rodents (excluding rats showing resistance), primates and humans leads to a parkinsonian syndrome, so that MPTP and $MPP^{+}$are widely used both in vivo and in vitro as tools of nigral degeneration [24]. In this regard there are numerous studies that identify a wide range of substances: metals (iron, copper, lithium, vanadium) [25–27], industrial chemicals (cypermethrin, polychlorinated biphenyls…) [28, 29] and exposure to pesticides (rotenone and paraquat) [14–16, 30, 31] that promote neuronal harm simulating the typical features of PD.

Genetic Factors

The existence of family history that has had PD is a risk factor for the development of PD [32, 33]. Thus, in 1997, an autosomal dominant mutation in the *PARK1* gene, was identified an inheritable form of PD in Italian and Greek families, which codifies for α-synuclein (α-syn) protein [34]. This fact, together with the discovery that the α-syn is the major component of Lewy bodies (LBs) [35], enables a greater interest in research on genetic aspects of PD. In subsequent years other genes involved in PD were found (Table 6.1). In 1998, *PARK2* gene encoding Parkin protein [36], which appears mutated in an inherited variant juvenile of PD was identified. Subsequent studies identified new key mutations in PD as in the case of a mutation in the DJ-1 protein in Dutch and Italian families [37–39], responsible for a variant autosomal recessive early-onset parkinsonism. Also it has been described a mutation in the *PARK6* gene encoding the protein phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), which could cause metabolic failure and neuronal death in the *substantia nigra* [40].

Also it has been investigating the role that leucine rich-repeat kinase 2 (LRRK2) protein may have on the development of PD. LRRK2 is encoded by the *PARK8* gene and in recent years has been postulated as one of the key proteins in the knowledge and understanding of PD.

Table 6.1 Identified Loci in familial Parkinsonism

Loci	Chromosome	Expressed protein	Hereditary	Year of discovery
PARK1	4q21-q23	α-synuclein	Dominant; sporadic	1996
PARK2	6q25.2-q27	Parkin	Recessive; sporadic	1997
PARK3	2p13	¿?	Dominant	1998
PARK4	4p15	α-synuclein (triplin)	Dominant; sporadic	1998
PARK5	4p14	UCHL1	Dominant	1998
PARK6	1p35-36	PINK1	Recessive	2001
PARK7	1p36.33-p36.12	DJ-1	Recessive	2001
PARK8	12p11.23-q13.11	LRRK2	Dominant	2002
PARK9	1p36	ATP13A2	Recessive	2001
PARK10	1p32	GIGYF2	Dominant	2002
PARK11	2q36-q37	HtrA2/Omi	¿?	2003
PARK12	Xq21-q25	¿?	¿?	2003
PARK13	2q12	¿?	¿?	2005
PARK14	22q13.1	PLA2G6	Recessive	2006
PARK15	22q12-q13	FBXO7	Recessive	2008
PARK16	1q32	RAB7L1?, NUCKS1?	¿?	2009
PARK17	16q11.2	VPS35	Dominant	2011
PARK18	3q27	EIF4G1	Dominant	2011

Autophagy

Definition

The cell is in a continuous process of replacement of organelles and proteins, as it is necessary to discard the material which has been synthesized but is no longer beneficial to the cell. Correct maintenance of the balance between synthesis and degradation of the cellular content is vital for its survival. The cell has two mechanisms of degradation, the ubiquitin-proteasome system (UPS) and autophagy [45, 46].

Autophagy is an intracellular catabolic mechanism, highly conserved through the evolution, whereby cell recycles or degrades damaged proteins or cytoplasmic organelles [47]. It was described by Christian de Duve in the 60 s, however, it was not until the 90 s when the genes involved in this process were identified in yeast. These genes were named thereafter as autophagy-related genes (Atg genes, Table 6.2), and its discovery was a breakthrough in understanding the autophagic mechanism [48, 49]. At present, the number of works related to autophagy grows exponentially, because such studies are revealing the importance of this mechanism in the development of many diseases, including neurodegenerative disorders.

There has been described a role for autophagy in the neonatal development [50], in other sense, there have also been related dysfunctions in autophagy with many diseases such as cancer [51], cardiomyopathies [52], problems with skeletal muscle [53] or adipose tissue [54]. Many studies have established a relationship between autophagy and neurodegeneration [15, 55, 56]. In this sense, an increase in autophagic vacuoles (AVs) was found in the *substantia nigra* of PD patients [57].

Paradoxically, although initially autophagy is a protecting process for the cell, for example, in the recycling of protein aggregates that might be toxic [58] also plays a key role in triggering cell death, the execution of a program of irreversible self-destruct [59].

There are also studies linking autophagy with aging processes. Thus, it has been demonstrated that a diet high in calories accelerates the aging process compared to a person with a low calorie diet without reaching malnutrition. Individuals with a low calorie diet have less impact on cancer, cardiovascular disease, diabetes and late mortality [60].

Autophagy is interrelated with apoptosis [61], this relationship is involved in the neurodegenerative process experienced by patients with PD.

Basic Machinery

During autophagy, elimination of certain proteins or cellular organelles takes place when they are damaged or are no longer needed for the cell. For this purpose, the content is enclosed in membranous structures, which have different structural stages and subsequently fuse with lysosomes to degrade their content through the action of lysosomal enzymes. The origin of these membranes can be varied, there are tomographic studies relating to ER (endoplasmic reticulum) with autophagosomes [62], although several studies point to the Golgi apparatus, the nucleus or mitochondria even as membrane sources.

Table 6.2 Atg genes

Yeast	Mammals	Functions
Atg1	ULK1,ULK2	Kinases that form the Atg1-Atg13-Atg17-Atg31-Atg29 complex (phagophore initiation and organization)
Atg2	Atg2	Atg9/Atg2-Atg18 complex (phagophore formation)
Atg3	Atg3	Enzime necessary for the Atg8 lipidation
Atg4	Atg4A, B, C, D	Cysteine protease: Atg8 activation and delipidation
Atg5	Atg5	Atg12-Atg5 complex, necessary for the Atg8 lipidation
Atg6	Beclin-1	Subunit of the Vps34-PI3K complex
Atg7	Atg7	Enzyme is conjugated to Atg12 and LC3
Atg8	LC3, GATE-16, GABARAP	Conjugation with PE in the autophagosome
Atg9	Atg9L1, L2	Interact with ATG2-Atg8 complex binding to the membrane
Atg10	Atg10	Conjugation with Atg12
Atg11	Atg11	Interaction with the phosphorylated Atg29. Important for phagophore elongation
Atg12	Atg12	Forms a complex with Atg5
Atg13	Atg13	From mTOR signaling: complex-Atg13-Atg17 ATG1-Atg29 (phagophore initiation and organization)
Atg14	Barkor	Vps34 subunit of PI3K complex, participates in the reticulophagy
Atg15	¿?	Lipase located in the RE and is required for fusion of vesicles derived from RE
Atg16	Atg16L	Forms a complex with Atg12-Atg5
Atg17	FIP200	Forms a complex with Atg1-Atg13-Atg17-Atg31-Atg29
Atg18	WIPI-1, 2, 3, 4	Forms a complex with Atg9/Atg2-Atg18
Atg29	¿?	Complex Atg17-Atg-31-Atg29 (phagophore initiation and organization)
Atg31	¿?	Complex Atg17-Atg-31-Atg29 (phagophore initiation and organization)

To get a better understanding of the autophagic process, we could differentiate autophagy in several phases:

1. Initiation: a start signal results in the implementation of autophagy. This initiation can occur by nutrient deprivation, lack of amino acids or fatty acids, or energy requirement [49, 63].
2. Nucleation: recruitment of Atg proteins necessary to the membrane, leading to the phagophore isolation.
3. Elongation: phagophore expansion occurs to engulf internalized material, resulting in a structure called autophagosome. The autophagosome is closed in a double-membrane structure.
4. Fusion: autophagosomes merge with endosomes and lysosomes to proceed to material elimination which has been captured by the autophagosome.
5. Maturation: the degradation and release of the material and gradient occurs.

This process is very complex and is highly regulated by many proteins.

Classification and Types

A classification of the different types of autophagy can be made, depending on the mechanism function to degrade the substrate into the lysosomal lumen [64]. We could distinguish three types of autophagy (Fig. 6.1):

1. Macroautophagy: often referred to as such term autophagy. In this process, the material to be degraded is sequestered into double-membrane vesicles called autophagosomes [65, 66]. Depending on the material to degrade within autophagosomes, it has been developed a classification of different types of macroautophagy:

   A. Mitophagy: kidnapping and selective degradation of mitochondria.
   B. Xenophagy: selective degradation of microbes (bacteria, fungi, parasites or virus).
   C. Ribophagy: kidnapping and selective degradation of ribosomes.
   D. Agrephagy: selective degradation of protein aggregates.
   E. Peroxiphagy: kidnapping and selective degradation of peroxisomes (this may also occur microautophagy).
   F. Crinophagy: direct fusion of secretory vesicles with lysosomes.
   G. Reticulophagy: kidnapping and selective degradation of ER.

2. Microautophagy: in this process, the material to be degraded is sequestered by lysosomes through invagination of its membrane. Once the cargo is introduced inside the lysosome, it is degraded by the action of lysosomal enzymes.
3. CMA: in this case, damaged material or misfolded proteins are translocated into the lumen through the lysosome membrane. This translocation is mediated by cytosolic chaperones, such as LAMP-2A (lysosomal associated membrane protein-2A), and is a very selective degradation.

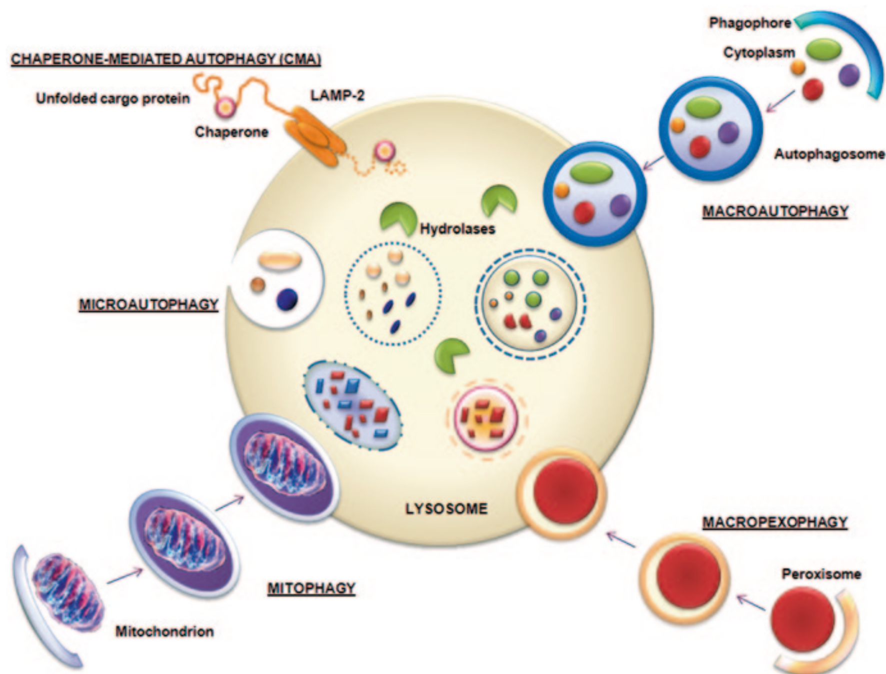

Fig. 6.1 Classification and types of autophagy

Regulation

Autophagy is a mechanism with a very complex regulation, and there are still many gaps in the understanding of this process. This is because there is an extensive network of routes with potential regulation of cellular recycling process.

mTOR (mammalian target of rapamycin) is one of the most studied inhibitors of autophagy. mTOR is a protein kinase that under favorable cellular conditions is active, suppressing autophagy by interacting with protein phosphatase type 2 (PP2A) [67]. The class I phosphoinositide 3-kinase (PI3K) pathway is also involved in the negative regulation of autophagy, by direct interaction with mTOR [68]. Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- kB), as class I PI3K, also exerts its negative regulation of mTOR activation [69]. Another molecule that downregulates autophagy is Bcl-2, which has the ability to inhibit the activation for Class III PI3K pathway (by interaction with Beclin-1) [70].

On the other hand, there are many other ways to positively regulate autophagy. The most known and studied is the path-dependent class III PI3K, Beclin-1, involved in the activation of the early stages of autophagosome formation [71]. It is also known that there is a stimulation of autophagy by way of extracellular signal-regulated kinases (ERK) [72] and others studies have also involved the production of reactive oxygen species (ROS) in the autophagy regulation [15, 16, 73, 74].

Dysfunction of Autophagy in Parkinson's Disease

The Importance of the Oxidative Stress in the Connection Between Parkinson's Disease and Autophagy: The Nrf2/keap1 Pathway

Free radicals are molecules with unpaired electrons. For this reason, they are highly reactive with others and, sometimes, the cell could be in a compromised situation. Among the most common free radicals are included: superoxide anion ($-O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($-OH^·$) or hydroxyperoxide ($-ROOH^-$). These molecules are consistently produced in cell metabolism, but their effect is normally neutralized by antioxidants (molecules which are able to receive electrons) [75]. This controlled production of ROS is very important, because it plays a critical role in immune reactions, growth factors stimulation, development of inflammatory response and even take part in the apoptosis process, regulating the cell population [76, 77]. The main problem occurs when the balance between the concentration of free radicals and antioxidants is broken and the cell status changes to "oxidative stress". In biochemical terms, oxidative stress is an increase in the potential cell reduction (becomes more negative) or a decrease in the redox pairs, reducing capacity of the cell [78–80]. This environment is too harmful to the cell and can cause irreparable damages in DNA [81], proteins and lipids [82], but also can disrupt the lysosomal membrane permeability, leading to the dysfunction of this organelle and hence of the processes in which it participates actively (CMA, microautophagy and macroautophagy) [83]. Interestingly, multiple studies suggest that in elderly people (80 years old), at least the 50% of the total protein has been subjected to an oxidation process [84] and the main sources of ROS production come from mitochondria and ER [85]. Therefore, it has been hypothesized that oxidative stress could generate permanent cell damage, which is a characteristic presented in all neurodegenerative diseases. Neurons and glial cells are much more sensitive to oxidative stress as compared to other body tissues [86], because these cells contain a larger amount of enzymes that generate free radicals, such as MAO-B and tyrosine hydroxylase, and dopaminergic neurons in the *substantia nigra* contain iron as well, which catalyzes the Fenton reaction (hydrogen peroxide catalysis in the presence of transition metals, generating superoxide radical) [87]. The difference among the neuronal axons metabolism and other cell lines is the requirement to adapt its structure to the functionality, but this fact makes it more susceptible to stress [88–90] degenerating without causing the soma death, which also could explain the chronicity in this pathology [91]. It seems that the principal sources of oxidative stress generated in the dopaminergic neurons derive from DA metabolism, mitochondrial dysfunction and neuroinflammation, as detailed below.

Dopamine Metabolism

Multiple evidences suggest that DA oxidation and its subsequent modification to quinone can be one of the main factors contributing to the vulnerability of dopaminergic cells against oxidative stress. Normally, DA is stored in vesicles, but

the neurotransmitter excess in the cytosol makes easily the spontaneous enzimatic oxidation to DA quinone. DA quinone species are able to modify cellular nucleophiles covalently, including low molecular weight groups as sulfhydryles or the cysteines residues present in some proteins such as gluthatione (GSH) and Kelch-like ECH-associated protein 1 (Keap1), which functions are important for cell survival. In particular, it has been shown that the DA quinone has the capacity to modify some proteins related to the pathophysiology of PD, among them the α-syn, Parkin, DJ-1, and ubiquitin C-terminal hydrolase L1 (ULC-H1), are found. The DA quinone modifies covalently the α-syn monomer and promotes their conversion to the cytotoxic form [92] which saturates the CMA [93]. Furthermore, an excess of α-syn can bind and permeabilize the membrane of the DA vesicles, causing a liberation of the neurotransmitter in the cytosol [94] and inducing the generation of further DA quinone. Parkin can also be modified by DA quinone, which transforms it into an insoluble protein with the consequent loss of E2 ubiquitin ligase activity, these findings have been detected in the *substantia nigra* of PD brains, but not in other human brain regions [95]. Other proteins that are modified by the DA quinone are UCH-L1 and DJ-1, because both proteins have a functional cysteine residue [96, 97] which makes them more sensitive to change in presence of DA quinone [98, 99]. DA quinone can inactivate tyrosine hydroxylase [100] and produce mitochondrial dysfunction [101, 102] with the consequent alteration of mitochondrial respiration, causing further ROS [103].

Mitochondrial Dysfunction

Mitochondrial dysfunction is another important source of ROS associated with the pathogenesis of PD. The functionality of neurons depends on the ATP produced by aerobic respiration, and some substances such as hydrogen peroxide and superoxide radicals are a waste product of oxidative phosphorylation that occurs in mitochondria to satisfy the energy demand, so any pathological situation leading to the dysfunction of this organelle can cause a dramatic increase in ROS and a saturation of cell antioxidant mechanisms. Dopaminergic neurons generate free radicals and they are inherently more vulnerable than others against ROS, in this sense, it was observed that intraperitoneal injection of MPTP and rotenone in mice, exerts a selective cytotoxicity in this cell group [104]. These results fit with findings in human PD brains, in which there is a large number of neurons with deficient mitochondrial complex I [105]. Mutations in genes related in mitochondrial proteins as Parkin, DJ-1 and PINK1 are linked to familial forms of PD, and their study could be a critical point to understand the pathology. Cells from patients with mutation in the Parkin gene show a decrease in mitochondrial complex I activity, and the same mice null for this gene show a reduced striatal chain with oxidative damaged [106]. PINK1 protein mutations induced mitochondrial dysfunction with excess free radical formation [107] and in the case of the DJ-1 protein, it has been observed that mice null for this gene, accumulate ROS and exhibit a fragmented mitochondrial phenotype [108].

Neuroinflammation

Neuroinflammation is mainly controlled by microglia and by innate immune cells in the central nervous system (CNS). Patients with sporadic or familial PD show in the *substantia nigra* a chronic inflammation caused by a microglial reaction [109], and these findings appear in animal models treated with MPTP as well [110]. Microglia activation is a defense mechanism against pathogens or cellular debris, and the process requires nitric oxide and superoxide release, which may contribute to an oxidative stress microenvironment. Therefore a chronic activation of this mechanism could cause an excessive and uncontrolled neuroinflammatory response, creating a vicious circle which ultimately ends with neurodegeneration [111].

Although the precise mechanism of the production of ROS in PD is still unknown, the presence of oxidative stress in PD is one of the constants of the pathology. For this reason, the theory that the neuronal degeneration may result from an excess of free radical concentration is becoming more and more important. In this sense, the study of antioxidants pathways as nuclear factor (erythroid-derived)-like 2 (Nrf2)/Keap1 axis as a preventive therapeutic target could be interesting in the coming years [112]. The transcription factor Nrf2 is one of the major cellular guards, being inducible its expression against oxidative stress, drug metabolism, inflammation and cytoprotection. Nrf2 has a complex spatio-temporal regulation in which different mechanisms are involved (inflammation, hipoxia, oxidative stress, xenobiotics signals, UPS and autophagy) [113]. Under normal conditions, Nrf2 cytosolic levels are low, because it is associated with its cytoplasmic inhibitor Keap1, which facilitates its degradation through the UPS, serving as an adapter complex for this degradative system. Conversely, under stress pressure or in the presence of compounds that target Nrf2 activation, this degradation is suppressed, allowing the translocation of Nrf2 transcription factor to the nucleus, where heterodimerizes with v-maf avian musculo-aponeurotic fibrosarcoma oncogene homolog K (MafK) protein. The Nrf2/MafK complex, aided by June dimerization protein 2 (JDP2) cofactor [114], interacts with antioxidant response element (ARE), from a promoter sequence in "*cis*" configuration [115] and begin to transcribe cytoprotective genes. These genes may encode enzymes, antioxidants, molecular chaperones, DNA repair enzymes, and proteins related to anti-inflammatory response. Anyway, they increase the cell's ability to repair damage. Apart of this, another thing that should be noted, is that Nrf2/Keap1 is closely related to DJ-1, so that the latter is capable to induce the synthesis of cytoprotective genes from this axis [116] enhancing the connection between PD and oxidative stress (Fig. 6.2).

Impairment of Mitophagy in Parkinson's Disease: Role of PINK1/Parkin

One of the key factor involved in aging, and especially in PD, is the mitochondrial dysfunction [117], and the maintenance of its homeostasis is essential. Mutations and deletions in mitochondrial DNA are common in PD patients [118] and induce impaired cellular respiration in aged neurons of the human substantia nigra

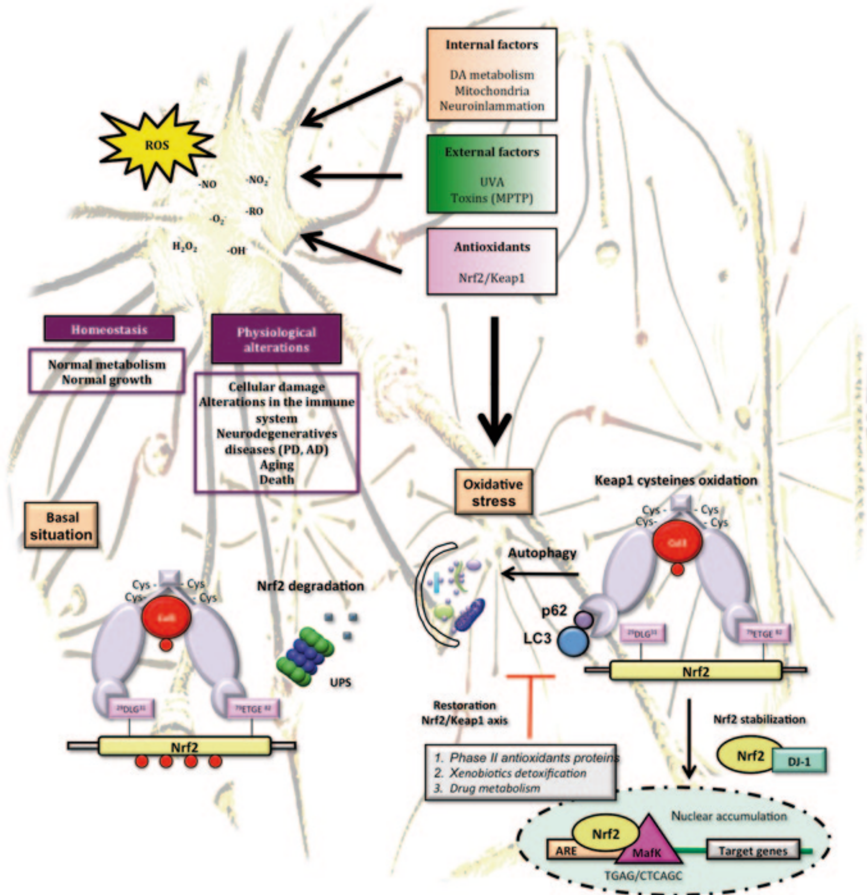

Fig. 6.2 Oxidative stress and its interaction with the Nrf2/Keap1 axis. Under basal conditions, Nrf2 is sequestered in the cytosol by Keap1 homodimer, facilitating its ubiquitination and proteasomal degradation. Conversely, when the cell encounters an insult (chemical or oxidative stress), a conformational change occurs in Keap1, following its cysteines oxidation. This causes the release of Nrf2, which will no longer be ubiquitinated and degraded, and after stabilization, translocates to the nucleus. Here heterodimerizes Nrf2 small Maf proteins (SMAF) and joins ARE, activating and promoting the expression of a battery of cytoprotective genes. When the cell reaches its basal state, this synthesis ceases and the route is restored

[119]. Some of PD-related genes encode proteins associated to this organelle, being PINK1 and Parkin the most relevant. Mutations of both genes are responsible for autosomal recessive forms of familial early-onset parkinsonism.

Briefly, PINK1 is a serine/threonine kinase and contains a N-terminal mitochondrial targeting sequence [120, 121]. Parkin is a RING-in-between-RING (RBR) E3 ubiquitin ligase [122], has recently been revealed [123, 124]. Several studies have shown that PD-related proteins PINK1 and Parkin work in a common pathway, and suggested that Parkin acts downstream of PINK1. In this sense,

parkin overexpression rescues the phenotype in *Drosophila melanogaster pink1*$^{-/-}$ (characterized by low mitochondrial mass, shortened lifespan, energy depletion, motor disturbances, muscle degeneration, reduction in the number of dopaminergic neurons) [125–127].

Different reports have shown that PINK1 regulates mitochondrial morphology, mitochondrial traffic and mitochondria-selective autophagy (mitophagy) [107, 128, 129], process [130] which we are going to describe deeply. These insights provide clear evidence that PINK1 develops a key role in the mitochondrial quality control, mainly through the interaction with Parkin.

In healthy mitochondria, full-length PINK1 (FL-PINK1) is imported to the inner mitochondrial membrane (Δψm-dependent step) and processed by the mitochondrial protease presenilin-associated rhomboid-like (PARL) [131–133]. The resulting cleaved PINK1, also called ΔN-PINK1, is subsequently degraded by the proteasome [134]. Some authors suggest that this mitochondrial cleavage is necessary for the PINK1 protective effect [131, 135]. Upon depolarization of the Δψm, induced in vitro by mitochondrial uncouplers, such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), cleavage of PINK1 is blocked and FL-PINK1 is accumulated on the mitochondrial outer membrane, recruiting Parkin to this organelle [128, 136–138]. Autophosphorylation of PINK1 is essential for this event [139]. Moreover, PINK1 phosphorylates mitofusin (Mfn) 2, acting as a mitochondrial receptor for Parkin [140] in depolarized mitochondria. Although, Parkin recruitment and mitophagy induction also occurs in absence of mitochondrial depolarization [128]. The loss of Δψm induces PINK1 calcium-dependent gene expression [141] and the mitochondrial localization of Parkin is PINK1 kinase activity-dependent [142]. Recently, studies have discovered that Parkin is phosphorylated at Ser65 [143, 144], being regulated by PINK1. Some mutations of *PINK1* associated to PD, such as G309D and L347P, and the synthetic kinase-dead *PINK1* triple mutant (K219A/D362A/D384A) abolish Parkin phosphorylation at serines in SH-SY5Y cells [145]. After translocation, Parkin ubiquitinates several mitochondrial substrates, such as voltage-dependent anion channel 1 (VDAC1) [146], Mfn1 and Mfn2 [147], as well as outer membrane proteins (Tom20, Tom40, Tom70) [148]. This event results in the fragmentation and isolation of damaged mitochondria, and is critical for the subsequent clearance by mitophagy. Curiously, Ambra1, protein involved in the formation of new phagophores, is recruited to mitochondria in a Parkin-dependent manner [149]. A recent study has identified that Fbxo7 (codified by *PARK15* gene) participates in mitochondrial recruitment of Parkin and ubiquitination of Mfn1, promoting mitophagy [150]. Once the VDAC1 is polyubiquitinated, it is recognized by the protein adaptor p62, allowing the recruitment of autophagosomes for the subsequent engulfment of mitochondria into these double-membrane vesicles.

A well-regulated balance between mitophagy and mitochondrial biogenesis is vital to achieve mitochondrial homeostasis. The disruption of these coordinated processes, marked by an accumulation of impaired mitochondria and a deregulated mitochondrial biogenesis, may contribute to PD pathogenesis [151, 152]. In this sense, PINK1 and Parkin mutations fail in mitochondrial elimination [128],

meanwhile higher levels of mitophagy produce neuroprotection [72]. However, in absence of mitochondrial biogenesis, excessive mitophagy could compromise the mitochondrial function, and thereby the cell survival [153–155].

As we described previously, PINK1 and Parkin are involved in mitocondrial remodeling. Several reports have shown that PINK1-Parkin pathway controls mitochondrial morphology. Thus, Parkin ubiquinitinates Mfn1 and Mfn2 [147, 156], blocking the mitochondrial fusion. Also, this couple of proteins regulates the mitochondrial traffic, because PINK1 phosphorylates Miro1 (a component of the motor complex responsible of the anterograde mitochondrial movement) for further Parkin-dependent ubiquitination and degradation by the proteasome [157], allowing the mitochondrial liberation from the microtubules. These events reduce the mitochondrial size and limit the mitochondrial motility, respectively, quarantining impaired mitochondria before their clearance via mitophagy (Fig. 6.3). Moreover, Parkin promotes mitochondrial biogenesis through the interaction with transcription factor A mitochondrial (TFAM) [158] and the ubiquitination of Parkin interacting substrate (PARIS), which represses the expression of the peroxisome proliferator-activated receptor gamma 1-α (PGC-1α) and its target gene, nuclear respiratory factor (NRF-1) [159]. Parkin also ubiquitinates Bax, playing a role in the apoptotic cell death regulation [160].

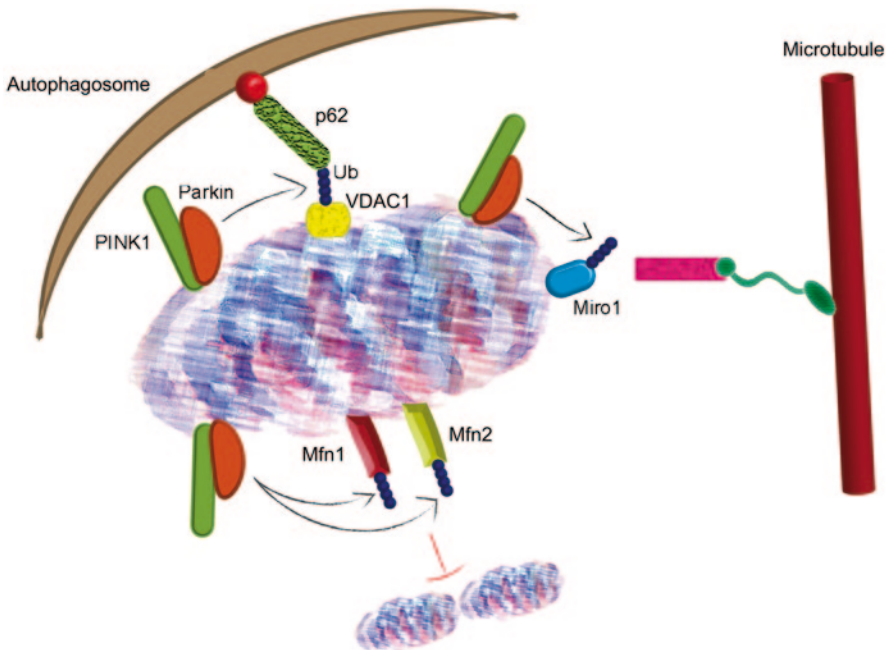

Fig. 6.3 Relationship between PINK1-Parkin and mitochondria**.** PINK1-Parkin are involved in different mitochondrial processes, such as mitophagy (through VDAC1 ubiquitination), and blockade of mitochondrial fusion (through Mfn1 and Mfn2 ubiquitination) and motility (through Miro1 ubiquitination)

Implication of Macroautophagy in Parkinson's Disease: Importance of LRRK2 and α-synuclein

LRRK2 is a serine/threonine kinase of 285 KDa, which belongs to the ROCO protein family [161]. The human ROCO proteins are a family of four proteins characterized by a conserved supradomain: a Ras-like GTPase domain. This domain consists of ROC (Ras of complex proteins) occurring in tandem with a COR (C-terminal of ROC) domain [162, 163].

LRRK2 plays several functions poorly understood, and is involved in a number of cellular process and signaling pathways [162]. LRRK2 autophosphorylates its own ROC and kinase domains on six residues (1 serine and 5 threonines); such autophosphorylation regulates the kinase/GTPase activity of LRRK2 [164, 165]. With its LRR domain, LRRK2 can form homodimer and is involved in protein-protein interactions [166], so interacts with heat shock proteins 90 (Hsp90), tubulin [167] and participates in the cytoskeleton organization [168].

LRRK2 domains are subjected to different mutations that alter the function and enzymatic activities of the protein. Mutations in GTPase (R1441G/C) and kinase (G2019S, I2020T) domains have been associated with the onset of PD, therefore are highly studied [162].

Many studies report that missense mutations in *LRRK2* are the most common cause of familial PD and have been identified in some sporadic cases [164, 166, 169]. Patients carrying *LRRK2* mutations display identical clinical symptoms to those sporadic cases [162, 170].

Nevertheless, all patients harboring *LRRK2* mutation do not display the symptoms in the same way, age at onset varies between and within family with mutation and may also vary following the mutated domain of the protein, such as the G2019S mutation that have been identified to manifest phenotype disease earlier than R1441C [170].

The mechanism whereby LRRK2 participates in PD pathogenesis remains unclear. Little is known about its regulation, its own substrates and of course which mutation throughout this protein really provokes PD [171].

About this, many studies stipulate that maybe it is not *LRRK2* mutations but the influence of others genetic and/or environment factors on the protein that trigger the pathogenesis of PD [170]. LRRK2 G2019S possesses a kinase activity at least 2–3 fold higher than wild type (WT) LRRK2 [165, 167, 172]. Moreover, it phosphorylates number of substrates and is involved in several signaling pathways that are connected directly or inderectly, for example vesicle trafficking [173], MAPK1/3 pathway [43], autophagy [172], etc.

In primary neuronal cells, LRRK2 regulates the distribution and endocytosis of synaptic vesicles. Experiments carried out on transfected SH-SY5Y and PC12 cells, showed an increasing extracellular DA level compared to control or others mutations of the protein; this releasing level is reduced by the inhibitor of LRRK2 GSK2578215A [173].

The role of LRRK2 in cellular regulation is very elusive, among others, cellular degradation mechanism; dysregulation of kinase activity affect the regulation of autophagy/lysosomal pathway [168, 174, 175]. LRRK2 phosphorylates ULK1 (unc-51-like kinase) [165], one of the proteins of autophagy initiation that participates in autophagosome formation together with the class III PI3K.

Autophagy is a catabolic process to degrade aggregated cytoplasmic proteins and damaged organelles, its dysfunction have been implicated in most of neurodegenerative diseases, particularly in PD [176].

Bravo-San Pedro et al. have observed in fibroblasts of parkinsonism patients a dysregulation of basal autophagy. Primary fibroblast G2019S have been identified to possess an increasing number of autophagic vacuoles compared to fibroblast control [172]. Consistent with it, G2019S LRRK2 in SH-SY5Y neuroblastoma cells displayed a high number of autophagic structures differently to cells transfected with vector or dead kinase K1906M-LRRK2 [174]

Hence, mutant G2019S fibroblasts exhibit an exacerbated autophagy respect to cells without mutation. The silencing of Atg5 gene reduce the level of autophagy and the cellular death. Indeed, in these cellular model, the MAPK pathway have been found overactivated; inhibition of this pathway by U0126 decreased basal autophagy level and was rised in cells control [172]. Fascinatingly, *LRRK2* knockdown shows an increased autophagy level death in both fibroblast control and mutant; suggesting the importance of LRRK2 in regulation of autophagy and also the playing role of G2019S mutation.

Moreover, Plowey et al. have shown by electron microscopy the number of AVs at differents stages of maturation. In G2019S LRRK2-transfected SH-SY5Y cells, autophagy induces neuritic pathology. In fact, shortened neuritic process occurs with G2019S LRRK2. Therefore, the inhibtion of autophagy by knocking-down LC3 or Atg7 genes reduce G2019S LRRK2-induced shortened neuritic [174].

Interestingly, treatment with rapamycin, an autophagy inducer, makes easier the neuritic shortening in G2019S neuroblastoma cells by increasing AVs, but not in cells control. However, co-treatment with U0126, a specific inhibitor of MAPK pathway, reduces autophagy observed and neurites shortening.

These findings suggest that there is a link between LRRK2 and MAPK pathway. So, LRRK2 kinase activity is responsible for the autophagy exacerbated in human G2019S fibroblast and shortened neuritic progress in G2019S-transfected neuroblastoma cells via MAPK route and the latter is also a regulator of autophagy (Fig. 6.4).

However, other studies reported that they did not observe any difference of autophagy basal between G2019S fibroblast and control; they suggest that the ratio LC3-II/LC3-I was more feeble in mutant than in control under starvation condition. Concluding, these mutations in LRRK2, including G2019S, perturb starvation induced-autophagy [177], but do not affect autophagy under normal condition.

PD is characterised by progressive degeneration of dopaminergic neurons and by the presence of intracellular proteins inclusions called LBs in surviving neurons [178]. α-syn is a major component of LB. *α-syn* is one of the *PARK* genes named *PARK1/4*, that is implicated in autosomal dominant form of PD. Triplication, duplication and mutations (A30P, A53T, E46K) of α-syn gene provoke PD progression due to its accumulation in neuronal cells [179].

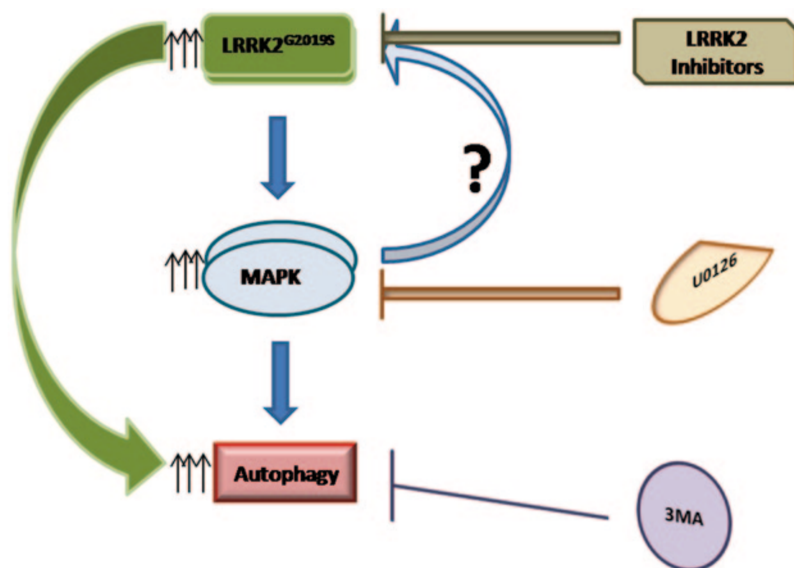

Fig. 6.4 Regulation of macroautophagy through MAPK pathway by G2019S LRRK2

Deletion of *Atg7* gene in mice promotes loss of dopaminergic neurons. The disruption of autophay is related to an accumulation of α-syn in axons terminal and presynaptics neurons leading to a dystrophic axons. α-syn inclusion was followed by an increasing level of LRRK2 due to its mRNA expression level. Consistent with, the enhanced level of LRRK2 proteins found in LB inclusions of postmortem PD brains [178].

It has been shown in transgenic mice expressing human G2019S LRRK2 the formation of autophagic vacuoles including early and late autophagosomes in neuronal soma, axons and synapses [180].

The acumulation of LRRK2 in autophagy impairment is observed in certain brains region and cell types. Overall, LRRK2 and α-syn has been implicated in pathogenesis of PD, as well as, associated with autophagy regulation [178].

As previously described, kinase activity of LRRK2 induces shortened neuritic [174, 180] further the biological effect of α-syn inclusions, both conduce to inflammation and neuronal degeneration (Fig. 6.5).

Involvement of Chaperone Mediated Autophagy in Parkinson's Disease: Implication of LRRK2 and α-synuclein

α-syn is a native unfolded protein localized in presinaptyc terminals in neurons and it is involved in neurotransmitter release [181]. It can also be found in different aberrant conformations such as oligomers, protofibrils amyloid fibrils, and receives different post-translational modifications [93]. Accumulation of this protein or defect in this

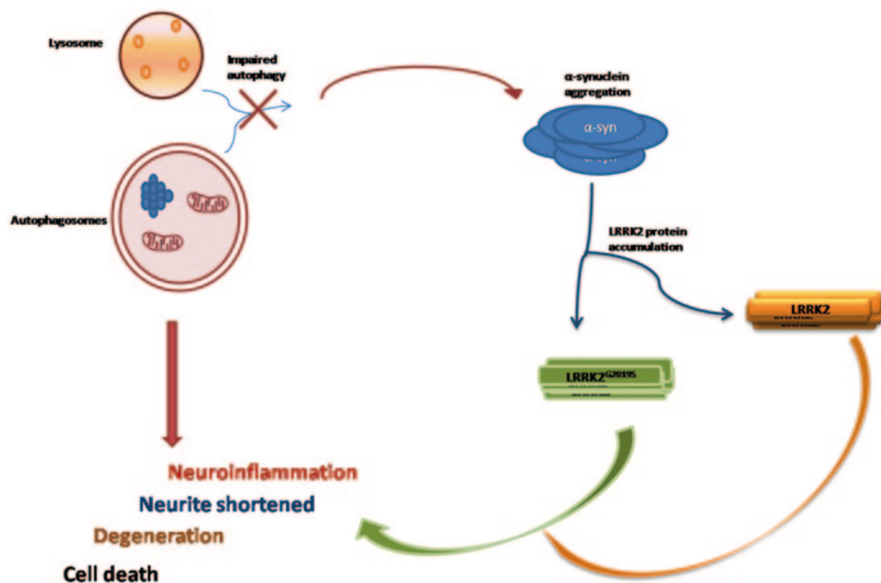

Fig. 6.5 Link between LRRK2 and α-syn in impaired autophagy

cleareance may contribute to PD [182]. In this sense, this protein is mainly degraded by UPS with a regulatory role of deubiquitinase USP9X [183], but, in cases of protein burden increased the autophagic pathway will be recruited [184].

Although its function remains undefined, as we have mentioned previously, several studies have described a regulation role between LRRK2 and the macroautophagic pathway [43, 184, 185].

Both *α-syn* and *LRRK2* genes may have autosomal dominant mutations (A53T and A30P in α-syn, and G2019S and R1441G in LRRK2) related to familial PD with clinical characteristics similar to sporadic PD. In addition, there is strong evidence of an interplay between these two proteins and a regulatory role of α-syn clearance mediated by LRRK2 [186]. In the last years, several studies have focused on the role of CMA in PD and α-syn clearance. Here we will point on what is known about these two proteins in this particular pathway.

First of all, both proteins have recognitions motifs to be degraded via CMA, eight putative motifs in LRRK2 and a single one in α-syn [187]. It has also been demonstrated that both proteins are degraded by this pathway realizing *in vitro* approaches [188, 189], and in vivo experiments in the case of α-syn [190].

Nevertheless, mutations or post-translational modifications may result in a blockade of clearance of both proteins by CMA pathway. In this sense, WT α-syn is selectively translocated into lysosomes via CMA [93, 188, 191], meanwhile A53T and A30P mutants appear to act as blockers of this pathway by inhibiting both another substrates and their own degradation [188]. Several post-translational modifications of this protein may result in a diminution of this degradation, or almost its inhibition in case of phosphorylation or exposure to DA [93]. Besides, G2019S *LRRK2* mutation is poorly degraded by CMA [189] (Fig. 6.6).

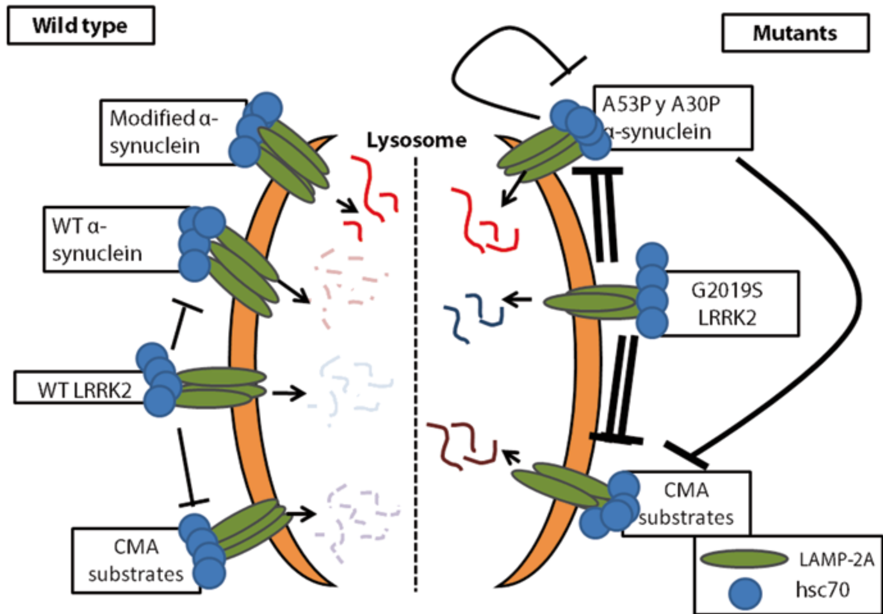

Fig. 6.6 Degradation of α-syn and LRRK2 in CAM. α-syn and LRRK2 can be degraded via CMA. In WT cells, LRRK2 inhibits degradation of other CMA substrates, including α-syn. Modified α-syn is poorly degraded by this pathway. In mutant cells, A53P and A30 P α-syn inhibit both other CMA substrates and their own degradation. Mutant G2019S LRRK2 is poorly degraded by this pathway, and it has stronger inhibitory effects on this pathway than observed in WT LRRK2

It has been described that only monomers or dimmers of α-syn are degraded via CMA, but not oligomers [93]. However, a work realized by the Stefanis group showed that CMA inhibition leads to an accumulation of soluble high molecular weight and detergent-insoluble species of α-syn [191]. These findings were reinforced by another study in which there were lower levels of LAMP-2A and heat-shock cognate 70 (hsc70) in PD patients' brains, thus finding a direct correlation between CMA diminution and illness [192].

In the case of LRKK2, little is known about its role in CMA in comparison of what is known about macroautophagy. Nonetheless, it has already been shown that LRRK2 can be specifically degraded via CMA. Additionally, this protein requires other CMA substrates for binding to LAMP-2A, and this union (both WT and G2019S) results in an inhibitory effect of this mechanism by blockade of translocation complex [189].

All these findings highlight one common feature: autosomal dominant mutations in both *LRRK2* and *α-syn* proteins results in an increase in the affinity of LAMP-2A with no formation of translocation complex, thus making an inhibitory effect of CMA pathway by blockade of LAMP-2 A receptor [188, 189]. In one hand, inhibition mediated by LRRK2, which is enhanced in mutants and, on the other hand, self-inhibition of α-syn mutants, contribute to decrease degradation of cytosolic substrates via CMA. As this feature is related to the formation of α-syn aberrant

species, CMA blockade may result in LB accumulation and PD establishment, although being a lesser via of degradation of this protein. In spite of these findings, functional interaction between CMA and PD needs to be further addressed for a better understanding of the role of this mechanism in the disease, especially in an in vivo approach [193].

Furthermore, it has been reported that CMA dysfunction previously described results in a macroautophagy buildup which lead to neuronal death when overexpressing WT α-syn, suggesting that this mechanism would be a possible therapeutic target [194]. The same group, in 2013, carried out an overexpression of LAMP-2A in her cell lines and they found a decrease in α-syn aggregation in their cell lines, highlighting the possibility of modulate this mechanism as a novel therapeutic target [195].

In conclusion, little are known about CMA function in α-syn clearance and the role of LRRK2 modulating this via. Nonetheless, first approaches strongly suggest a role of this pathway avoiding formation of aberrant species of α-syn that may contribute to LB appearance and PD development. In this sense, LRRK2 appears to have a regulatory role in the degradation of cytosolic substrates by CMA pathway. Mutations in α-syn and LRRK2 inhibit this mechanism, thus avoiding the proper degradation of CMA substrates, including both α-syn and LRRK2. A better comprehension of this mechanism in PD will reveal the relevance of this mechanism and possibilities of enhancing this mechanism as a therapeutical target.

Autophagy as an Adaptative Response in Parkinson's Disease Neuroprotector Role

The three types of autophagy coexist in neurons to maintain intracellular homeostasis and also serve to support the development and neuronal plasticity. Disturbances in these pathways could be responsible for the accumulation of misfolded proteins in the PD. Some studies have shown that autophagy activation allows the degradation of α-syn in PD, through CMA [196], whereas the mutant forms of this protein block this degradation pathway. Furthermore, it has been observed that in studies on post-mortem brains from parkinsonian patients, there is a decrease in the activity of the CMA [188] and accumulation of damaged mitochondria and lysosomes [197]. Furthermore, it was found that activation of autophagy decreases α-syn deposition [198]. Another protein associated with PD, which seems essential in autophagic cell response, is DJ-1. It has been shown that silencing of this protein induced autophagy decreases upon exposure to the herbicide PQ [199]. Furthermore, LRRK2 is involved in the control of autophagic process and the presence of pathogenic mutations in LRRK2 leads to the accumulation of autophagic structures [174] and deregulation of this degradative process [43, 172]. Autophagy inhibition promotes upregulation of antioxidant response genes, suggesting the activation of the transcription factor Nrf2. In addition, these expression changes are due to increased p62 [200, 201].

Therefore, modulation of autophagy is very useful as a therapeutic strategy. In fact, in some cases activation of autophagy is an adaptive compensatory response to increased protein load that in the instance has a neuroprotective effect.

Pharmacological activation of autophagy reduces the levels of soluble and aggregated forms of mutant forms of α-syn; in this sense, it has been described that curcumin, a natural compound derived from the curry spice turmeric and with low toxicity in normal cells, could efficiently reduce the accumulation of A53T α-syn through downregulation of the mTOR signaling and recovery of macroautophagy which was suppressed. These findings suggested that the regulation of mTOR/p70S6K signaling may be a participant of the accumulation of A53T α-syn protein-linked parkinsonism. Meanwhile curcumin could be a candidate neuroprotective agent by inducing macroautophagy [202].

The role of autophagy in protection against neurodegenerative diseases is established in animal models but not yet in patients. Nonetheless, preclinical animal data provide a strong rationale for proceeding with clinical trials with autophagy stimulatory agents; this is especially true as agents shown to be beneficial in reducing neurotoxicity of mutant aggregate prone proteins are already in clinical use to treat other diseases.

Rapamycin analogs, which are approved for the use of preventing organ transplant rejection and postangioplasty coronary artery restenosis and are in phase II oncology trials, protect against neurodegeneration [203]. However, because mTOR also affects protein synthesis, cell proliferation, cell growth, cell death, and immune function, its inhibition has some adverse effects; perhaps intermittent rather than continuous stimulation of autophagy with rapamycin may reduce such side effects while maintaining therapeutic efficacy.

Another group of new agents, small molecule enhancers of rapamycin (SMERs), enhance the clearance α-syn and protect against neurodegeneration [198]. Of long-term interest, both for the treatment of neurodegenerative diseases and, potentially, the prevention of aging, would be drugs that can reverse age-dependent declines that occur in central nervous system autophagy protein expression and lysosomal clearance of autophagosomes.

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References

1. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. Lancet Neurol. 2006;5(6):525–35.
2. Rajput AH. Frequency and cause of Parkinson's disease. Can J Neurol Sci. 1992;19(1 Suppl):103–7.
3. Hughes AJ, et al. A clinicopathologic study of 100 cases of Parkinson's disease. Arch Neurol. 1993;50(2):140–8.

4. Lee CS, et al. Clinical observations on the rate of progression of idiopathic parkinsonism. Brain. 1994;117(Pt 3):501–7.
5. Ehringer H, Hornykiewicz O. Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. Parkinsonism Relat Disord. 1998;4(2):53–7.
6. Agid Y, et al., The efficacy of levodopa treatment declines in the course of Parkinson's disease: do nondopaminergic lesions play a role? Adv Neurol. 1990;53:83–100.
7. Aarsland D, et al. Frequency of dementia in Parkinson disease. Arch Neurol. 1996;53(6):538–42.
8. Jankovic J. Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry. 2008;79(4):368–76.
9. Benmoyal-Segal L, Soreq H. Gene-environment interactions in sporadic Parkinson's disease. J Neurochem. 2006;97(6):1740–55.
10. Langston JW, Ballard PA Jr. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983;309(5):310.
11. Gerlach M, Riederer P. Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man. J Neural Transm. 1996;103(8–9):987–1041.
12. Bove J, et al. Toxin-induced models of Parkinson's disease. NeuroRx. 2005;2(3):484–94.
13. Gonzalez-Polo RA, Soler G, Fuentes JM. MPP+: mechanism for its toxicity in cerebellar granule cells. Mol Neurobiol. 2004;30(3):253–64.
14. Gonzalez-Polo RA, et al. Paraquat-induced apoptotic cell death in cerebellar granule cells. Brain Res. 2004;1011(2):170–6.
15. Gonzalez-Polo RA, et al. Relationship between autophagy and apoptotic cell death in human neuroblastoma cells treated with paraquat: could autophagy be a "brake" in paraquat-induced apoptotic death? Autophagy. 2007;3(4):366–7.
16. Gonzalez-Polo RA, et al. Inhibition of paraquat-induced autophagy accelerates the apoptotic cell death in neuroblastoma SH-SY5Y cells. Toxicol Sci. 2007;97(2):448–58.
17. Gonzalez-Polo RA, et al. Protection against MPP+ neurotoxicity in cerebellar granule cells by antioxidants. Cell Biol Int. 2004;28(5):373–80.
18. Niso-Santano M, et al. Low concentrations of paraquat induces early activation of extracellular signal-regulated kinase 1/2, protein kinase B, and c-Jun N-terminal kinase 1/2 pathways: role of c-Jun N-terminal kinase in paraquat-induced cell death. Toxicol Sci. 2006;92(2):507–15.
19. Gitler AD, et al. Alpha-synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity. Nat Genet. 2009;41(3):308–15.
20. Poskanzer DC, Schwab RS. Cohort analysis of Parkinson's syndrome: evidence for a single etiology related to subclinical infection about 1920. J Chronic Dis. 1963;16:961–73.
21. Schapira AH, Jenner P. Etiology and pathogenesis of Parkinson's disease. Mov Disord. 2011;26(6):1049–55.
22. Langston JW, et al. 1-Methyl-4-phenylpyridinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neurosci Lett. 1984;48(1):87–92.
23. Lotharius J, O'Malley KL. Role of mitochondrial dysfunction and dopamine-dependent oxidative stress in amphetamine-induced toxicity. Ann Neurol. 2001;49(1):79–89.
24. Tetrud JW, Langston JW. MPTP-induced parkinsonism as a model for Parkinson's disease. Acta Neurol Scand Suppl. 1989;126:35–40.
25. Farhoudi M, et al. Serum iron and ferritin level in idiopathic Parkinson. Pak J Biol Sci. 2012;15(22):1094–7.
26. Jellinger KA. The relevance of metals in the pathophysiology of neurodegeneration, pathological considerations. Int Rev Neurobiol. 2013;110:1–47.
27. Ngwa HA, et al. Vanadium exposure induces olfactory dysfunction in an animal model of metal neurotoxicity. Neurotoxicology. 2013;43:73–81.
28. Winneke G. Developmental aspects of environmental neurotoxicology: lessons from lead and polychlorinated biphenyls. J Neurol Sci. 2011;308(1–2):9–15.
29. Singh AK, et al. A current review of cypermethrin-induced neurotoxicity and nigrostriatal dopaminergic neurodegeneration. Curr Neuropharmacol. 2012;10(1):64–71.

30. Niso-Santano M, et al. Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis. Free Radic Biol Med. 2010;48(10):1370–81.
31. Tamilselvam K, et al. Neuroprotective effects of hesperidin, a plant flavanone, on rotenone-induced oxidative stress and apoptosis in a cellular model for Parkinsons' disease. Oxid Med Cell Longev. 2013;2013:102741.
32. De Michele G, et al. Etiology of Parkinson's disease. The role of environment and heredity. Adv Neurol. 1996;69:19–24.
33. De Michele G, et al. Environmental and genetic risk factors in Parkinson's disease: a case-control study in southern Italy. Mov Disord. 1996;11(1):17–23.
34. Polymeropoulos MH, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997;276(5321):2045–7.
35. Spillantini MG, et al. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A. 1998;95(11):6469–73.
36. Kitada T, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;392(6676):605–8.
37. van Duijn CM, et al. Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. Am J Hum Genet. 2001;69(3):629–34.
38. Bonifati V, et al. DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurol Sci. 2003;24(3):159–60.
39. Bonifati V, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. 2003;299(5604):256–9.
40. Morais VA, et al. Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. EMBO Mol Med. 2009;1(2):99–111.
41. Chan SL, Angeles DC, Tan EK. Targeting leucine-rich repeat kinase 2 in Parkinson's disease. Expert Opin Ther Targets. 2013;17(12):1471–82.
42. Reinhardt P, et al. Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. Cell Stem Cell. 2013;12(3):354–67.
43. Bravo-San Pedro JM, et al. Possible involvement of the relationship of LRRK2 and autophagy in Parkinson's disease. Biochem Soc Trans. 2012;40(5):1129–33.
44. Tsika E, Moore DJ. Mechanisms of LRRK2-mediated neurodegeneration. Curr Neurol Neurosci Rep. 2012;12(3):251–60.
45. Leroy E, et al. The ubiquitin pathway in Parkinson's disease. Nature. 1998;395(6701):451–2.
46. Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol. 2005;6(1):79–87.
47. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004;6(4):463–77.
48. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. Nat Rev Mol Cell Biol. 2001;2(3):211–6.
49. Suzuki K, Ohsumi Y. Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. FEBS Lett. 2007;581(11):2156–61.
50. Komatsu M, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880–4.
51. Kondo Y, Kondo S. Autophagy and cancer therapy. Autophagy. 2006;2(2):85–90.
52. Nakai A, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med. 2007;13(5):619–24.
53. Masiero E, et al. Autophagy is required to maintain muscle mass. Cell Metab. 2009;10(6):507–15.
54. Singh R, et al. Autophagy regulates adipose mass and differentiation in mice. J Clin Invest. 2009;119(11):3329–39.
55. Ravikumar B, Rubinsztein DC. Can autophagy protect against neurodegeneration caused by aggregate-prone proteins? Neuroreport. 2004;15(16):2443–5.

56. Martinez-Vicente M, Cuervo AM. Autophagy and neurodegeneration: when the cleaning crew goes on strike. Lancet Neurol. 2007;6(4):352–61.
57. Anglade P, et al. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol Histopathol. 1997;12(1):25–31.
58. Baehrecke EH. Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol. 2005;6(6): 505–10.
59. Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ. 2001;8(6):569–81.
60. Colman RJ, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009;325(5937):201–4.
61. Bursch W, et al. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann N Y Acad Sci. 2000;926:1–12.
62. Yla-Anttila P, et al. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy. 2009;5(8):1180–5.
63. Mizushima N, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol. 2001;152(4):657–68.
64. Klionsky DJ, et al. How shall I eat thee? Autophagy. 2007;3(5):413–6.
65. Baba M, et al. Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. J Cell Biol. 1994;124(6):903–13.
66. Fengsrud M, et al. Ultrastructural and immunocytochemical characterization of autophagic vacuoles in isolated hepatocytes: effects of vinblastine and asparagine on vacuole distributions. Exp Cell Res. 1995;221(2):504–19.
67. Reggiori F, Klionsky DJ. Autophagy in the eukaryotic cell. Eukaryot Cell. 2002;1(1):11–21.
68. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol. 2004;36(12):2445–62.
69. Djavaheri-Mergny M, et al. NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy. J Biol Chem. 2006;281(41):30373–82.
70. Pattingre S, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122(6):927–39.
71. Petiot A, et al. Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J Biol Chem. 2000;275(2):992–8.
72. Dagda RK, et al. Mitochondrially localized ERK2 regulates mitophagy and autophagic cell stress: implications for Parkinson's disease. Autophagy. 2008;4(6):770–82.
73. Kulich SM, Chu CT. Role of reactive oxygen species in extracellular signal-regulated protein kinase phosphorylation and 6-hydroxydopamine cytotoxicity. J Biosci. 2003;28(1):83–9.
74. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 2009;16(7):1040–52.
75. Jezek P, Hlavata L. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. Int J Biochem Cell Biol. 2005;37(12):2478–503.
76. Brieger K, et al. Reactive oxygen species: from health to disease. Swiss Med Wkly. 2012;142:w13659.
77. Krause KH, Bedard K. NOX enzymes in immuno-inflammatory pathologies. Semin Immunopathol. 2008;30(3):193–4.
78. Danielson SR, Andersen JK. Oxidative and nitrative protein modifications in Parkinson's disease. Free Radic Biol Med. 2008;44(10):1787–94.
79. Seet RC, et al. Oxidative damage in Parkinson disease: measurement using accurate biomarkers. Free Radic Biol Med. 2010;48(4):560–6.
80. Tsang AH, Chung KK. Oxidative and nitrosative stress in Parkinson's disease. Biochim Biophys Acta. 2009;1792(7):643–50.
81. Henle ES, Linn S. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem. 1997;272(31):19095–8.
82. Lee DH, Gold R, Linker RA. Mechanisms of oxidative damage in multiple sclerosis and neurodegenerative diseases: therapeutic modulation via fumaric acid esters. Int J Mol Sci. 2012;13(9):11783–803.

83. Vila M, et al. Lysosomal membrane permeabilization in Parkinson disease. Autophagy. 2011;7(1):98–100.
84. Starke-Reed PE, Oliver CN. Protein oxidation and proteolysis during aging and oxidative stress. Arch Biochem Biophys. 1989;275(2):559–67.
85. Harman D. The biologic clock: the mitochondria? J Am Geriatr Soc. 1972;20(4):145–7.
86. Kumar H, et al. The role of free radicals in the aging brain and Parkinson's disease: convergence and parallelism. Int J Mol Sci. 2012;13(8):10478–504.
87. Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem. 1992;59(5):1609–23.
88. Lopez-Erauskin J, et al. Antioxidants halt axonal degeneration in a mouse model of X-adrenoleukodystrophy. Ann Neurol. 2011;70(1):84–92.
89. Sherer TB, et al. Mechanism of toxicity in rotenone models of Parkinson's disease. J Neurosci. 2003;23(34):10756–64.
90. Testa CM, Sherer TB, Greenamyre JT. Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. Brain Res Mol Brain Res. 2005;134(1):109–18.
91. Bjartmar C, Trapp BD. Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. Curr Opin Neurol. 2001;14(3):271–8.
92. Conway KA, et al. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science. 2001;294(5545):1346–9.
93. Martinez-Vicente M, et al. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J Clin Invest. 2008;118(2):777–88.
94. Lotharius J, Brundin P. Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease. Hum Mol Genet. 2002;11(20):2395–407.
95. LaVoie MJ, et al. Dopamine covalently modifies and functionally inactivates parkin. Nat Med. 2005;11(11):1214–21.
96. Nishikawa K, et al. Alterations of structure and hydrolase activity of parkinsonism-associated human ubiquitin carboxyl-terminal hydrolase L1 variants. Biochem Biophys Res Commun. 2003;304(1):176–83.
97. Petersen P. The dialogue principle in medical management. Diskussionsforum Med Ethik. 1991;7:XXXVII–XXXVIII.
98. Choi J, et al. Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. J Biol Chem. 2004;279(13):13256–64.
99. Choi J, et al. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. J Biol Chem. 2006;281(16):10816–24.
100. Kuhn DM, et al. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: possible relevance to Parkinson's disease. J Neurochem. 1999;73(3):1309–17.
101. Berman SB, Hastings TG. Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J Neurochem. 1999;73(3):1127–37.
102. Lee HJ, et al. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J Biol Chem. 2002;277(7):5411–7.
103. Van Laar VS, et al. Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. Neurobiol Dis. 2009;34(3):487–500.
104. Betarbet R, et al. Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. Neurobiol Dis. 2006;22(2):404–20.
105. Muftuoglu M, et al. Mitochondrial complex I and IV activities in leukocytes from patients with parkin mutations. Mov Disord. 2004;19(5):544–8.
106. Bekris LM, Mata IF, Zabetian CP. The genetics of Parkinson disease. J Geriatr Psychiatry Neurol. 2010;23(4):228–42.

107. Gandhi S, et al. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Mol Cell. 2009;33(5):627–38.
108. Irrcher I, et al. Loss of the Parkinson's disease-linked gene DJ-1 perturbs mitochondrial dynamics. Hum Mol Genet. 2010;19(19):3734–46.
109. McGeer PL, et al. Microglia in degenerative neurological disease. Glia. 1993;7(1):84–92.
110. Wang L, et al. Metabolic inflammation exacerbates dopaminergic neuronal degeneration in response to acute MPTP challenge in type 2 diabetes mice. Exp Neurol. 2014;251:22–9.
111. Qian L, Flood PM, Hong JS. Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy. J Neural Transm. 2010;117(8):971–9.
112. Zhang M, et al. Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog Neurobiol. 2013;100:30–47.
113. Tong KI, et al. Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. Biol Chem. 2006;387(10–11):1311–20.
114. Tanigawa S, et al. Jun dimerization protein 2 is a critical component of the Nrf2/MafK complex regulating the response to ROS homeostasis. Cell Death Dis. 2013;4:e921.
115. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol. 2003;43:233–60.
116. Milani P, et al. SOD1 and DJ-1 converge at Nrf2 pathway: a clue for antioxidant therapeutic potential in neurodegeneration. Oxid Med Cell Longev. 2013;2013:836760.
117. Lopez-Otin C, et al. The hallmarks of aging. Cell. 2013;153(6):1194–217.
118. Bender A, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38(5):515–7.
119. Kraytsberg Y, et al. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet. 2006;38(5):518–20.
120. Beilina A, et al. Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. Proc Natl Acad Sci U S A. 2005;102(16):5703–8.
121. Silvestri L, et al. Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. Hum Mol Genet. 2005;14(22):3477–92.
122. Shimura H, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet. 2000;25(3):302–5.
123. Trempe JF, et al. Structure of parkin reveals mechanisms for ubiquitin ligase activation. Science. 2013;340(6139):1451–5.
124. Riley BE, et al. Structure and function of Parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases. Nat Commun. 2013;4:1982.
125. Clark IE, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature. 2006;441(7097):1162–6.
126. Park J, et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature. 2006;441(7097):1157–61.
127. Yang Y, et al. Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci U S A. 2006;103(28):10793–8.
128. Narendra DP, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8(1):e1000298.
129. Matenia D, et al. Microtubule affinity-regulating kinase 2 (MARK2) turns on phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) at Thr-313, a mutation site in Parkinson disease: effects on mitochondrial transport. J Biol Chem. 2012;287(11):8174–86.
130. Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res. 2005;8(1):3–5.
131. Deas E, et al. PINK1 cleavage at position A103 by the mitochondrial protease PARL. Hum Mol Genet. 2011;20(5):867–79.
132. Meissner C, et al. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. J Neurochem. 2011;117(5):856–67.

133. Whitworth AJ, et al. Rhomboid-7 and HtrA2/Omi act in a common pathway with the Parkinson's disease factors Pink1 and Parkin. Dis Model Mech. 2008;1(2–3):168–74; discussion 173.
134. Takatori S, Ito G, Iwatsubo T. Cytoplasmic localization and proteasomal degradation of N-terminally cleaved form of PINK1. Neurosci Lett. 2008;430(1):13–7.
135. Shi G, et al. Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease. Hum Mol Genet. 2011;20(10):1966–74.
136. Jin SM, et al. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J Cell Biol. 2010;191(5):933–42.
137. Matsuda N, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol. 2010;189(2):211–21.
138. Vives-Bauza C, et al. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc Natl Acad Sci U S A. 2010;107(1):378–83.
139. Okatsu K, et al. PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. Nat Commun. 2012;3:1016.
140. Chen Y, Dorn GW 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science. 2013;340(6131):471–5.
141. Gomez-Sanchez R, et al. Mitochondrial impairment increases FL-PINK1 levels by calcium-dependent gene expression. Neurobiol Dis. 2014;62:426–40.
142. Kim Y, et al. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem Biophys Res Commun. 2008;377(3):975–80.
143. Kondapalli C, et al. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating serine 65. Open Biol. 2012;2(5):120080.
144. Shiba-Fukushima K, et al. PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. Sci Rep. 2012;2:1002.
145. Sha D, Chin LS, Li L. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum Mol Genet. 2010;19(2):352–63.
146. Geisler S, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol. 2010;12(2):119–31.
147. Gegg ME, et al. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet. 2010;19(24):4861–70.
148. Yoshii SR, et al. Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. J Biol Chem. 2011;286(22):19630–40.
149. Van Humbeeck C, et al. Parkin interacts with Ambra1 to induce mitophagy. J Neurosci. 2011;31(28):10249–61.
150. Burchell VS, et al. The Parkinson's disease-linked proteins Fbxo7 and Parkin interact to mediate mitophagy. Nat Neurosci. 2013;16(9):1257–65.
151. Exner N, et al. Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. EMBO J. 2012;31(14):3038–62.
152. Su YC, Qi X. Inhibition of excessive mitochondrial fission reduced aberrant autophagy and neuronal damage caused by LRRK2 G2019S mutation. Hum Mol Genet. 2013;22(22):4545–61.
153. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res. 2012;111(9):1208–21.
154. Yan J, et al. Enhanced autophagy plays a cardinal role in mitochondrial dysfunction in type 2 diabetic Goto-Kakizaki (GK) rats: ameliorating effects of (-)-epigallocatechin-3-gallate. J Nutr Biochem. 2012;23(7):716–24.
155. Zhu JH, et al. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. Am J Pathol. 2007;170(1):75–86.
156. Poole AC, et al. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One. 2010;5(4):e10054.

157. Wang X, et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell. 2011;147(4):893–906.
158. Kuroda Y, et al. Parkin enhances mitochondrial biogenesis in proliferating cells. Hum Mol Genet. 2006;15(6):883–95.
159. Shin JH, et al. PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease. Cell. 2011;144(5):689–702.
160. Johnson BN, et al. The ubiquitin E3 ligase parkin regulates the proapoptotic function of Bax. Proc Natl Acad Sci U S A. 2012;109(16):6283–8.
161. Li X, et al. Leucine-rich repeat kinase 2 (LRRK2)/PARK8 possesses GTPase activity that is altered in familial Parkinson's disease R1441C/G mutants. J Neurochem. 2007;103(1):238–47.
162. Seol W. Biochemical and molecular features of LRRK2 and its pathophysiological roles in Parkinson's disease. BMB Rep. 2010;43(4):233–44.
163. Waxman EA, et al. Leucine-rich repeat kinase 2 expression leads to aggresome formation that is not associated with alpha-synuclein inclusions. J Neuropathol Exp Neurol. 2009;68(7):785–96.
164. Kamikawaji S, Ito G, Iwatsubo T. Identification of the autophosphorylation sites of LRRK2. BioChemistry. 2009;48(46):10963–75.
165. Pungaliya PP, et al. Identification and characterization of a leucine-rich repeat kinase 2 (LRRK2) consensus phosphorylation motif. PLoS One. 2010;5(10):e13672.
166. Vancraenenbroeck R, et al. Expression, purification and preliminary biochemical and structural characterization of the leucine rich repeat namesake domain of leucine rich repeat kinase 2. Biochim Biophys Acta. 2012;1824(3):450–60.
167. Civiero L, et al. Biochemical characterization of highly purified leucine-rich repeat kinases 1 and 2 demonstrates formation of homodimers. PLoS One. 2012;7(8):e43472.
168. Bravo-San Pedro JM, et al. Parkinson's disease: leucine-rich repeat kinase 2 and autophagy, intimate enemies. Parkinsons Dis. 2012;2012:151039.
169. Zimprich A, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 2004;44(4):601–7.
170. Golub Y, et al. Genetic factors influencing age at onset in LRRK2-linked Parkinson disease. Parkinsonism Relat Disord. 2009;15(7):539–41.
171. Nichols RJ, et al. Substrate specificity and inhibitors of LRRK2, a protein kinase mutated in Parkinson's disease. Biochem J. 2009;424(1):47–60.
172. Bravo-San Pedro JM, et al. The LRRK2 G2019S mutant exacerbates basal autophagy through activation of the MEK/ERK pathway. Cell Mol Life Sci. 2013;70(1):121–36.
173. Migheli R, et al. LRRK2 affects vesicle trafficking, neurotransmitter extracellular level and membrane receptor localization. PLoS One. 2013;8(10):e77198.
174. Plowey ED, et al. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. J Neurochem. 2008;105(3):1048–56.
175. Schapansky J, et al. Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy. Hum Mol Genet. 2014;23(16):4201–14. doi:10.1093/hmg/ddu138. First published online: March 27, 2014.
176. Park HJ, et al. Neuroprotective effects of mesenchymal stem cells through autophagy modulation in a parkinsonian model. Neurobiol Aging. 2014;35(8):1920–28. doi:10.1016/j.neurobiolaging.2014.01.028. Epub 29 Jan 2014..
177. Manzoni C, et al. Pathogenic Parkinson's disease mutations across the functional domains of LRRK2 alter the autophagic/lysosomal response to starvation. Biochem Biophys Res Commun. 2013;441(4):862–6.
178. Friedman LG, et al. Disrupted autophagy leads to dopaminergic axon and dendrite degeneration and promotes presynaptic accumulation of alpha-synuclein and LRRK2 in the brain. J Neurosci. 2012;32(22):7585–93.
179. Chen LL, et al. Corynoxine, a natural autophagy enhancer, promotes the clearance of alpha-synuclein via Akt/mTOR Pathway. J Neuroimmune Pharmacol. 2014;9(3):380–87. doi:10.1007/s11481-014-9528-2. Epub 13 Feb 2014.

180. Ramonet D, et al. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. PLoS One. 2011;6(4):e18568.
181. Lynch-Day MA, et al. The role of autophagy in Parkinson's disease. Cold Spring Harb Perspect Med. 2012;2(4):a009357.
182. Spillantini MG, et al. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839–40.
183. Rott R, et al. Alpha-synuclein fate is determined by USP9X-regulated monoubiquitination. Proc Natl Acad Sci U S A. 2011;108(46):18666–71.
184. Pan PY, Yue Z. Genetic causes of Parkinson's disease and their links to autophagy regulation. Parkinsonism Relat Disord. 2014;20(Suppl 1);S154–7.
185. Ebrahimi-Fakhari D, Wahlster L, McLean PJ. Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol. 2012;124(2):153–72.
186. Hyun CH, et al. LRRK2 as a potential genetic modifier of synucleinopathies: interlacing the two major genetic factors of Parkinson's disease. Exp Neurobiol. 2013;22(4):249–57.
187. Dice JF. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci. 1990;15(8):305–9.
188. Cuervo AM, et al. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science. 2004;305(5688):1292–5.
189. Orenstein SJ, et al. Interplay of LRRK2 with chaperone-mediated autophagy. Nat Neurosci. 2013;16(4):394–406.
190. Mak SK, et al. Lysosomal degradation of alpha-synuclein in vivo. J Biol Chem. 2010;285(18):13621–9.
191. Vogiatzi T, et al. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. J Biol Chem. 2008;283(35):23542–56.
192. Alvarez-Erviti L, et al. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch Neurol. 2010;67(12):1464–72.
193. Yue Z, Yang XW. Dangerous duet: LRRK2 and alpha-synuclein jam at CMA. Nat Neurosci. 2013;16(4):375–7.
194. Xilouri M, et al. Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. PLoS One. 2009;4(5):e5515.
195. Xilouri M, et al. LAMP2A as a therapeutic target in Parkinson disease. Autophagy. 2013;9(12):2166–8.
196. Masliah E, et al. Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease. Neuron. 2005;46(6):857–68.
197. Dehay B, et al. Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci. 2010;30(37):12535–44.
198. Sarkar S, et al. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. 2007;282(8):5641–52.
199. Gonzalez-Polo R, et al. Silencing DJ-1 reveals its contribution in paraquat-induced autophagy. J Neurochem. 2009;109(3):889–98.
200. Ashoor R, et al. The contribution of lysosomotropism to autophagy perturbation. PLoS One. 2013;8(11):e82481.
201. Darvekar SR, et al. SPBP is a sulforaphane induced transcriptional coactivator of NRF2 regulating expression of the autophagy receptor p62/SQSTM1. PLoS One. 2014;9(1):e85262.
202. Jiang TF, et al. Curcumin ameliorates the neurodegenerative pathology in A53T alpha-synuclein cell model of Parkinson's disease through the downregulation of mTOR/p70S6K signaling and the recovery of macroautophagy. J Neuroimmune Pharmacol. 2013;8(1):356–69.
203. Rubinsztein DC, et al. Potential therapeutic applications of autophagy. Nat Rev Drug Discov. 2007;6(4):304–12.

Chapter 7
Autophagy in Alzheimer's disease: A Cleaning Service Out-of-order?

Sónia C. Correia, Paula I. Moreira and George Perry

Abstract Autophagy is a major route for the degradation of protein aggregates and damaged organelles. Alzheimer's disease (AD) is mainly characterized by two distinctive neuropathological lesions, the accumulation of amyloid-β (Aβ) deposits and the presence of neurofibrillary tangles composed of hyperphosphorylated tau protein, which clearly indicates that the mechanisms of neuronal housekeeping and protein quality control are compromised in AD pathology. Indeed, the AD brain is marked by defects in the retrograde transport of autophagosomes and their maturation to lysosomes, which trigger a massive accumulation of autophagic vacuoles within large swellings along dystrophic and degenerating neurites. The combination of altered induction of the autophagic process and hampered lysosomal clearance of autophagic substrates creates conditions favorable for Aβ and tau accumulation in AD. Understanding the step(s) affected during the autophagic process in the different stages of AD is essential for the development of novel therapeutic approaches. In this chapter, the current knowledge pertaining to the cellular and molecular mechanisms involved in autophagy and its important role in the progression of AD pathology will be highlighted. The ongoing drug discovery strategies for therapeutic modulation of autophagy in the context of AD will also be discussed.

Keywords Alzheimer's disease · AβPP · Autophagic vacuoles · Autophagy · Aβ · Lysosomes · Mitophagy · Tau · Therapeutics

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Introduction

Alzheimer's disease (AD), the most prevalent age-related neurodegenerative disorder, affects approximately 35 million people worldwide [1]. The prevalence of AD increases exponentially with age, rising from 3 % in people aged 65–74 to almost 50 % in people aged 85 or older [2]. Additionally, it has been predicted that in 2050 more than 140 million people worldwide will suffer from AD [2]. The clinical symptoms of AD comprise the progressive deterioration of cognitive functions, together with impairments in behavior, language, and visuospatial skills, culminating in the premature death of the individual typically 3–9 years after diagnosis [1]. However, the definitive diagnosis requires post-mortem detection of two distinctive pathological lesions: the presence of extracellular deposits of amyloid-β (Aβ) in brain parenchyma and cerebral blood vessels and intracellular accumulation of neurofibrillary tangles composed of hyperphosphorylated tau protein [3]. AD is also marked by severe neuronal atrophy, which initially occurs in the entorhinal region and the temporal lobe progressing to the limbic system and, subsequently, extending to major areas of the neocortex [4]. Notably, AD-related changes in brain structure and function precede the clinical manifestations of the disease by 20–30 years [4]. Thus, there is a critical need to unveil the pathological events that trigger the "beginning" of AD pathology, as well as the emergence of cognitive symptoms.

The two major degradation and recycling pathways in eukaryotic cells are the ubiquitin-proteasome system (UPS) and the autophagic-lysosomal pathway. The ubiquitin–proteasome system is responsible for the degradation of short-lived proteins, whereas autophagy regulates the levels of long-lived proteins and organelles [5]. An emerging consensus is that autophagy is a neuroprotective response and defective or inadequate autophagy promotes neuronal cell death in several neurodegenerative diseases, including AD [6]. In light of this evidence, the first part of this chapter is aimed to provide a brief overview on the molecular machinery that controls the autophagic process and highlight the physiological role of autophagy on neuronal homeostasis. Based on clinical and experimental findings, the second part of this chapter discusses the involvement of autophagy in AD pathology, putting a special focus in the close connection between defective autophagy and Aβ and tau pathologies and the selective degradation of dysfunctional mitochondria by autophagy—mitophagy. Lastly, this chapter outlines the pharmacological approaches to target autophagy and their beneficial effects against the symptomatic and neuropathological features of AD.

Autophagy: the Core Molecular Machinery

Autophagy is a catabolic process involving the degradation of cellular components, damaged organelles and misfolded proteins through the lysosomal machinery [7, 8]. Depending on the route of delivery to the lysosome, three distinct forms of autophagy have been identified: microautophagy, chaperone-mediated autophagy

(CMA) and macroautophagy [9]. In microautophagy, substrates and the surrounding cytosol are directly engulfed by invagination or protrusion of arm-like structures of the lysosomal membrane [10]. CMA involves the selective degradation of cytosolic proteins containing a KFERQ-like peptide motif that is recognized by the heat shock cognate protein 70 (Hsc70). Hsc70 traffics the cargo to the lysosome surface, and the subsequent interaction with lysosome-associated membrane protein 2A (LAMP2A) imports the substrate into the lumen, being degraded by proteases [11]. The last but most predominant type of autophagy is macroautophagy (hereafter autophagy), in which cytoplasmic components destined for degradation are encapsulated within a double-membrane bounded sequestering compartment termed the phagophore. During this process, the phagophore expands to generate an autophagosome. Then autophagossome fuses with a lysosome to form a autophagolysosome, where the cargos are degraded by lysosomal hydrolases [12].

The process of autophagy encompasses several key steps including initiation and nucleation of the autophagic vesicle, elongation and closure of the autophagosome membrane to envelop cytoplasmic constituents, docking of the autophagosome with the lysosome, and degradation of the cytoplasmic material inside the autolysosome (Fig. 7.1). All these steps are controlled by a set of products of autophagy-related genes (Atg) [13, 14]. So far, more than 30 Atg genes have been identified in yeast, and many of them have homologues in mammalian cells [15].

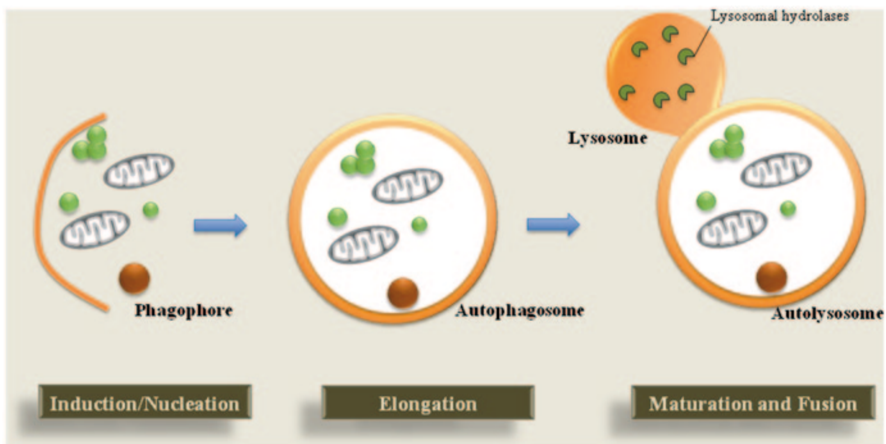

Fig. 7.1 Schematic illustration of the autophagic pathway. Under conditions of nutrient starvation or stress, the autophagic process is activated. The initial steps of the autophagic process leads to vesicle nucleation and involves the formation of the phagophore or isolation membrane. In turn, the concerted action of the autophagy core machinery proteins at the phagophore assembly site (*PAS*) induces the expansion of the phagophore into an autophagosome—elongation step. The autophagosome engulfs cytosolic protein aggregates and damaged organelles. Thereafter, the outer membrane of the autophagosome fuses with a lysosome to form an autophagolysosome, and the sequesteres cytosolic components are degraded by the action of lysosomal hydrolases

Initiation of Autophagosome Formation

Although typically low under basal conditions, autophagy can be markedly stimulated by a variety of physiological stimuli such as nutrient starvation, hypoxia, endoplasmic reticulum (ER) stress, damaged mitochondria and protein aggregates. Once activated, autophagy requires the synthesis of a phagophore, the precursor of the autophagosomes, from the ER, Golgi complex, mitochondria, or plasma membrane via clathrin-mediated endocytosis [16–19].

The core machinery of the initiation stage is the Unc-51-like kinase (ULK) complex consisting of ULK1 (the mammalian homolog to yeast Atg1), Atg13, FIP200 (a putative counterpart of yeast Atg17) and Atg101, an Atg13-binding protein [20, 21]. The mammalian target of rapamycin (mTOR) is one of the key negative regulators of autophagy induction. Indeed, activation of mTOR complex 1 (mTORC1) acts as an upstream negative regulator of ULK 1 and Atg 13 complex [22, 23]. Under nutrient-rich conditions, the active TORC1 associates with the ULK complex, phosphorylates ULK1, and hyperphosphorylates Atg13, which inhibits the kinase activity of ULK1, and thus blocks autophagy induction. Activated ULK complex could further activate the other important complex involved in the initiation of the autophagic process, beclin1-Vps34-Atg14L-p150 complex, by phosphorylating beclin-1 [24, 25]. The activity of Vps34, a class III phosphatidylinositol-3-kinase (PI3K), is essential for the formation of new autophagosomes, since Vps34 generates phosphatidylinositol-3-phosphate (PI3-P) at the phagophore-assembly-site(s) (PAS), allowing the recruitment of other Atg proteins [26]. The activity of Vps34 is enhanced by its binding to beclin-1. AMBRA1, UVRAG and Bif-1 are positive regulators of beclin-1 function and autophagy, whereas the anti-apoptotic proteins Bcl-2 and Bcl-X_L negatively regulate beclin-1 [27]. Inhibitors of Vps34 such as 3-methyladenine (3-MA) or wortmannin can be used to inhibit macroautophagy since they prevent autophagosome nucleation [28].

Elongation

The elongation and expansion of the autophagosomal membrane is dependent on two ubiquitin-like conjugation systems: microtubule-associated protein 1 light chain 3 (LC3) and Atg12 systems [29]. In the first conjugation event, Atg12 is conjugated to Atg5 in a reaction that requires Atg7 and Atg10 [30]. Thereafter, Atg12-Atg5 forms a complex with Atg16L that modulates the next process, the ubiquitin-like conjugation of LC3-I (mammalian orthologue of Atg8). The protein LC3 is proteolytically activated by Atg4, which cleaves the C-terminus of LC3, thereby generating a cytosolic LC3-I, which subsequently conjugates with phosphatidylethanolamine (PE). This process requires Atg7 and Atg3, and the Atg16L complex modulates the LC3-I lipidation by acting like an E3-like enzyme [31]. PE-conjugated LC3 becomes in nonsoluble form (LC3-II) that is stably inserted into the autophagosomal membrane [32]. Cargo selection is partly achieved by the recognition of ubiquitin-interacting

domains in the autophagic cargo receptor proteins such as p62/SQSTM1. Then the cargo is targeted to the autophagosome via LC3-interacting regions of p62 [33].

Maturation and Fusion

After completion of autophagosome formation, autophagosomes can be fused with lysosomes resulting in the formation of the autolysosome [34]. Fusion firstly requires the movement of autophagosomes into lysosomes along microtubules by using the dynein-dynactin complex [35]. The fusion of autophagosomes with lysosomes, is regulated by N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), Rab proteins, and ATPase [36, 37]. In this final stage, the cargoes are degraded by lysosomal hydrolases in the autolysosomes. Importantly, the acidification by proton pump or vacuolar ATPase (v-ATP-ase) is essential for the fusion of autophagosomes with lysosomes and degradation of cargoes in autolysosomes [38].

Role of Autophagy in Neuronal Homeostasis

Autophagy plays a vital role in maintaining cellular metabolic balance, influencing cellular death and survival and guarding the genome [39]. Importantly, autophagy represents one major quality control pathway involved in neuronal surveillance. Because neurons are post-mitotic cells, the effective degradation of protein aggregates and damaged organelles by autophagy acts as a local housekeeping process [40]. Autophagic vacuoles (AVs) are scarce in neurons of the healthy brain [41–43]. Using mice expressing green fluorescent protein-tagged LC3 (GFP-LC3) as an autophagosome marker, it was demonstrated that GFP-LC3-labeled autophagosomes do not occur in the brain, even after 48 h of food withdrawal [42]. An electron microscopic study of brain biopsy specimens also confirmed the scarcity of autophagosomes in healthy neurons [43]. Furthermore, AVs may be rarely detected, even though autophagic flux—the net rate of AVs formation and clearance - could be also very rapid [44, 45]. The relation between the autophagic flux and the magnitude of the autophagy induction is illustrated by the autophagosome clearance blockage through inhibition of lysosomal proteolysis, which causes a rapid accumulation of AVs without altering autophagy induction in cultured primary neurons [44]. Furthermore, rapamycin and starvation accelerates the generation of autophagosomes and the autophagic flux [46]. In this way, autophagy has been proposed to be constituently active, inducible and highly efficient in healthy neurons [46].

The importance of constitutive autophagy for neuronal protection has been highlighted by two landmark studies demonstrating that Atg5- and Atg7-deficient mice exhibit progressive neuronal degeneration and massive neuronal loss within the first few months of life [47, 48]. Additionally, in Atg5- and Atg7-deficient neurons, it was initially observed to be a diffuse abnormal accumulation of intracellular proteins, and then a massive number of aggregates and inclusions developed [47, 48].

Accordingly, the expression of several autophagic genes in neuronal tissue is decreased in aged *Drosophila*, a phenomenon positively correlated with the increase in the accumulation of insoluble ubiquitinated protein aggregates [49]. Autophagy is also required for the maintenance of axonal homeostasis and the prevention of axonal degeneration. Specific ablation of an essential autophagy gene, Atg7, in Purkinje cells initially causes cell-autonomous, progressive dystrophy (manifested by axonal swellings) and degeneration of the axon terminals [50]. Consistent with suppression of autophagy, no autophagosomes were observed in these dystrophic swellings, contrasting with the accumulation of autophagosomes in the axonal dystrophic swellings under pathological conditions [50]. Axonal dystrophy of mutant Purkinje cells proceeds with little sign of dendritic or spine atrophy, indicating that axon terminals are much more vulnerable to autophagy impairment than dendrites. This early pathological event in the axons is followed by cell-autonomous Purkinje cell death and mouse behavioral deficits [50]. Overall, these findings suggest that Atg7 is required for membrane trafficking and turnover in the axons. Additionally, Hollenbeck and collaborators [51] reported that the basal level of autophagy is crucial for the remodeling of neurite and growth-cone structure during neurite extension. Neural-specific deletion of FIP200, which is involved in autophagosome biogenesis also causes neuronal cell death and axon degeneration leading to cerebellar degeneration [52]. Finally, autophagy participates in synapse development. In fact, experimental evidence reveals that autophagy promotes synapse growth in the *Drosophila* neuromuscular junction by downregulating Highwire [53]. Furthermore, the overexpression of Atg1 stimulates autophagy and the subsequent enhancement of synaptic growth, while suppressing autophagy by genetic manipulation culminates in the reduction of synapse size [53]. So, the modulation of autophagy results in corresponding effects on synaptic size.

Defective Autophagy in Alzheimer's disease: What is Known So Far?

The abnormal accumulation of AVs in hippocampal and prefrontal cortical pyramidal neurons represents the first clue of defective autophagy in AD pathology [54, 55]. More recently, ultrastructural analysis of the AD brain confirmed that dystrophic neurites display a "traffic jam" of autophagosomes and other AVs, including cathepsin-containing autophagolysosomes [43]. The accumulation of AVs in dystrophic neurites occurs even before the appearance of extracellular Aβ deposition, indicating that AV accumulation is an early event in AD [56]. Early autophagic markers, such as Atg5, Atg12, and LC3 co-localize with tau-positive dystrophic neurites and neurofibrillary tangles and are increased in the capillary endothelial cells in close proximity to Aβ plaques; however, the pattern of autophagy activation changes with the progression of the disease [57]. In this sense, the major challenge is to uncover what are the precise defective steps along the autophagic pathway and their pathological relevance in the development of AD pathology. Recent breakthroughs states that the combination of impaired autophagy initiation, retrograde transport of AVs and their maturation to lysosomes, with the concomitant reduction

in lysosomal clearance of autophagic substrates are underlying the massive accumulation of immature AVs in AD [44, 46, 58].

In this scenario, it is tempting to speculate that up-regulation of autophagy initiation is a primary cause of this massive accumulation of AVs in AD, however, there are some conflicting findings. In 2008, Pickford and collaborators [59] observed that the expression levels of beclin-1, a critical component in the class III PI3K complex that initiates the formation of autophagosomes, were reduced in early stages of AD in brain areas affected by the disease, including entorhinal cortex and hippocampus. Furthermore, this reduction in beclin-1 levels is further potentiated with the progression of the disease from mild cognitive impairment to severe AD [59]. The loss of function of beclin-1 in the brains of AD subjects has been attributed to its proteolytic cleavage by caspase-3 [60]. Conversely, it was demonstrated that autophagy is upregulated in AD, due both reactive oxygen species (ROS)-dependent activation of the type III PI3K, which is critical for the initiation of autophagy, and the transcriptional activation of positive regulators of autophagy [61].

Defective maturation and retrograde transport of AVs towards the soma is an important aspect involved in the accumulation of AVs within large swelling along dystrophic and degenerating neuritis [62]. Experimental findings noted a selective transport deficit of autophagy-related compartments in *in vitro* and *in vivo* models of AD [46, 63]. The involvement of defective axonal transport of AVs in AD is further corroborated by data demonstrating that the blockage of autophagosome delivery to the lysosome with vinblastine induces a rapid accumulation of AVs in neurites with morphology similar to that seen in the AD brain [44].

Progressing in the autophagic process, the components of the lysosomal machinery are upregulated in the early stages of AD [64, 65]. However, immature AVs accumulate in dystrophic neurites even though acid-hydrolase-containing dense lysosomes are abundant, which suggests that autophagosomes have access to hydrolase-containing compartments but may not fuse efficiently. A relatively high proportion of double-membrane-limited 'immature' AVs contain cathepsin immunoreactivity but also abundant partially digested substrates, suggesting impaired maturation of AVs to lysosomes [43]. On the other hand, presenilin 1 (PS1), a component of the γ- secretase complex and whose mutations result in early-onset forms of autosomal dominant AD [56], has also been associated with impaired AVs clearance by interfering with lysosome acidification. Indeed, PS1 acts as a chaperone essential for the *N*-glycosylation and delivery of v-ATPase to lysosomes, which is essential for lysosome acidification and protease activation. PS1 mutations in familial AD compromise macroautophagic turnover of proteins [62]. Lee and collaborators [66] reported that fibroblasts from PS1 mutant AD subjects exhibit defective autophagy, resulting in Aβ accumulation caused by impairment in the maturation of V0a1 subunit of the bimodular v-type H^+-ATPase proton pump that achieves autolysosomal acidification. Moreover, PS1-null cells exhibit an excessive accumulation of immature unglycosylated v-ATPase and abnormal accumulation of late-stage autophagosomes with undigested contents, resembling the ultrastructures present the AD brain [43].

Overall, these observations suggest that the defective transport and maturation of AVs might be an even more crucial factor than defective autophagy induction in the development of AD pathology.

Autophagy and Aβ Metabolism

The local accumulation of autophagosomes and other AVs in dystrophic neurites constitutes a major intracellular reservoir of the Aβ peptide [43]. Aβ is proteolitically derived from a larger integral membrane protein, the amyloid-β protein precursor protein (AβPP) [67]. Briefly, AβPP is sequentially matured in the ER and Golgi apparatus and then delivered to the plasma membrane through the trans-Golgi networks. Within minutes of arrival at the cell surface, AβPP is internalized in clathrin-coated vesicles through endocytosis. The internalized AβPP is delivered to endosomes, where the β-secretase activity initiates Aβ generation by shedding a large part of the ectodomain of AβPP (AβPPsβ) and generating an AβPP carboxy-terminal fragment (AβPP -CTF), which is then cleaved by γ-secretase [68]. The endosomal-lysosome pathway is a critical regulator of Aβ generation and clearance, its dysfunction being considered a major pathological "trigger" underlying Aβ peptide overproduction and neuronal loss in AD pathology [69, 70].

Using human posmortem brain tissue and experimental *in vitro* and *in vivo* models of AD, it was demonstrated the AVs contain Aβ peptides (both Aβ40 and Aβ42), AβPP, and β- and γ-secretases [56, 71]. A clear correlation between autophagic activity and Aβ generation has been established. Notably, upregulation of autophagy by starvation or mTOR inhibition accelerates Aβ generation, whereas autophagy inhibition by 3-MA or an excess of amino acids suppresses Aβ generation [56]. Indeed, autophagy failure is a crucial Aβ-generating mechanism, in part by increasing the levels of mature AβPP and its paralogue amyloid beta precursor-like protein 1 (APLP1), whereas autophagy blockage inhibits the expression of both AβPP and APLP1 [72]. Under stress conditions, such as proteasome inhibition, it was reported there is an autophagy-dependent accumulation of Aβ in lysosomes, and increased levels of intracellular and secreted Aβ in a neuronal cell line overexpressing mutant AβPP [73]. This increase in Aβ burden results from the inefficient degradation of AβPP -CTF by the proteasome, that is then cleaved by the γ-secretase and Aβ produced [73]. Furthermore, inhibition of autophagy further increases the AβPP -CTF to AβPP ratio, supporting the involvement of the autophagosome in Aβ generation [73]. Mechanistically, beclin-1, a protein involved in the initiation and execution of autophagy, regulates AβPP processing and turnover. Jaegger and collaborators [74] found that the knockdown of beclin-1 increases the levels of AβPP and AβPP -CTF, which are accompanied by impaired autophagosomal clearance. In contrast, beclin-1 overexpression reduces cellular AβPP levels, indicating that beclin-1 deficiency disrupts cellular autophagy and autophagosomal-lysosomal degradation and alters AβPP metabolism [74]. Similarly, another study demonstrated that the genetic ablation of beclin-1 in AβPP transgenic mice model for AD increased intraneuronal Aβ accumulation, extracellular Aβ deposition, and neurodegeneration and caused

profound neuronal ultrastructural abnormalities. Administration of a lentiviral vector expressing beclin-1 reduced Aβ pathology in AβPP transgenic mice [59].

Another important link between autophagy and Aβ metabolism is the stimulation of γ- secretase. When autophagy is induced in mouse fibroblasts by inhibition of mTOR by rapamycin or starvation, the γ-secretase complex translocates from a predominantly endosomal/ER pool to AVs, which accumulate transiently and become the largest cellular pool of γ-secretase activity. Under these conditions, Aβ production rises two-fold over that in autophagy-suppressed cells and Aβ immunoreactivity appears within AVs [56]. Additionally, γ-secretase activity is enhanced in basal autophagy-disturbed cells through the alpha subunit of eukaryotic translation initiation factor 2 (eIF2alpha) kinase, general control nonderepressible 2 (GCN2) [75]. PS1 expression was increased even in the presence of nutrients in autophagy-related 5 knockdown (Atg5KD) human embryonic kidney (HE K293) cells expressing a short hairpin RNA as well as in chloroquine-treated HE K293 cells. However, PS1 expression induction was prevented in GCN2KD and ATF4KD cells. Furthermore, Atg5KD cells showed an increase in Aβ production and Notch1 cleavage—these effects being abrogated by resveratrol, an autophagy inducer [75]. Overall, these findings suggest that the autophagy-lysosomal system regulates γ-secretase activity through GCN2.

Aβ is also an autophagic substrate, being a target of autophagy-mediated clearance as noticed by the co-localization of AβPP and Aβ peptide with LC3-positive autophagosomes in an AβPP-overexpressing cell line and AD mouse models [76]. Recently, the complex adaptor protein 2/phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia (AP2/PICALM) was also identified as a mechanistic bridge between the AβPP endocytic and autophagic pathways [77]. The adaptor complex AP2/PICALM functions as an autophagic cargo receptor that interacts with AβPP-CTF and LC3. This interaction, in turn, directly recruits AβPP-CTF from endosomes to autophagosomes [77]. The autophagic proteins Atg5, Beclin-1, and Ulk1 were shown to be involved in the housekeeping clearance of Aβ and AβPP -CTF [78]. Taking advantage of the small-molecule enhancers of rapamycin 28 (SMER28), an enhancer of autophagy, these authors observed a drastic reduction in levels of Aβ and AβPP-CTF in an Atg5-dependent fashion [78]. The physiological importance of the autophagic-lysosomal pathway in Aβ clearance is further highlighted in a study developed in the TgCRND8 mouse model of AD, which exhibits defective proteolytic clearance of autophagy substrates, robust intralysosome Aβ accumulation, extracellular Aβ deposition, and cognitive deficits [79]. Genetic deletion of the endogenous cathepsin D inhibitor, cystatin B, rescues autophagic-lysosomal dysfunction and reduces Aβ levels [79].

However, a crucial question remains: could Aβ interfere with the autophagic process? Compelling evidences demonstrate that Aβ peptide induces autophagy [80, 81]. Particularly, confocal microscopy reveals that the exogenous application of $A\beta_{42}$ in medium causes the co-localization of $A\beta_{42}$ with the autophagic marker LC3, in neuronal cells [81]. Aβ was also shown to activate autophagy by inducing autophagic vesicle formation and Atg5, and up-regulate the lysosomal machinery for the degradation of autophagosomes, events that occur prior to Aβ-induced apoptosis.

Notably, under Aβ toxicity, inhibition of autophagy by 3-MA promotes neuronal apoptosis, suggesting that autophagy can protect against Aβ-mediated neurotoxicity [82]. Consistently, the overexpression of $A\beta_{42}$ in *Drosophila* neurons causes an extensive accumulation of autophagic vesicles that become increasingly dysfunctional with age [83]. $A\beta_{42}$-induces impairment of the degradative function, as well as the structural integrity, of post-lysosomal AVs triggers a neurodegenerative cascade that can be enhanced by autophagy activation or partially rescued by autophagy inhibition [83]. Compromise and leakage from post-lysosomal vesicles result in cytosolic acidification, additional damage to membranes and organelles, and erosive destruction of cytoplasm leading to eventual neuronal loss [83]. Prompted by these findings, it was suggested that autophagy initially plays a pro-survival role that changes in an age-dependent way to a pro-death role in the context of $A\beta_{42}$ expression [83]. Similarly, $A\beta_{40}$ was found to impair the autophagic protein degradation pathway due to failure in the autophagic flux in brain endothelial cells, which is a causal factor underlying cell demise [84]. But, how does Aβ impair autophagy? Silva and collaborators [85] proposed that Aβ disrupts the mitochondrial metabolism, which in turn compromises mitochondrial network dynamics and consequent Aβ clearance by the autophagic-lysosomal pathway. Importantly, the microtubule stabilizer taxol is able to restore the microtubule network and to prevent Aβ toxicity [85].

Collectively, this evidences pinpoint the pivotal role autophagy plays not only in Aβ generation by regulating AβPP turnover and stimulating γ- secretase, but also in Aβ clearance [56, 71, 86]. Furthermore, reduced autophagic flux promotes an escalating cycle of AβPP/AβPP-CTF/Aβ accumulation, which could further hamper the autophagic process.

Autophagy and Tau

Tau is a microtubule-binding protein that plays a primary role in microtubule assembly and stabilization; however, new functions in the modulation of signaling pathways through scaffolding, adult neurogenesis and cytoskeletal organization have recently emerged [87]. Under pathological conditions, tau is hyperphosphorylated and aggregates to form insoluble inclusions that lead to the development of neurofibrillary tangles [88]. A flurry of recent findings reveals that abnormal processing and altered clearance by autophagic-lysosomal machinery of tau protein is implicated in the formation of neurofibrillary tangles in the AD brain [89]. As a long-lived protein, tau is expected to be degraded in lysosomes. Indeed, postmortem studies demonstrated that Alz-50 immunoreactivity, an early indicator of tau misfolding, co-localizes with lysosomes [90, 91]. Furthermore, the treatment of rat organotypic entorhino-hippocampal slices with chloroquine, which raises the pH of lysosomes to impair their enzymatic function, induces meganeurites and tangle-like structures [92], fostering the role of the lysosomal pathway in tau degradation. Using inducible neuronal cell models of tauopathy, it was further observed that autophagy contributes to both tau fragmentation into pro-aggregating forms and clearance of tau aggregates, whereas its inhibition enhances tau protein aggregation

and neurotoxicity[93, 94]. Conversely, CMA was shown to allow the delivery of tau fragments to lysosomes, contributing to the generation of more aggregation-prone fragments, and subsequent tau aggregation [93]. Thus, this finding suggests the role of (macro)autophagy and CMA in tau aggregation is antagonistic. Also, the cathepsin D stimulator ZPAD increases the proteolysis of full-length tau resulting in the production of smaller fragments, including a phosphorylated 29 kDa fragment [95, 96]. In this scenario, a study performed in a *Drosophila* tauopathy model highlighted the critical role of cathepsin D in the degradation of tau protein. For instance, authors found elevated levels of cathepsin D in neurons expressing mutant human tau, with the genetic ablation of cathepsin D being responsible for enhanced neurotoxicity and a shorter lifespan [89]. Directly targeting directly autophagy through genetic and pharmacological approaches, Caccamo and collaborators found that mTOR, a negative regulator of autophagy, regulates tau phosphorylation [97]. Particularly, the stimulation of TOR activity provoked an increase in both total and AT8-positive phosphorylated tau in a mouse model overexpressing mutant human tau [97]. Mechanistically, impaired autophagic function is underlying this association between mTOR and tau. Consistently, an age-dependent neurodegeneration with accumulation of phosphorylated tau within intracellular inclusions was observed in the forebrain neurons of the Atg7 conditional knockout mice [98]. These inclusions specifically contained tau phosphorylated at AT8, AT100, and TG3 epitopes, but not PHF1 [98]. Interestingly, the treatment of primary neurons with the proteasomal inhibitor MG-132 reduces total tau levels, in part due to a compensatory upregulation of autophagy, as noticed by increased LC3-II protein levels and number of autophagosomes [99]. In line with this, an elegant study designed to decipher the contributions of the UPS and autophagy to tau degradation revealed that in primary cortical neurons, phosphomutants E18tau and E27tau, when compared with wild type tau, are targets for autophagy, while endogenous tau is not [100]. In the absence of autophagy, degradation of tau by the UPS in neurons is minimal [100], which reinforces the idea that autophagy is the preferential primary route for clearing phosphorylated tau in neurons.

At this point, it is clear that autophagy is intimately involved in tau processing and degradation. However, it is important to be aware that tau hyperphosphorylation *per se* could also affect the autophagic process by hampering the trafficking of autophagosomes, generating a vicious cycle [101]. Autophagosomes carrying the cellular cargoes are transported along the microtubule tracks toward the soma (which contains the highest concentration of lysosomes), where they fuse with lysosomes to form the autophagolysosome [102, 103]. It was demonstrated that tau hyperphosphorylation is one of the key elements underlying the disruption of microtubule network in AD pathology [103].

Mitophagy in Alzheimer's disease

Autophagy was initially considered to be a nonselective process; however cargo-specific autophagy is a major player. Mitophagy is one type of cargo-specific

autophagy, which selectively removes damaged mitochondria [104]. Neurons are, owing to their limited glycolytic capacity, highly reliant on mitochondrial function for energy production [105]. High levels of ATP are required to sustain axonal transport of macromolecules and organelles, including mitochondria, to maintain ionic gradients and a resting membrane potential, and to load neurotransmitters into the synaptic cleft, a site distant from the soma [106]. Mitochondria are generated from the division of pre-existing organelles in the soma—a process called mitochondrial biogenesis—and have a particularly long half-life, increasing the probability of accumulating damage [107]. Therefore, the efficient removal of dysfunctional mitochondria by mitophagy is a vital quality control mechanism to ensure normal neuronal functioning and integrity.

But, how can autophagy selectively target damaged mitochondria for degradation? The specific labeling of damaged mitochondria and their subsequent degradation seem to be mediated by a PTEN-induced putative kinase protein 1 (PINK1)/parkin-dependent pathway [108]. Briefly, reduction in mitochondrial potential ($\Delta\Psi_m$), which indicates a loss-of-function of the mitochondrial respiratory chain or the permeabilization of the inner membrane, function as a "eat me" signal that stimulates the Lys63-linked ubiquitylation of proteins anchored in the outer mitochondrial membrane, including voltage-dependent anion-selective channel 1 (VDAC1), mitofusin 1 (MFN1) and MFN2, which tags the organelle for mitophagy [109]. Then, PINK 1 recruits the E3 ubiquitin ligase parkin from the cytosol to the damaged mitochondria, whereas it induces p62 recruitment to the mitochondria via ubiquitination, thus co-recruiting LC3 [108].

Mitochondrial dysfunctional has been posited as a central piece in the AD puzzle. Indeed, converging evidence shows that defective mitochondrial metabolism and dynamics sets up a cascade of pathological events that underlies neuronal degeneration in AD pathology [105]. The most consistent mitochondrial abnormalities reported in AD are the robust decline in cytochrome c oxidase (COX) activity, loss of ΔΨm and exacerbated production of mitochondrial-derived ROS [105]. Incited by these exciting findings, the next question to come to mind: Is mitophagy involved in AD? The first sign for an involvement of mitophagy in AD came from a study reporting that in AD brains much of the mtDNA and COX were located in the neuronal cytoplasm and in the case of mtDNA, it was found in vacuoles associated with lipofuscin [110]. Similarly, subsequent studies demonstrated that AD brains presented an increased concentration of mitochondrial components, namely COX and lipoic acid, within autophagosomes, suggesting an increase in the rate of mitochondrial degradation by autophagy [111, 112]. Mitophagy markers were shown to reach maximum level and return to basal level as apoptotic markers start to increase in the hippocampus of Aβ-injected rats [113]. Additionally, an elegant study performed in a mouse model of AD revealed that the overexpression of parkin stimulates a beclin-1-dependent molecular cascade of autophagy that facilitates the clearance AVs containing debris and defective mitochondria [114]. Furthermore, parkin overexpression decreases intracellular Aβ levels and extracellular plaque deposition, prevents mitochondrial dysfunction and oxidative stress and restores neurotransmitter synthesis [114]. Postmortem studies also revealed an increased

immunostaining of parkin in AD brains [115], which indicates that the increase in parkin levels might be an intrinsic protective mechanism to limit/counteract the effects of AD-related beclin-1 reduction to avoid the accumulation of damaged mitochondria. More recently, AD-linked, toxic NH2 human tau was shown to adversely affect the interplay between mitochondria dynamics and their selective autophagic clearance [116]. Particularly, NH2 human tau induces fragmentation and perinuclear mislocalization of mitochondria, these effects being accompanied by (1) net reduction in mitochondrial mass correlated with a general parkin-mediated remodeling of the membrane proteome; (2) mitochondrial co-localization with LC3 and LAMP1 autophagic markers; (3) bioenergetic deficits and (4) synaptic pathology [116].

Targeting Autophagy in Alzheimer's disease

The signs of defective autophagic process in AD pathology are undeniable. Nevertheless, a deeper knowledge on the precise steps underlying autophagy malfunction in AD is required. However, modulation of autophagy by genetic and pharmacological approaches has promising effects in AD pathology, by rescuing both Aβ and tau pathologies and AD-realted symptomatic features [39].

As aforementioned, mTOR is a critical negative regulator of autophagy. The most familiar drug used to treat AD in alter the mTOR-dependent pathway is rapamycin. Using the triple transgenic mouse model of AD (3xTg-AD), that pharmacologically restoring mTOR signaling with rapamycin was shown to rescue cognitive deficits and ameliorate Aβ and tau pathology by increasing autophagy [117, 118]. Notably, Liu and collaborators [119] found that rapamycin decreases tau phosphorylation at Ser214 residue through regulation of cAMP-dependent kinase (PKA). Because tau phosphorylation at Ser214 residue may prime tau for further phosphorylation by other kinases, this could represent a possible mechanism by which rapamycin reduces or prevents tau hyperphosphorylation [119]. Additionally, genetic ablation of mTOR ameliorates AD-related phenotype by increasing autophagy induction and restoring hippocampal gene expression [120]. In opposition, the treatment of *Drosophila* overexpressing $A\beta_{42}$ with rapamycin culminated in a significantly shortened lifespan, indicating that enhancement of autophagy may potentiate $A\beta_{42}$ neurotoxicity [83]. Furthermore, only the induction of autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits in the 3xTg-AD mouse [118]. In this sense, the timing of intervention in AD progression should be considered when implementing autophagy inducers as therapeutic approaches. Temsirolimus, an analog of rapamycin was also shown to promote the autophagic clearance of Aβ and attenuate tau hyperphosphorylation in both *in vitro* and *in vivo* models of AD pathology [121, 122]. Lithium, another pharmacological inducer of autophagy, possesses disease-modifying effects in AD. A clinical trial performed in amnestic mild cognitive impairment (MCI) subjects showed that long-term lithium treatment slows the progression of cognitive and functional deficits, and also attenuate tau hyperphosphorylation in the MCI-AD continuum [123]. Induction of autophagy in a mTOR-independent manner by overexpressing

beclin-1 also reduces both intracellular and extracellular deposits of Aβ in an APP-transgenic mouse model of AD [59]. The anti-epileptic drug carbamazepine also alleviates memory deficits and cerebral Aβ pathology in the same AD mouse model by enhancing the autophagic flux [124].

Besides targeting the initiation of the autophagic process, the improvement of the lysosomal proteolysis is another important therapeutic strategy to "fight" AD pathology. In the TgCRND8 mouse model of AD, which exhibits defective proteolytic clearance of autophagy substrates, enhancement of lysosomal activity by genetic ablation of cystatin B (an endogenous inhibitor of lysosomal cysteine proteases) enhances the clearance of the autophagic substrates, and ameliorates Aβ pathology and memory deficits [79].

Despite these beneficial effects, the therapeutic use of autophagy modulation requires some important considerations. First and foremost, the clearance of autophagosome is impaired in AD, in part due to a defective lysosomal proteolysis. Thus, when using autophagy inducers, such as rapamycin, the autophagic efficiency should be firstly restored to avoid abnormal accumulation of AVs. Secondly, there is a temporal window to use autophagy inducers in AD pathology. As mentioned, the increase in the induction of autophagy could be a valid therapeutic strategy during the early stages of AD pathology. However, increasing autophagy induction alone may be ineffective when AD-related neuropathology is well-established.

Final Remarks

Substantial progress has been made in the past few years in understanding the mechanisms underlying defective autophagy in AD pathology. Autophagic vacuoles accumulated in dystrophic neurites are major sites of Aβ generation, where autophagy regulates AβPP turnover and stimulates γ-secreatase activity and Aβ clearance. Abnormal processing and altered clearance by autophagic-lysosomal machinery of tau protein is also implicated AD pathology. Recent breakthroughs also revealed that dysfunctional mitochondria are tagged for degradation by autophagy (mitophagy) in an attempt to maintain an healthy mitochondrial pool. In line with this, therapeutic modulation of autophagy has been shown to mitigate Aβ and tau pathologies and cognitive deficits in experimental models of AD. However, during the implementation of this therapeutics, the nature of the autophagic defect, the timing of intervention, and the optimal level and duration of autophagy stimulation should be considered. Although up-regulation of autophagy might be beneficial during the early stages of AD, increasing autophagic input may backfire if applied to later stages of AD, in which the lysosomal blockage is preeminent.

A better understanding of the precise role of autophagy in the different stages of AD progression will show promise as an avenue for future therapy against this incurable disease.

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References

1. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med. 2010;362(4):329–44.
2. Pahnke J, Walker LC, Scheffler K, Krohn M. Alzheimer's disease and blood-brain barrier function-Why have anti-beta-amyloid therapies failed to prevent dementia progression? Neurosci Biobehav Rev. 2009;33(7):1099–108.
3. Weller RO, Boche D, Nicoll JA. Microvasculature changes and cerebral amyloid angiopathy in Alzheimer's disease and their potential impact on therapy. Acta Neuropathol. 2009;118(1):87–102.
4. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging. 1995;16(3):271–8 (discussion 8–84).
5. Moreira PI, Santos RX, Zhu X, Lee HG, Smith MA, Casadesus G. Autophagy in Alzheimer's disease. Expert Rev Neurother. 2010;10(7):1209–18.
6. Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci. 2010;13(7):805–11.
7. Mizushima N. Autophagy: process and function. Genes Dev. 2007;21(22):2861–73.
8. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000;290(5497):1717–21.
9. Yamamoto A, Yue Z. Autophagy and its normal and pathogenic states in the brain. Annu Rev Neurosci. 2014 (in press).
10. Santambrogio L, Cuervo AM. Chasing the elusive mammalian microautophagy. Autophagy. 2011;7(6):652–4.
11. Kaushik S, Cuervo AM. Chaperone-mediated autophagy: a unique way to enter the lysosome world. Trends Cell Biol. 2012;22(8):407–17.
12. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol. 2010;12(9):814–22.
13. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol. 2009;10(7):458–67.
14. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67–93.
15. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol. 2007;9(10):1102–9.
16. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol. 2008;182(4):685–701.
17. Geng J, Nair U, Yasumura-Yorimitsu K, Klionsky DJ. Post-Golgi Sec proteins are required for autophagy in Saccharomyces cerevisiae. Mol Biol Cell. 2010;21(13):2257–69.
18. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;141(4):656–67.
19. Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol. 2010;12(8):747–57.
20. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009;20(7):1992–2003.
21. Mercer CA, Kaliappan A, Dennis PB. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. Autophagy. 2009;5(5):649–62.

22. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. FEBS Lett. 2010;584(7):1287–95.
23. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol. 2011;13(2):132–41.
24. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol. 2010;20(6):355–62.
25. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol. 2013;15(7):741–50.
26. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell. 2008;19(12):5360–72.
27. Sinha S, Levine B. The autophagy effector Beclin 1: a novel BH3-only protein. Oncogene. 2008;27(Suppl 1):S137–48.
28. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem. 2010;285(14):10850–61.
29. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep. 2008;9(9):859–64.
30. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem. 1998;273(51):33889–92.
31. Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, et al. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J Biol Chem. 2007;282(52):37298–302.
32. Tanida I, Sou YS, Ezaki J, Minematsu-Ikeguchi N, Ueno T, Kominami E. HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates. J Biol Chem. 2004;279(35):36268–76.
33. Moscat J, Diaz-Meco MT, Wooten MW. Signal integration and diversification through the p62 scaffold protein. Trends Biochem Sci. 2007;32(2):95–100.
34. Gordon PB, Seglen PO. Prelysosomal convergence of autophagic and endocytic pathways. Biochem Biophys Res Commun. 1988;151(1):40–7.
35. Jahreiss L, Menzies FM, Rubinsztein DC. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. Traffic. 2008;9(4):574–87.
36. Eskelinen EL. Maturation of autophagic vacuoles in Mammalian cells. Autophagy. 2005;1(1):1–10.
37. Furuta N, Fujita N, Noda T, Yoshimori T, Amano A. Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Mol Biol Cell. 2010;21(6):1001–10.
38. Mousavi SA, Kjeken R, Berg TO, Seglen PO, Berg T, Brech A. Effects of inhibitors of the vacuolar proton pump on hepatic heterophagy and autophagy. Biochim Biophys Acta. 2001;1510(1–2):243–57.
39. Zhu XC, Yu JT, Jiang T, Tan L. Autophagy modulation for Alzheimer's disease therapy. Mol Neurobiol. 2013;48(3):702–14.
40. Harris H, Rubinsztein DC. Control of autophagy as a therapy for neurodegenerative disease. Nat Rev Neurol. 2011;8(2):108–17.
41. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069–75.
42. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell. 2004;15(3):1101–11.

43. Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, et al. Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. J Neuropathol Exp Neurol. 2005;64(2):113–22.
44. Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci. 2008;28(27):6926–37.
45. Chu CT. Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol. 2006 May;65(5):423–32.
46. Nixon RA, Yang DS. Autophagy failure in Alzheimer's disease—locating the primary defect. Neurobiol Dis. 2011;43(1):38–45.
47. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441(7095):885–9.
48. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880–4.
49. Simonsen A, Cumming RC, Brech A, Isakson P, Schubert DR, Finley KD. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. Autophagy. 2008;4(2):176–84.
50. Komatsu M, Wang QJ, Holstein GR, Friedrich VL, Jr., Iwata J, Kominami E, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci USA. 2007;104(36):14489–94.
51. Hollenbeck PJ. Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport. J Cell Biol. 1993;121(2):305–15.
52. Liang CC, Wang C, Peng X, Gan B, Guan JL. Neural-specific deletion of FIP200 leads to cerebellar degeneration caused by increased neuronal death and axon degeneration. J Biol Chem. 2010;285(5):3499–509.
53. Shen W, Ganetzky B. Autophagy promotes synapse development in Drosophila. J Cell Biol. 2009;187(1):71–9.
54. Okamoto K, Hirai S, Iizuka T, Yanagisawa T, Watanabe M. Reexamination of granulovacuolar degeneration. Acta Neuropathol. 1991;82(5):340–5.
55. Cataldo AM, Hamilton DJ, Barnett JL, Paskevich PA, Nixon RA. Properties of the endosomal-lysosomal system in the human central nervous system: disturbances mark most neurons in populations at risk to degenerate in Alzheimer's disease. J Neurosci. 1996;16(1):186–99.
56. Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, et al. Macroautophagy–a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J Cell Biol. 2005;171(1):87–98.
57. Ma JF, Huang Y, Chen SD, Halliday G. Immunohistochemical evidence for macroautophagy in neurones and endothelial cells in Alzheimer's disease. Neuropathol Appl Neurobiol. 2010;36(4):312–9.
58. Tan CC, Yu JT, Tan MS, Jiang T, Zhu XC, Tan L. Autophagy in aging and neurodegenerative diseases: implications for pathogenesis and therapy. Neurobiol Aging. 2014;35(5):941–57.
59. Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, et al. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. J Clin Invest. 2008;118(6):2190–9.
60. Rohn TT, Wirawan E, Brown RJ, Harris JR, Masliah E, Vandenabeele P. Depletion of Beclin-1 due to proteolytic cleavage by caspases in the Alzheimer's disease brain. Neurobiol Dis. 2011;43(1):68–78.
61. Lipinski MM, Zheng B, Lu T, Yan Z, Py BF, Ng A, et al. Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc Natl Acad Sci USA. 2010;107(32):14164–9.
62. Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci. 2007;120(Pt 23):4081–91.
63. Sanchez-Varo R, Trujillo-Estrada L, Sanchez-Mejias E, Torres M, Baglietto-Vargas D, Moreno-Gonzalez I, et al. Abnormal accumulation of autophagic vesicles correlates with

axonal and synaptic pathology in young Alzheimer's mice hippocampus. Acta Neuropathol. 2011;123(1):53–70.
64. Cataldo AM, Barnett JL, Berman SA, Li J, Quarless S, Bursztajn S, et al. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. Neuron. 1995;14(3):671–80.
65. Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell. 2010;141(7):1146–58.
66. Placido AI, Pereira CM, Duarte AI, Candeias E, Correia SC, Santos RX, et al. The role of endoplasmic reticulum in amyloid precursor protein processing and trafficking: Implication's for Alzheimer's disease. Biochim Biophys Acta. 2014;1842:1444–53.
67. Cataldo AM, Barnett JL, Pieroni C, Nixon RA. Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. J Neurosci. 1997;17(16):6142–51.
68. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. Cold Spring Harb Perspect Med. 2012;2(5):a006270.
69. Mathews PM, Guerra CB, Jiang Y, Grbovic OM, Kao BH, Schmidt SD, et al. Alzheimer's disease-related overexpression of the cation-dependent mannose 6-phosphate receptor increases Abeta secretion: role for altered lysosomal hydrolase distribution in beta-amyloidogenesis. J Biol Chem. 2002;277(7):5299–307.
70. Koo EH, Squazzo SL. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J Biol Chem. 1994;269(26):17386–9.
71. Yu WH, Kumar A, Peterhoff C, Shapiro Kulnane L, Uchiyama Y, Lamb BT, et al. Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for beta-amyloid peptide over-production and localization in Alzheimer's disease. Int J Biochem Cell Biol. 2004;36(12):2531–40.
72. Zhou F, van Laar T, Huang H, Zhang L. APP and APLP1 are degraded through autophagy in response to proteasome inhibition in neuronal cells. Protein Cell. 2011;2(5):377–83.
73. Agholme L, Hallbeck M, Benedikz E, Marcusson J, Kagedal K. Amyloid-beta secretion, generation, and lysosomal sequestration in response to proteasome inhibition: involvement of autophagy. J Alzheimers Dis. 2012;31(2):343–58.
74. Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T. Regulation of amyloid precursor protein processing by the Beclin 1 complex. PLoS One. 2010;5(6):e11102.
75. Ohta K, Mizuno A, Ueda M, Li S, Suzuki Y, Hida Y, et al. Autophagy impairment stimulates PS1 expression and gamma-secretase activity. Autophagy. 2010;6(3):345–52.
76. Lunemann JD, Schmidt J, Schmid D, Barthel K, Wrede A, Dalakas MC, et al. Beta-amyloid is a substrate of autophagy in sporadic inclusion body myositis. Ann Neurol. 2007;61(5):476–83.
77. Tian Y, Chang JC, Fan EY, Flajolet M, Greengard P. Adaptor complex AP2/PICALM, through interaction with LC3, targets Alzheimer's APP-CTF for terminal degradation via autophagy. Proc Natl Acad Sci USA. 2013;110(42):17071–6.
78. Tian Y, Bustos V, Flajolet M, Greengard P. A small-molecule enhancer of autophagy decreases levels of Abeta and APP-CTF via Atg5-dependent autophagy pathway. FASEB J. 2011;25(6):1934–42.
79. Yang DS, Stavrides P, Mohan PS, Kaushik S, Kumar A, Ohno M, et al. Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. Brain. 2011;134(Pt 1):258–77.
80. Pajak B, Songin M, Strosznajder JB, Orzechowski A, Gajkowska B. Ultrastructural evidence of amyloid beta-induced autophagy in PC12 cells. Folia Neuropathol. 2009;47(3):252–8.
81. Hung SY, Huang WP, Liou HC, Fu WM. Autophagy protects neuron from Abeta-induced cytotoxicity. Autophagy. 2009;5(4):502–10.
82. Cheung YT, Zhang NQ, Hung CH, Lai CS, Yu MS, So KF, et al. Temporal relationship of autophagy and apoptosis in neurons challenged by low molecular weight beta-amyloid peptide. J Cell Mol Med. 2010;15(2):244–57.

83. Ling D, Song HJ, Garza D, Neufeld TP, Salvaterra PM. Abeta42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in Drosophila. PLoS One. 2009;4(1):e4201.
84. Fonseca AC, Oliveira CR, Pereira CF, Cardoso SM. Loss of proteostasis induced by amyloid beta peptide in brain endothelial cells. Biochim Biophys Acta. 2014;1843(6):1150–61.
85. Silva DF, Esteves AR, Arduino DM, Oliveira CR, Cardoso SM. Amyloid-beta-induced mitochondrial dysfunction impairs the autophagic lysosomal pathway in a tubulin dependent pathway. J Alzheimers Dis. 2011;26(3):565–81.
86. Mizushima N. A(beta) generation in autophagic vacuoles. J Cell Biol. 2005;171(1):15–7.
87. Morris M, Maeda S, Vossel K, Mucke L. The many faces of tau. Neuron. 2011;70(3):410–26.
88. Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci. 2007;8(9):663–72.
89. Khurana V, Elson-Schwab I, Fulga TA, Sharp KA, Loewen CA, Mulkearns E, et al. Lysosomal dysfunction promotes cleavage and neurotoxicity of tau in vivo. PLoS Genet. 2010;6(7):e1001026.
90. Ikeda K, Akiyama H, Arai T, Kondo H, Haga C, Tsuchiya K, et al. Neurons containing Alz-50-immunoreactive granules around the cerebral infarction: evidence for the lysosomal degradation of altered tau in human brain? Neurosci Lett. 2000;284(3):187–9.
91. Ikeda K, Akiyama H, Arai T, Kondo H, Haga C, Iritani S, et al. Alz-50/Gallyas-positive lysosome-like intraneuronal granules in Alzheimer's disease and control brains. Neurosci Lett. 1998;258(2):113–6.
92. Bi X, Zhou J, Lynch G. Lysosomal protease inhibitors induce meganeurites and tangle-like structures in entorhinohippocampal regions vulnerable to Alzheimer's disease. Exp Neurol. 1999;158(2):312–27.
93. Wang Y, Martinez-Vicente M, Kruger U, Kaushik S, Wong E, Mandelkow EM, et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. Hum Mol Genet. 2009;18(21):4153–70.
94. Hamano T, Gendron TF, Causevic E, Yen SH, Lin WL, Isidoro C, et al. Autophagic-lysosomal perturbation enhances tau aggregation in transfectants with induced wild-type tau expression. Eur J Neurosci. 2008;27(5):1119–30.
95. Bi X, Haque TS, Zhou J, Skillman AG, Lin B, Lee CE, et al. Novel cathepsin D inhibitors block the formation of hyperphosphorylated tau fragments in hippocampus. J Neurochem. 2000;74(4):1469–77.
96. Bednarski E, Lynch G. Cytosolic proteolysis of tau by cathepsin D in hippocampus following suppression of cathepsins B and L. J Neurochem. 1996;67(5):1846–55.
97. Caccamo A, Magri A, Medina DX, Wisely EV, Lopez-Aranda MF, Silva AJ, et al. mTOR regulates tau phosphorylation and degradation: implications for Alzheimer's disease and other tauopathies. Aging Cell. 2013;12(3):370–80.
98. Inoue K, Rispoli J, Kaphzan H, Klann E, Chen EI, Kim J, et al. Macroautophagy deficiency mediates age-dependent neurodegeneration through a phospho-tau pathway. Mol Neurodegener. 2012;7:48.
99. Kruger U, Wang Y, Kumar S, Mandelkow EM. Autophagic degradation of tau in primary neurons and its enhancement by trehalose. Neurobiol Aging. 2012;33(10):2291–305.
100. Rodriguez-Martin T, Cuchillo-Ibanez I, Noble W, Nyenya F, Anderton BH, Hanger DP. Tau phosphorylation affects its axonal transport and degradation. Neurobiol Aging. 2013;34(9):2146–57.
101. Li L, Zhang X, Le W. Autophagy dysfunction in Alzheimer's disease. Neurodegener Dis. 2010;7(4):265–71.
102. Maday S, Wallace KE, Holzbaur EL. Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. J Cell Biol. 2012;196(4):407–17.
103. Maday S, Holzbaur EL. Autophagosome assembly and cargo capture in the distal axon. Autophagy. 2012;8(5):858–60.

104. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys. 2007;462(2):245–53.
105. Correia SC, Santos RX, Cardoso S, Carvalho C, Candeias E, Duarte AI, et al. Alzheimer disease as a vascular disorder: where do mitochondria fit? Exp Gerontol. 2012;47(11):878–86.
106. Saxton WM, Hollenbeck PJ. The axonal transport of mitochondria. J Cell Sci. 2012;125(Pt 9):2095–104.
107. DuBoff B, Feany M, Gotz J. Why size matters—balancing mitochondrial dynamics in Alzheimer's disease. Trends Neurosci. 2013;36(6):325–35.
108. Wild P, Dikic I. Mitochondria get a Parkin' ticket. Nat Cell Biol. 2010;12(2):104–6.
109. Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol. 2011;12(1):9–14.
110. Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, et al. Mitochondrial abnormalities in Alzheimer's disease. J Neurosci. 2001;21(9):3017–23.
111. Moreira PI, Siedlak SL, Wang X, Santos MS, Oliveira CR, Tabaton M, et al. Autophagocytosis of mitochondria is prominent in Alzheimer disease. J Neuropathol Exp Neurol. 2007;66(6):525–32.
112. Moreira PI, Siedlak SL, Wang X, Santos MS, Oliveira CR, Tabaton M, et al. Increased autophagic degradation of mitochondria in Alzheimer disease. Autophagy. 2007;3(6):614–5.
113. Shaerzadeh F, Motamedi F, Minai-Tehrani D, Khodagholi F. Monitoring of neuronal loss in the hippocampus of Abeta-injected rat: autophagy, mitophagy, and mitochondrial biogenesis stand against apoptosis. Neuromolecular Med. 2014;16(1):175–90.
114. Khandelwal PJ, Herman AM, Hoe HS, Rebeck GW, Moussa CE. Parkin mediates beclin-dependent autophagic clearance of defective mitochondria and ubiquitinated Abeta in AD models. Hum Mol Genet. 2011;20(11):2091–102.
115. Witte ME, Bol JG, Gerritsen WH, van der Valk P, Drukarch B, van Horssen J, et al. Parkinson's disease-associated parkin colocalizes with Alzheimer's disease and multiple sclerosis brain lesions. Neurobiol Dis. 2009;36(3):445–52.
116. Amadoro G, Corsetti V, Florenzano F, Atlante A, Ciotti MT, Mongiardi MP, et al. AD-linked, toxic NH2 human tau affects the quality control of mitochondria in neurons. Neurobiol Dis. 2014;62:489–507.
117. Caccamo A, Majumder S, Richardson A, Strong R, Oddo S. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. J Biol Chem. 2010;285(17):13107–20.
118. Majumder S, Richardson A, Strong R, Oddo S. Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. PLoS One. 2011;6(9):e25416.
119. Liu Y, Su Y, Wang J, Sun S, Wang T, Qiao X, et al. Rapamycin decreases tau phosphorylation at Ser214 through regulation of cAMP-dependent kinase. Neurochem Int. 2013;62(4):458–67.
120. Caccamo A, De Pinto V, Messina A, Branca C, Oddo S. Genetic reduction of Mammalian target of rapamycin ameliorates Alzheimer's disease-like cognitive and pathological deficits by restoring hippocampal gene expression signature. J Neurosci. 2014;34(23):7988–98.
121. Jiang T, Yu JT, Zhu XC, Tan MS, Wang HF, Cao L, et al. Temsirolimus promotes autophagic clearance of amyloid-beta and provides protective effects in cellular and animal models of Alzheimer's disease. Pharmacol Res. 2014;81:54–63.
122. Jiang T, Yu JT, Zhu XC, Zhang QQ, Cao L, Wang HF, et al. Temsirolimus attenuates tauopathy in vitro and in vivo by targeting tau hyperphosphorylation and autophagic clearance. Neuropharmacology. 2014 (in press).
123. Forlenza OV, de Paula VJ, Machado-Vieira R, Diniz BS, Gattaz WF. Does lithium prevent Alzheimer's disease? Drugs Aging. 2012;29(5):335–42.
124. Li L, Zhang S, Zhang X, Li T, Tang Y, Liu H, et al. Autophagy enhancer carbamazepine alleviates memory deficits and cerebral amyloid-beta pathology in a mouse model of Alzheimer's disease. Curr Alzheimer Res. 2013;10(4):433–41.

Chapter 8
Autophagy as a Neuroprotective Mechanism Against 3-Nitropropionic Acid-Induced Cell Death

Maria F. Galindo, Sara Saez-Atienzar, Luis Bonet-Ponce and Joaquín Jordan

Abstract The natural environmental toxin 3-Nitropropionic acid (3-NP) is used to induce situations somehow similar to those that take place in human patients suffering the progressive neurodegenerative illness known as Huntington's disease (HD). Biochemical studies have shown reduced activities of complex II-III in the striatum of human HD brain tissues. 3-NP, structurally similar to succinate, is an inhibitor of succinate dehydrogenases or respiratory complex II. As a consequence, 3-NP stimulates Ca^{2+}release from mitochondria, decreases mitochondrial membrane potential, induces mitochondrial swelling, stimulates cytochrome c release from mitochondria and leads to a rapid decline of ATP levels. Autophagy, defined as the lysosomal digestion of a cell's own cytoplasmic material, exerts its functions under conditions that require energy and use of intracellular nutrients. Under these conditions, autophagy shows a rapid increase, permitting the release of substrates used for the maintenance of ATP levels and the new synthesis of proteins that plays a fundamental role in stress adaptation. Autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stress, including ATP depletion, calcium

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oxidative stress and mitochondrial disruption. Autophagy participates in the turnover of mitochondria, a strictly regulated process called mitophagy. So, autophagy maintaining cell metabolic balance plays an important role in cell fate. Indeed, autophagy is essential for the survival of neurons, since they cannot dilute the level of altered proteins and damaged organelles by means of cell division. We herein summarize and discuss the relevance of intracellular autophagic pathways activated by 3-NP, thus trying to shed light on the potential mechanism underlying the involvement of autophagy in HD model. Finally, we summarize the role of pharmacological modulators in changing 3-NP-induced autophagy and their effects on cell survival.

Keywords Mitophagy · Mitochondrial dynamic · Huntington disease · Neurodegeneration · Apoptosis · Necrosis · Mitochondria · Reactive oxygen species

Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
3-MA	3-methyladenine
3-NP	3-nitropropionic acid
AMPK	AMP-activated protein kinase
ATG	Autophagic related gene
CMA	Chaperone-mediated autophagy
ETC	Electron transport chain
HD	Huntington disease
HDAC6	histone deacetylase 6 protein
htt	huntingtin protein
JNK	Jun N-terminal kinase
LKB1 kinase	Liver kinase BQ
MPT	Membrane permeability transition
MPTP	Mitochondrial permeability transition pores
mTOR	Mammalian Target of Rapamycin
ROS	Reactive oxygen species
TCA	Tricarboxylic acid cycle

Introduction

Experimental models provide the pharmacologist with the opportunity to explore key features underlying neurodegenerative diseases. So, neuroscientists use drugs that, either in animal model or in vitro conditions, induce situations somehow similar to those that take place in human patients with neurodegenerative diseases. We have to say that these tools are far from real clinical scenarios, and under some conditions, the investigators use them in ideal settings that do not resemble pathological conditions. Even so, they further investigation into questions that cannot otherwise be easily addressed, either because they are too expensive, unethical or logistically

too complex. Also, they are the best way to learn and reach conclusions on what could be happening in the brain or in a neuron.

Huntington's disease (HD) is an autosomal dominant, progressive neurodegenerative disease in which a single mutation in the gene responsible for the huntingtin protein (htt) leads to a primarily striatal and cortical neuronal loss. The clinical symptoms of HD include progressive motor, cognitive, emotional deficits and psychiatric disability and ultimately death. Several studies suggest involvement of mitochondrial dysfunction in HD [1–3]. Biochemical studies have shown reduced activities of complex II-III in the striatum of human HD brain tissues [4]. Administration of 3-nitropropionic acid (3-NP) in rodents and non-human primates has provided useful experimental models for Huntington disease[5–7].

Autophagy has an important role in cell fate as well as in maintaining cell metabolic balance. The absence of growth factors, or amino acids, particularly leucine, represents promoting conditions for autophagy. Amino acids do not need hormones to regulate such a dynamic process and the type of amino acid involved depends on the cell and tissues [8]. Autophagy exerts functions in conditions that require energy and use of intracellular nutrients, as shown by its induction under conditions of hypoxia and metabolic or oxidative stress [9]. Under these conditions, autophagy shows a rapid increase, permitting the release of substrates used for maintenance of ATP levels and the new synthesis of proteins that plays a fundamental role in stress adaptation [9].

Herein we provide an overview of the role of autophagy in Huntington disease, focusing particularly on 3-NP model.

The 3-Nitropropionic Acid

3-nitropropionic acid ($C_3H_5NO_4$) is also known as 3-Nitropropanoic acid, Bovinocidin, Hiptagenic acid, beta-Nitropropionic acid, Propanoic acid, and 3-nitro-, 3-Nitropropionate. 3-NP is a natural environmental toxin produced by various plants (i.e. Fabaceae family) and fungal (i.e. Aspergillus and Pennicillium) species.

3-NP is structurally similar to succinate (Fig. 1), having succinate dehydrogenase (also known as succinate-coenzyme Q reductase or respiratory Complex II) as pharmacological target. Succinate dehydrogenase, located in the inner mitochondrial membrane, is a member of the Krebs tricarboxylic acid cycle (TCA) oxidizing succinate to fumarate and an entry point for electrons into the respiratory chain at the level of ubiquinol. It consists of a large flavoprotein subunit containing covalently bound FAD, an iron-sulfur protein [10]. The normal reaction pathway involves a

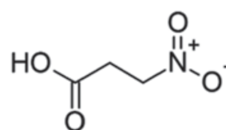

Fig. 1 3-Nitropropionic acid. Chemical structure

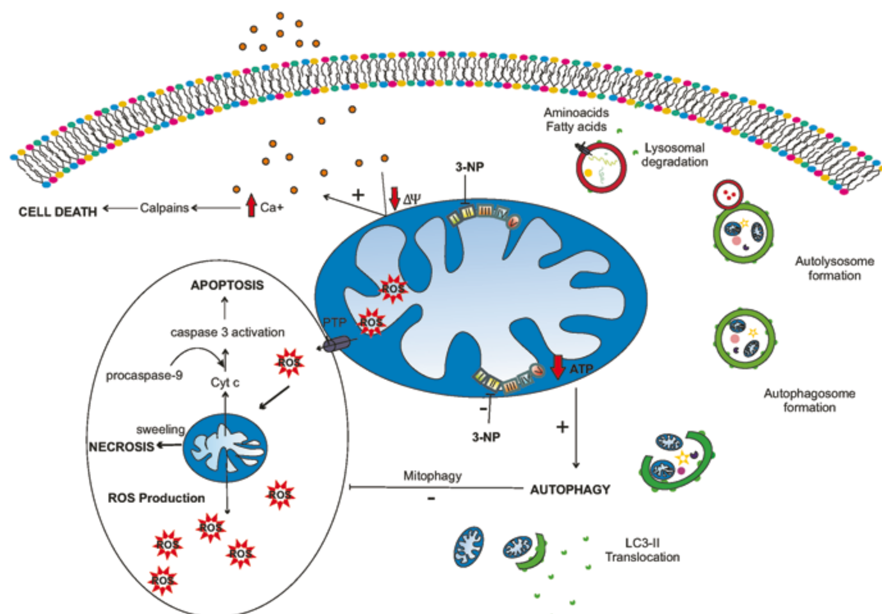

Fig. 2 Intracellular autophagic pathways activated by 3-Nitropropionic acid

temporary adduct with the N-5 nitrogen of flavin, which in the case of 3-NP collapses to a stable adduct resulting in permanent inactivation [10]. As a consequence, 3-NP induces the generation of reactive oxygen species (ROS), mitochondrial DNA damage, loss of mitochondrial function [11] and leads also to a rapid decline of ATP levels [12] (Fig. 2). Other mitochondrial inhibitors such as rotenone, paraquat and malonate share these effects. 3-NP significantly induces disruption of mitochondrial energy metabolism, and the subsequent decrease in cellular ATP levels can induce p53 protein expression in striatum probably preceding the DNA damage [13].

In rats treatment with 3-NP result in a dose-dependent decrease in the number of viable neurons but 3-NP did not affect the viability of non-neuronal cells. In primary cultures of rat hippocampal neurons, 3-NP induces rapid necrosis and a delayed apoptosis. The balance between the two depends on extracellular glutamate levels [14]. Other studies, with synaptic vesicle preparations from brain of adult rats indicate that low concentrations of 3-NP are able to selectively prevent vesicular glutamate storage, and this may represent at least one of the mechanisms responsible for the neurotoxic effects of 3-NP [15].

Huntington-Induced Autophagic-Pathway

Autophagy, a term coined by Christian De Duve in the 1960s, is taken from the Greek words *auto* “self” *phagy* “eating” to describe a intracellular degradation process [16]. Since its very early description, autophagy has been defined as the

lysosomal digestion of a cell´s own cytoplasmic material. Based on how substrates are delivered from the lysosomal compartment, autophagy is classified into three general subtypes: micro-autophagy, chaperone-mediated autophagy (CMA) and macro-autophagy [17, 18]. It is reported that micro- and macro-autophagy can occur in the same organism and at the same time, just like mitochondrial degradation [19].

Macroautophagy is referred to as autophagy. Autophagy is characterized by the sequestration of cytoplasmic material in a double-membrane structure to form a unique vesicle called the autophagosome for bulk degradation by lysosomes (Fig. 2). Under basal conditions, lysosomal degradation clears the autophagosome and other contents from the cell, thus maintaining a "balance" between autophagosome formation and degradation. An accumulation of autophagosomes could represent either an increase in formation or a decrease in fusion events.

Autophagy is essential for the survival of neurons. Indeed, in neurons, as in any other post-mitotic cell, the role of autophagy in maintaining neuronal homeostasis is crucial, since they cannot dilute the level of altered proteins and damaged organelles by means of cell division. Wide arrays of human diseases and conditions have been shown to be intimately linked to alterations in autophagy such as cancer, heart disease, aging and neurodegenerative diseases. In this line, autophagic dysfunctions are referred as a secondary pathological mechanisms for various neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, and Huntington disease [20].

Autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stress, including ATP depletion, calcium oxidative stress and mitochondrial disruption.

- **ATP depletion.** Partial reduction of intracellular ATP causes a decrease in proteolytic flux, and in the uptake of cytosolic components into the autophagic-lysosomal compartment [21]. This bulk form of degradation generates free amino and fatty acids that can be recycled in a cell-autonomous fashion or delivered systemically to distant sites within the organism. The amino acids released from autophagic degradation can be further processed and, together with the fatty acids, used by the TCA to maintain cellular ATP production. Reduction in cellular ATP levels dramatically changes the ATP:ADP:AMP ratio, thus increasing AMP and ADP levels. These changes are detected by AMPK. As a matter of fact, ADP and AMP can directly activate AMPK, leading to an increase of AMPK which inhibits mammalian target of rapamycin (mTOR). ULK1 is no longer repressed and activates the different autophagy-complexes (Beclin1, VPS34…) initiating the phagofore formation.

A number of studies have shown that HD patients are in negative energy balance [22, 23]. In addition, well-recognized metabolic deficits occur in the brain and muscle of HD patients. There is glucose hypometabolism on positron emission tomography imaging, even in pre-symptomatic gene carriers [24, 25]. NMR spectroscopy reveals increased lactate in the cerebral cortex and basal ganglia, and impaired phosphocreatine and ATP production in muscle, in both HD patients and pre-symptomatic gene carriers [26–28].

- Calcium (Ca^{2+}) acts as a critical second messenger for extracellular and intracellular signaling and is fundamental in cell life and death decisions [29, 30].

Høyer-Hansen et al. showed that after increasing cytoplasmic Ca^{2+} by using different Ca^{2+} mobilizing agents (vitamin D_3 compounds, ionomycin, ATP, and thapsigargin), autophagy flux dramatically increased [31]. Ca^{2+} seems to regulate autophagy by activating the CaMKK-β/AMPK pathway, which finally inhibits mTOR-inducing autophagy. Moreover, Ca^{2+}-induced autophagy appears to be beclin1/PI3K class III and Atg5 dependent [32] like most classic autophagy machinery proteins [33]. In addition, calmodulin enables production of Ca^{2+}/calmodulin-PI3 kinase hVPS34 cascade, which is essential for phagosome formation [34]. 3NP induces elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) [35, 36].

- **Oxidative stress**. Many stimuli that induce reactive oxygen species generation also trigger autophagy, including nutrient starvation, mitochondrial toxins and oxidative stress [37]. Oxidative stress has deep effect on the lysosomal membrane damage by oxidizing proteins. Specific forms of ROS and reactive nitrogen species include hydrogen peroxide (H_2O_2), superoxide (O_2^-) and nitric oxide (NO) among others. Superoxide anions are mainly produced from electron transport chain (ETC) complexes I and III, and seems to be a major ROS regulator of autophagy. In fact, O_2^- production (by starvation, exogenous H_2O_2 addition, SOD inhibition or mETC blockade) leads to an increase of autophagy by activation of the beclin1/PI3K class III pathway [38] or through activating AMPK, then inhibiting mTOR negative regulatory pathway [38]. Besides, H_2O_2 activates autophagy by inhibiting Atg4 and therefore preventing the LC3-II to LC3-I conversion, hence increasing the number of autophagosomes [39]. So, H_2O_2 could bind to the Cys 81 site of Atg4 and help generate thiol, a reduced form of Atg4 that promotes autophagosome maturation and its fusion with lysosome [40]. On the contrary, NO inhibits autophagy by decreasing JNK activity and Bcl2 phosphosrylation and activating mTORC1 [41]. We and others have shown 3-NP increases intracellular ROS, which could be blocked by the presence of the *mitochondrial permeability transition pores (MPTP)* inhibitor cyclosporine A [42]. These data together with those published by Liot et al., place the ROS production downstream of mitochondrial fragmentation [12].
- **Mitophagy.** Autophagy also participates in the turnover of mitochondria, whose dysfunction represents an important pathogenic mechanism of cell death in HD. Mitochondria degradation is a strictly regulated process called mitophagy [43]. Other organelle-specific autophagy has been described as ribophagy, pexophagic [19, 44, 45]. Depolarized mitochondria produced by fission process are an easy target to mitophagy [43]. The specificity of mitophagy pathway was first demonstrated by using the proton ionophore CCCP to uncouple the respiratory chain or photo-irradiation and depolarize individual mitochondria [46, 47]. Modulation of mitochondrial shape is one of the many important steps in mitophagy and has been proven by manipulation of pro-fission proteins Fis1 and Drp1 and appearance of fragmented mitochondria prior to mitophagy [48]. Mitophagy controls the mitochondrial quality by removing of unhealthy or damaged mitochondria. Indeed, altered mitochondria release apoptotic factors to the cytosol, thereby inducing apoptosis [49]. Somehow, mitophagy has a cytoprotective effect, which is not simply a function of autophagy liberating fuels for cells, but appears to be

related to a decrease in the amount of mitochondria. Mitophagy, in its turn, results in a weaker release of toxic molecules like cytochrome c from mitochondria in response to pro-apoptotic insults.

3-NP induces a membrane permeability transition (MPT), which plays an important role in the mechanism of cell death [42]. 3-NP stimulates Ca^{2+} release from mitochondria, decreases membrane potential, induces mitochondrial swelling and stimulates cytochrome c release from mitochondria [50–54].

- **Misfolded and aggregate proteins.** Studies have found that autophagy is involved in degradation of disease-causing misfolded proteins in certain neurodegenerative diseases and thus may exert beneficial influence on neuronal survival [55, 56]. A classic hallmark of HD and other poliQ diseases is the accumulation of mutant htt proteins aggregated in a perinuclear structure called aggresome. Briefly, mutant ubiquitinated htt proteins that cannot be degraded by the UPS system are recognized by the histone deacetylase 6 protein (HDAC6) and travel through microtubules to the microtubule-organizing center in the vicinity of the nucleus. Then, LC3-II in the autophagosome membrane recognizes p62 protein attached to ubiquitinated-htt proteins, in order to degrade those aggresomes, after fusion with the lysosome. Another crucial cell mechanism involved in HD related aggrephagy is acetylation of the lysine residue 444 of the mutant htt. Indeed, mutant htt resistant to acetylation accumulates as aggresomes and increases neurodegeneration in different HD models. Interestingly, HDAC inhibition decreases the number of aggresomes and ameliorates HD-related phenotypes in mouse models of HD.

At basal levels autophagy plays a vital role in keeping cell homeostasis [57] Autophagy may promote cell survival as it removes potentially toxic substances from cells [55, 58].

Autophagic Machinery in Huntington Disease

Through genetic analysis, autophagy is found as a multidirectional pathway. Autophagy is evolutionarily well preserved from early eukaryotes to mammals with as many as 30 autophagic related genes (ATGs) identified in yeasts, and their human orthologs. In conditions of cellular energy deprivation, autophagy is triggered by the AMP-activated protein kinase (AMPK), whose activation is, in turn, induced by the liver kinase BQ (LKB1 kinase). AMPK activation induces ULK1 forming AMPK/ULK1. This interaction plays a critical role in the autophagic process through the phosphorylation of rapto by AMPK that blocks the inhibitory effects exerted by mTOR on the ULK complex [59].

An exhaustive albeit clear explanation of the mechanisms involved in autophagy under 3-NP challenge would be too intricate, all the more so if we take into account that autophagy is a multifaceted process. Therefore, it is crucial to understand the

potential mechanism underlying the involvement of autophagy in Huntington disease model.

- The ATG genes are organized into five functional groups, consisting of Atg1/ULK initiation complex, Vps34/PI3-kinase nucleation complex, phohpatidylinositol 3-phphospate (PI3P)-binding Atg18/Atg2 complex, the Atg5-Atg12 conjugation system, and the Atg8/LC3-PE (Atg8/LC3-phospohatidylethanolamine) conjugation system, respectively [60]. The mitochondrial outer membrane recruits the autophagy proteins ATG5 and LC3. They are recruited not for the autophagic removal of mitochondria, but to provide the anchorage and share lipid moieties required for the elongation of the initial phagophore [61]. Loss of ATG5 and ATG7 also significantly increases accumulation of damaged mitochondria [62, 63]. Additionally, the lack of the ATG3-ATG12 conjugation leads to mitochondrial fragmentation and loss of mitophagy [64].
- LC3 is a mammalian homologue of Apg8p yeast. In its cytosolic form it is known as LC3-I, however when proteolitically processed and covalently conjuged to phosphatidylethanolamine on the autophagosomal membrane it is known as LC3-II [65]. LC3-II is required for the formation of autophagosomes and has been defined as a marker of autophagosomes in mammalian cells [66]. In SH-SY5Y cell cultures, 3-NP addition increased the number of LC3 dots [42]. Moreover, increased LC3-II protein expression is noted at 9 weeks and sustained throughout the disease course in HdhQ150 knock-in mice [67].
- P62 is a multifunctional protein that has been shown to bind ubiquitin and LC3, possibly providing a molecular shuttle for misfolded aggregated proteins to promote their clearance by autophagy. Rue et al studied protein levels and intracellular distribution of p62 in R6/1 mice, showing that protein levels decreased in all brain regions analyzed early in the disease, whereas at late stages they accumulated in the striatum and hippocampus, but not in the cortex [68]. In addition, 3-NP induced depletion in p62 protein levels in SH-SY5Y cell cultures [42].
- Beclin1, a component of the PI3 kinase complex [69], is the orthologous of yeast Apg6/Vps30 gene, and postulated to be the major inducer of the autophagy [70]. Beclin-1 is bound to the anti-apoptotic protein Bcl-2 in nutrient-rich condition. But, when nutrients are scarce, Bcl-2 is phosphorylated by Jun N-terminal kinase 1 (JNK1), thereby achieving the dissociation of Bcl-2 from beclin1 [71]. Meanwhile, down-regulation of Bcl-2 amplifies autophagy activation and apoptotic signaling. Bcl-2 thus plays important roles in mitochondria dysfunction-induced apoptotic death of striatal neurons by modulating both autophagic and apoptotic processes [72]. 3-NP up-regulates the expression of beclin1 and down-regulates Bcl-2 expression. 3-NP-induced striatal death is controlled at least in part by a genetic program involving the activation of the JNK pathway. Garcia and coworkers using a dominant negative c-Jun blocked model showed how chronic infusion of 3-NP activates the JNK pathway specifically in the striatum [73]. Furthermore, 3-NP also induced Beclin-1 levels in rat primary cortical astrocyte cultures [74].

- The mammalian target of rapamycin threonine is cytoplasmic signaling in negative manner for autophagy by kinase. The positive regulator of mTOR is Akt, which is involved in the formation of autophagosomes and also the in autophagy inhibition [75]. Intracellular signals including nutrient starvation, low energy levels, hypoxia, and DNA damage inhibit mTOR activity. Inhibition of mTOR complex 1 (mTORC1), which leads to dephosphorylation and activation of the ULK1-ATG13-FIP200 complex is a crucial initiating step in autophagy [76].

Sequestration of mTOR, in polyglutamine aggregates, impairs its kinase activity and induces autophagy. In fact, the specific mTOR inhibitor rapamycin attenuates huntingtin accumulation and cell death. Furthermore, rapamycin protects against neurodegeneration in a fly model of Huntington disease, and the rapamycin analog CCI-779 improved performance on four different behavioral tasks and decreased aggregate formation in a mouse model of Huntington disease [77].

- The evolutionarily preserved lysosomal protein DNA Damage Regulated Autophagy Modulator1 (DRAM1) has been reported to play an essential role in TP53-dependent autophagy activation [78]. In addition, 3NP significantly induced p53 protein expression in striatum probably preceding the DNA damage [13]. The TP53 specific inhibitor pifithrin-α (PFT-α) blocked induction of autophagic proteins including LC3-II and beclin1, and apoptotic proteins including TP53-upregulated modulator of apoptosis (PUMA) and BAX.

Some other pathways regulating autophagy are standardized by cyclic AMP, inositol triphosphate (IP3) receptor and calpains [79].

Pharmacological Modulation of 3-np-Induced Autophagy

Taking all the above described information together, it shows the potential to become a remarkable therapeutic approach via the pharmacologic manipulation of autophagy. Several studies revealed that induction of autophagy leads to a decrease in both aggregated and soluble monomeric htt as well as mutant huntingtin fragments, and mitigates their toxicity in various HD models [77].

Although Htt does not directly contain "KFERQ" Hsc70-binding motif in its sequence, phosphorylation of Htt serine 16 where the phosphorylated serine resembles a glutamic acid can provide a similar motif. The ability of phosphomimetic Htt to interact with Hsc70 in vitro was tested and it has been found that mimicking the phosphorylation of Htt serines 13 and 16 on the unexpanded polyQ htt increases the in vitro binding of Htt to Hsc70 by a specific ADP-dependent mechanism [80]. In addition, acetylation at K444 or K9 of Htt can directly influence mHtt in HD by facilitating the autophagic clearance [80, 81].

Next, we briefly try to summarize the role of pharmacological modulators on changed 3-NP-induced autophagy and their effects on cell survival, so:

- Rapamycin, an antifungal macrolide, stimulates autophagy by inhibiting mTOR. By doing so, rapamycin enhances the clearance of aggregate-prone proteins and reduces the appearance of aggregates and cell death [82]. In this line, rapamycin reduces Htt aggregate formation in x57 cell s [77]. Still, rapamycin protects against neurodegeneration in a HD fly model, and the rapamycin analog CCI-779 improved performance on four different behavioral tasks and decreased aggregate formation in a mouse model of Huntington disease [77]. Floto et al. identified compounds that enhance the growth-inhibitory effects of rapamycin in the budding yeast Saccharomyces cerevisiae, which we termed small molecule enhancers of rapamycin (SMERs). Of them, three SMERs induced autophagy independently, or downstream of mTOR, in mammalian cells, and furthermore enhanced the clearance of a mutant huntingtin fragment in Huntington's disease cell models. These SMERs also protected against mutant huntingtin fragment toxicity in Drosophila [83].
- Bafilomycin A1, known inhibitor of the late phase of autophagy. Bafilomycin A1, inhibiting vacuolar H^+ATPase, prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes [84]. Bafilomycin A1 reduces the size of 3-NP-induced striatal damage in rats [13].
- 3-Methyladenine (3-MA) is a nucleotide derivative that blocks class III PI3K activity and has been widely used for identifying autophagy functions. [85, 86]. 3-MA significantly inhibited the 3-NP-induced alterations in beclin 1 and Bcl-2 protein levels and attenuated the 3-NP-induced release of cytochrome c [72]. The addition of 3-MA increased Htt aggregate formation in x57 cells [77].
- Trehalose, a disaccharide present in many non-mammalian species, is an mTOR-independent autophagy activator. Trehalose-induced autophagy enhanced the clearance of autophagy substrates like mutant Htt. Furthermore, trehalose together with rapamycin exerted an additive effect on the clearance of aggregate-prone proteins. By inducing autophagy, trehalose also protects cells against subsequent pro-apoptotic insults via the mitochondrial pathway [87].
- Minocycline, a tetracycline member family, has been shown to induce autophagy [88], perhaps by inhibition of the mTOR complex [89]. Minocycline effects on neurological disorders remain controversial (for review see [90]). Minocycline is being tested in HD patients and preliminary observations are encouraging [91].
- Nutraceuticals, such as the flavonoid polyphenol EGCG, curcumin and resveratrol, may modulate autophagy in other neurodegenerative diseases including Parkinson and function as neuroprotective agents [92, 93].

References

1. Chaturvedi RK, Flint Beal, M. Mitochondrial diseases of the brain. Free Radic Biol Med. 2013;63:1–29.
2. Jordan J, Cena V, Prehn JH Mitochondrial control of neuron death and its role in neurodegenerative disorders. J Physiol Biochem. 2003;59:129–41.
3. Galindo MF, Ikuta I, Zhu X, Casadesus G, Jordan J. Mitochondrial biology in Alzheimer's disease pathogenesis. J Neurochem. 2010;114:933–45.

4. Browne SE, Beal MF. The energetics of Huntington's disease. Neurochem Res. 2004;29:531–46.
5. Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci. 1993;13:4181–92.
6. Ludolph AC, He F, Spencer PS, Hammerstad J, Sabri, M. 3-Nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. Can J Neurol Sci. 1991;18:492–8.
7. Brouillet E. The 3-NP model of striatal neurodegeneration. Curr Protoc Neurosci. 2014;67:9.48.1–9.48.14.
8. Lleo A, Invernizzi P, Selmi C, Coppel RL, Alpini G, Podda M, Mackay IR, Gershwin ME. Autophagy: highlighting a novel player in the autoimmunity scenario. J Autoimmun. 2007;29:61–8.
9. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132:27–42.
10. Huang LS, Sun G, Cobessi D, Wang AC, Shen JT, Tung EY, Anderson VE, Berry EA. 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. J Biol Chem. 2006;281:5965–72.
11. Kim GW, Chan PH. Involvement of superoxide in excitotoxicity and DNA fragmentation in striatal vulnerability in mice after treatment with the mitochondrial toxin, 3-nitropropionic acid. J Cereb Blood Flow Metab. 2002;22:798–809.
12. Liot G, Bossy B, Lubitz S, Kushnareva Y, Sejbuk N, Bossy-Wetzel E. Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA- and ROS-dependent pathway. Cell Death Differ. 2009;16:899–909.
13. Zhang XD, Wang Y, Wang Y, Zhang X, Han R, Wu JC, Liang ZQ, Gu ZL, Han F, Fukunaga K, Qin ZH. p53 mediates mitochondria dysfunction-triggered autophagy activation and cell death in rat striatum. Autophagy. 2009;5:339–50.
14. Pang Z, Geddes JW. Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis. J Neurosci. 1997;17:3064–73.
15. Maurer FD. Livestock, a world food resource threatened by disease. J Am Vet Med Assoc. 1975;166:920–3.
16. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ. 2009;16:966–75.
17. Cuervo AM. Autophagy: many paths to the same end. Mol Cell Biochem. 2004;263:55–72.
18. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. Biochem J. 2012;441:523–40.
19. Kissova I, Salin B, Schaeffer J, Bhatia S, Manon S, Camougrand N. Selective and non-selective autophagic degradation of mitochondria in yeast. Autophagy. 2007;3:329–36.
20. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013;19:983–97.
21. Schellens JP, Meijer AJ. Energy depletion and autophagy. Cytochemical and biochemical studies in isolated rat hepatocytes. Histochem J. 1991;23:460–6.
22. Djousse L, Knowlton B, Cupples LA, Marder K, Shoulson I, Myers RH. Weight loss in early stage of Huntington's disease. Neurology. 2002;59:1325–30.
23. Pratley RE, Salbe AD, Ravussin E, Caviness JN. Higher sedentary energy expenditure in patients with Huntington's disease. Ann Neurol. 2000;47:64–70.
24. Antonini A, Leenders KL, Spiegel R, Meier D, Vontobel P, Weigell-Weber M, Sanchez-Pernaute R, de Yebenez JG, Boesiger P, Weindl A, Maguire RP. Striatal glucose metabolism and dopamine D2 receptor binding in asymptomatic gene carriers and patients with Huntington's disease. Brain. 1996;119(Pt 6):2085–95.
25. Feigin A, Leenders KL, Moeller JR, Missimer J, Kuenig G, Spetsieris P, Antonini A, Eidelberg D. Metabolic network abnormalities in early Huntington's disease: an [(18)F]FDG PET study. J Nucl Med. 2001;42:1591–5.
26. Jenkins BG, Koroshetz WJ, Beal MF, Rosen BR. Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized 1H NMR spectroscopy. Neurology. 1993;43:2689–95.

27. Koroshetz WJ, Jenkins BG, Rosen BR, Beal MF. Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. Ann Neurol. 1997;41:160–5.
28. Lodi R, Schapira AH, Manners D, Styles P, Wood NW, Taylor DJ, Warner TT. Abnormal in vivo skeletal muscle energy metabolism in Huntington's disease and dentatorubropallidoluysian atrophy. Ann Neurol. 2000;48:72–6.
29. Berridge MJ, Lipp P, Bootman MD. Signal transduction. The calcium entry pas de deux. Science. 2000;287:1604–5.
30. Perez-Alvarez S, Solesio ME, Cuenca-Lopez MD, Melero-Fernandez de Mera RM, Villalobos C, Kmita H, Galindo MF, Jordan J. Pharmacological characterization of the mechanisms involved in delayed calcium deregulation in SH-SY5Y cells challenged with methadone. Int J Cell Biol. 2012;2012:642482.
31. Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, Bianchi K, Fehrenbacher N, Elling F, Rizzuto R, Mathiasen IS, Jaattela M. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell. 2007;25:193–205.
32. Gao W, Ding WX, Stolz DB, Yin XM. Induction of macroautophagy by exogenously introduced calcium. Autophagy. 2008;4:754–61.
33. Chen X, Li M, Chen D, Gao W, Guan JL, Komatsu M, Yin XM. Autophagy induced by calcium phosphate precipitates involves endoplasmic reticulum membranes in autophagosome biogenesis. PloS one. 2012;7:e52347.
34. Vergne I, Chua J, Deretic V. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. J Exp Med. 2003;198:653–9.
35. Rosenstock TR, Carvalho AC, Jurkiewicz A, Frussa-Filho R, Smaili SS. Mitochondrial calcium, oxidative stress and apoptosis in a neurodegenerative disease model induced by 3-nitropropionic acid. J Neurochem. 2004;88:1220–8.
36. Milakovic T, Johnson GV. Mitochondrial respiration and ATP production are significantly impaired in striatal cells expressing mutant huntingtin. J Biol Chem. 2005;280:30773–82.
37. Jangamreddy JR, Ghavami S, Grabarek J, Kratz G, Wiechec E, Fredriksson BA, Rao Pariti RK, Cieslar-Pobuda A, Panigrahi S, Los MJ. Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: differences between primary and cancer cells. Biochim Biophys Acta. 2013;1833:2057–69.
38. Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 2009;16:1040–52.
39. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J. 2007;26:1749–60.
40. Scherz-Shouval R, Shvets E, Elazar Z. Oxidation as a post-translational modification that regulates autophagy. Autophagy. 2007;3:371–3.
41. Sarkar S, Korolchuk VI, Renna M, Imarisio S, Fleming A, Williams A, Garcia-Arencibia M, Rose C, Luo S, Underwood BR, Kroemer G, O'Kane CJ, Rubinsztein DC. Complex inhibitory effects of nitric oxide on autophagy. Mol Cell. 2011;43:19–32.
42. Solesio ME, Saez-Atienzar S, Jordan J, Galindo MF. 3-Nitropropionic acid induces autophagy by forming mitochondrial permeability transition pores rather than activating the mitochondrial fission pathway. Br J Pharmacol. 2013;168:63–75.
43. Novak I. Mitophagy: a complex mechanism of mitochondrial removal. Antioxid Redox Signal. 2012;17:794–802.
44. Manjithaya R, Nazarko TY, Farre JC, Subramani S. Molecular mechanism and physiological role of pexophagy. FEBS lett. 2010;584:1367–73.
45. Suzuki K. Selective autophagy in budding yeast. Cell Death Differ. 2013;20:43–8.
46. Kim I, Lemasters JJ. Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. Antioxid Redox Signal. 2011;14:1919–28.
47. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol. 2008;183:795–803.
48. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, Shirihai OS. Fission and

selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 2008;27:433–46.
49. Lemasters JJ, Qian T, He L, Kim JS, Elmore SP, Cascio WE, Brenner DA. Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. Antioxid Redox Signal. 2002;4:769–81.
50. Nishimura M, Okimura Y, Fujita H, Yano H, Lee J, Suzaki E, Inoue M, Utsumi K, Sasaki J. Mechanism of 3-nitropropionic acid-induced membrane permeability transition of isolated mitochondria and its suppression by L-carnitine. Cell Biochem Funct. 2008;26:881–91.
51. Bayram-Weston Z, Jones L, Dunnett SB, Brooks SP. Light and electron microscopic characterization of the evolution of cellular pathology in the R6/1 Huntington's disease transgenic mice. Brain Res Bull. 2012;88:104–12.
52. Bayram-Weston Z, Jones L, Dunnett SB, Brooks SP. Light and electron microscopic characterization of the evolution of cellular pathology in YAC128 Huntington's disease transgenic mice. Brain Res Bull. 2012;88:137–47.
53. Bayram-Weston Z, Jones L, Dunnett SB, Brooks SP. Light and electron microscopic characterization of the evolution of cellular pathology in HdhQ92 Huntington's disease knock-in mice. Brain Res Bull. 2012;88:171–81.
54. Bayram-Weston Z, Torres EM, Jones L, Dunnett SB, Brooks SP. Light and electron microscopic characterization of the evolution of cellular pathology in the Hdh(CAG)150 Huntington's disease knock-in mouse. Brain Res Bull. 2012;88:189–198.
55. Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biology. 2005;25:1025–40.
56. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8:741–52.
57. Mizushima N. Autophagy: process and function. Genes Dev. 2007;21:2861–73.
58. Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pangalos MN, Schmitt I, Wullner U, Evert BO, O'Kane CJ, Rubinsztein DC. Rapamycin alleviates toxicity of different aggregate-prone proteins. Hum Mol Genet. 2006;15:433–442.
59. Gianchecchi E, Delfino DV, Fierabracci A. Recent insights on the putative role of autophagy in autoimmune diseases. Autoimmun Rev. 2014;13:231–41.
60. Gelino S, Hansen M. Autophagy—an emerging anti-aging mechanism. J Clin Exp Pathol. 2012;Suppl 4.
61. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;141:656–67.
62. Stephenson LM, Miller BC, Ng A, Eisenberg J, Zhao Z, Cadwell K, Graham DB, Mizushima NN, Xavier R, Virgin HW, Swat W. Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes. Autophagy. 2009;5:625–35.
63. Mortensen M, Soilleux EJ, Djordjevic G, Tripp R, Lutteropp M, Sadighi-Akha E, Stranks AJ, Glanville J, Knight S, Jacobsen SE, Kranc KR, Simon AK. The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. J Exp Med. 2011;208:455–67.
64. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, Fung C, Debnath J. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. Cell. 2010;142:590–600.
65. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. Nat Cell Biol. 2007;9:1102–9.
66. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19:5720–8.
67. Heng MY, Detloff PJ, Paulson HL, Albin RL. Early alterations of autophagy in Huntington disease-like mice. Autophagy. 2010;6:1206–8.
68. Rue L, Lopez-Soop G, Gelpi E, Martinez-Vicente M, Alberch J, Perez-Navarro E. Brain region- and age-dependent dysregulation of p62 and NBR1 in a mouse model of Huntington's disease. Neurobiol Dis. 2013;52:219–28.

69. Funderburk SF, Wang QJ, Yue Z. The Beclin 1-VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol. 2010;20:355–62.
70. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol. 2007;9:218–24.
71. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol Cell 2008;30:678–88.
72. Zhang XD, Wang Y, Wu JC, Lin F, Han R, Han F, Fukunaga K, Qin ZH. Down-regulation of Bcl-2 enhances autophagy activation and cell death induced by mitochondrial dysfunction in rat striatum. J Neurosci Res. 2009;87:3600–10.
73. Garcia M, Vanhoutte P, Pages C, Besson MJ, Brouillet E, Caboche J. The mitochondrial toxin 3-nitropropionic acid induces striatal neurodegeneration via a c-Jun N-terminal kinase/c-Jun module. J Neurosci. 2002;22:2174–84.
74. Pereira GJ, Tressoldi N, Hirata H, Bincoletto C, Smaili, SS Autophagy as a neuroprotective mechanism against 3-nitropropionic acid-induced murine astrocyte cell death. Neurochem Res. 2013;38:2418–26.
75. Ferte C, Andre F, Soria JC. Molecular circuits of solid tumors: prognostic and predictive tools for bedside use. Nat Rev Clin Oncol. 2010;7:367–80.
76. Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. Journal Biol Chem. 2009;284:12297–305.
77. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet. 2004;36:585–95.
78. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell. 2006;126:121–34.
79. Metcalf DJ, Garcia-Arencibia M, Hochfeld WE, Rubinsztein DC. Autophagy and misfolded proteins in neurodegeneration. Exp Neurol. 2012;238:22–8.
80. Thompson LM, Aiken CT, Kaltenbach LS, Agrawal N, Illes K, Khoshnan A, Martinez-Vincente M, Arrasate M, O'Rourke JG, Khashwji H, Lukacsovich T, Zhu YZ, Lau AL, Massey A, Hayden MR, Zeitlin SO, Finkbeiner S, Green KN, LaFerla FM, Bates G, Huang L, Patterson PH, Lo DC, Cuervo AM, Marsh JL, Steffan JS. IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. J Cell Biol. 2009;187:1083–99.
81. Jeong H, Then F, Melia TJ Jr., Mazzulli JR, Cui L, Savas JN, Voisine C, Paganetti P, Tanese N, Hart AC, Yamamoto A, Krainc D. Acetylation targets mutant huntingtin to autophagosomes for degradation. Cell. 2009;137:60–72.
82. Ravikumar B, Duden R, Rubinsztein DC. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum Mol Genet. 2002;11:1107–17.
83. Floto RA, Sarkar S, Perlstein EO, Kampmann B, Schreiber SL, Rubinsztein DC. Small molecule enhancers of rapamycin-induced TOR inhibition promote autophagy, reduce toxicity in Huntington's disease models and enhance killing of mycobacteria by macrophages. Autophagy. 2007;3:620–2.
84. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct. 1998;23:33–42.
85. Blommaart EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem/FEBS. 1997;243:240–6.
86. Xue L, Fletcher GC, Tolkovsky AM. Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. Mol Cell Neurosci. 1999;14:180–98.

87. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. 2007;282:5641–52.
88. Liu WT, Lin CH, Hsiao M, Gean PW. Minocycline inhibits the growth of glioma by inducing autophagy. Autophagy. 2011;7:166–75.
89. Bruning A, Brem GJ, Vogel M, Mylonas I. Tetracyclines cause cell stress-dependent ATF4 activation and mTOR inhibition. Exp Cell Res. 2014;320:281–9.
90. Jordan J, Fernandez-Gomez FJ, Ramos M, Ikuta I, Aguirre N, Galindo MF. Minocycline and cytoprotection: shedding new light on a shadowy controversy. Curr Drug Deliv. 2007;4:225–31.
91. Bonelli RM, Heuberger C, Reisecker F. Minocycline for Huntington's disease: an open label study. Neurology. 2003;60:883–4.
92. Wu Y, Li X, Zhu JX, Xie W, Le W, Fan Z, Jankovic J, Pan T. Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. Neuro-Signals. 2011;19:163–74.
93. Giordano S, Darley-Usmar V, Zhang J. Autophagy as an essential cellular antioxidant pathway in neurodegenerative disease. Redox Biol. 2014;2:82–90.

Chapter 9
Paraquat: Molecular Mechanisms of Neurotoxicity and its Relation with Autophagy

Maria Teresa Baltazar, Ricardo Jorge Dinis-Oliveira, Maria de Lourdes Bastos and Félix Carvalho

Abstract Paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride; PQ) is an effective and widely used herbicide in Asiatic and American countries with a claimed safety record when appropriately applied to eliminate weeds. However, over the last decades a growing body of epidemiologic evidence has been linking long-term/low-dose PQ exposure to the development of Parkinson's disease (PD). PQ is well known for its ability to induce oxidative stress, mitochondrial dysfunction, α-synuclein fibrillation and neuronal cell loss. More recently, more attention has been given to the role of autophagy in several major neurodegenerative diseases and the influence of environmental toxins in this pathway. This chapter provides an overview of the main mechanisms of neurotoxicity of PQ with an emphasis in the autophagic process and its possible relationship to PD.

Keywords Paraquat · Oxidative stress · Autophagy · Neurotoxicity · Neurodegenerative diseases

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Abbreviations

AP-1	Activator protein 1
ASK1	Apoptosis signal regulating kinase
JNK	c-Jun N-terminal kinase
DA	Dopamine
MPP^+	1-methyl-4-phenylpyridine
NF-κB	Nuclear factor kappa B
PD	Parkinson's disease
PQ	Paraquat
ROS	Reactive oxygen species
SN	*Substantia nigra*

Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride; PQ) [CAS number 1910-42-5] was one of the most commonly used herbicides of the bipyridylium quaternary ammonium family. PQ was introduced in the market in 1962 by the Imperial Chemical Industries, and since its introduction it has been used all over the world in more than 130 countries, although nowadays it is only registered and sold in about two thirds of those countries. In the past decade, the use of PQ has been controversial, as it appears to be, in one hand, a cheap option for farmers to control weeds and, in another hand, extremely toxic to humans, animals and aquatic organisms. For this reason, PQ was banned from the European Union in 2007, and recently other countries have been restricting its use or applying a phase-out procedure. The fatalities due to PQ poisoning continue to be a heavy burden worldwide with a rate of fatalities between 30–80% [1, 2]. PQ poisoning is usually characterized by multi-organ failure, but the toxicity is particularly severe in the lung [3] due to selective and energy-dependent accumulation in this organ through a polyamine uptake system [4]. It is generally accepted that the primary mechanism of PQ toxicity is associated with its capacity to undergo redox-cycling, through activation by diaphorases, and subsequent generation of superoxide radical and related reactive oxygen species (ROS), and activation of important inflammatory signaling pathways involving the nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) [5, 6].

Besides the toxicity of PQ on peripheral organs, other observations have led to the hypothesis that long-term exposure to pesticides, and to PQ in particular, may be an etiological factor of Parkinson's disease (PD) and other neurodegenerative diseases [4, 7]. Recently, case-control studies, cohort studies and cross-sectional studies were combined in two meta-analyses [8, 9]. Their systematic review indicates that PD is associated to pesticide exposure, namely to herbicides and insecticides [8, 9]. Despite previous epidemiological studies showing a strong association between PQ exposure and development of PD, the causal relationship remains to be fully accepted [10–16]. The main reasons for the skeptical opinions regarding PQ neurotoxicity is the lack of fully unbiased epidemiological studies and the inherent

properties of PQ as an herbicide. Firstly, PQ is a hydrophilic compound, poorly absorbed through the skin and the respiratory tract when properly applied. Secondly, even if PQ is systemically absorbed, the concentrations that might reach the brain (and the specific nigrostriatal system) are likely to be much lower than the doses used in experimental studies [7]. Corroborating this, the few occupational studies performed have shown that even after a dermal exposure of PQ during application, urine levels were either undetectable or very low [17, 18]. Additionally, other occupational studies do not report the quantification of PQ in biological samples and therefore the extension of the PQ absorbed through skin and by the respiratory tract is unknown [19, 20]. In addition, the follow up of survivors of PQ poisoning have reported that ingestion of high doses of PQ does not lead to long-term deleterious effects on brain [21].

In spite of the scientific disputes concerning the linking of long-term/low-dose PQ exposure to the development PD, it is an unsurmountable fact that experimental data confirms the neurotoxic potential of PD. This chapter provides an overview of the main mechanisms of neurotoxicity of PQ with an emphasis in the autophagic process and its possible relationship to PD.

Mechanisms of PQ Neurotoxicity

Experimental data have shown that PQ is able to reproduce some of the features of PD such as specific cell loss, accumulation and increased aggregation of α-synuclein in dopaminergic neurons of *substantia nigra* (SN), but does not induce clear motor deficits [22]. Several pathways have been proposed to be involved in the neurotoxicity of PQ: (i) induction of oxidative stress, (ii) microglia activation, (iii) mitochondrial dysfunction, (iv) apoptosis, (v) induction of synucleinopathy and tauopathy, and (v) autophagy and inhibition of the ubiquitin-proteasome system.

Oxidative Stress

PQ neurotoxicity is related to its ability to undergo redox cycling, accepting an electron from several cellular diaphorases (enzymes that transfer one electron from NAD(P)H), mainlycytoplasmic NADPH oxidases of Nox family and NADPH-cytochrome P450 reductase, to form the monocation radical ($PQ.^{+}$)with subsequent reduction of O_2 to superoxide (O_2^{-}) [23]. The $O_2{\bullet}^{-}$ produced can react with nitric oxide (NO) to form peroxynitrite ($ONOO^{-}$)or be dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). In the presence of iron (II), H_2O_2 is reduced to hydroxyl radical ($HO^{\cdot}$), a far more damaging ROS that rapidly oxidizes DNA, proteins and lipids [4, 7, 24]. Accordingly, *in vitro* experiments show that PQ increases the levels of malondialdehyde and protein carbonyls, as well as DNA fragmentation in neuroblastoma SH-SY5Y cells [25]. PQ exposure was also shown to cause depletion of glutathione and an increase in the biomarkers of lipid peroxidation and nitrosylation

(4-hydroxinonenal and nitrotyrosine, respectively), accompanied by a dose-dependent loss of nigrostriatal dopaminergic neurons in the SN of C57BL/6 mice [26, 27].

In recent years, the involvement of mitochondria in the mechanism of ROS production by PQ has emerged. Animal models overexpressing mitochondrial antioxidant enzymes (catalase and peroxiredoxin 5) or deficient in mitochondrial manganese superoxide dismutase and glutathione peroxidase have shown the important role of mitochondria in PQ-induced toxicity [28–30]. Also at the neuronal level PQ has been shown to cause mitochondrial damage [31, 32] by inhibiting the complexes I and III, although this concept is not consensual between authors [33, 34]. In addition, PQ was shown to induce mitochondrial-aconitase-dependent increase in H_2O_2, Fe^{2+} and death of the rat dopaminergic cell line, N27, as seen by the attenuation of H_2O_2 production when mitochondrial-aconitase expression was reduced by RNA interference [35].

The other major source of ROS and inflammatory signaling molecules derive from activated microglia, the resident macrophages in the brain [36]. Within microglia PQ increases NADPH oxidase expression possibly due to the ROS produced by the PQ redox cycle, mitochondria or after activation of NF-κB [36, 37]. Activated microglia releases inflammatory cytokines and O_2^- radicals, which damage the adjacent neurons. This mechanism might explain the intriguing feature that when microglial activation is induced by pre-treatment with lipopolysaccharide, a single PQ exposure is sufficient trigger loss of dopaminergic neurons [38].

Apoptosis

PQ induces selective neurodegeneration in dopaminergic neurons in the substantia nigra pars compacta, triggering different mechanisms of cell death. Apoptosis induced by PQ has been shown to involve mainly the intrinsic mitochondrial pathway, and, more recently, some evidences suggest the contribution of endoplasmatic reticulum stress and an autophagic process in neuronal cell death [reviewed in [7]]. Release of cytochrome *c* and activation of caspase-9 was shown to be associated to the induction of Bcl-2 family members such as Bak, Bid, BNip3, and NOXA in neuroblastoma cells suggesting that PQ neurotoxicity is mediated by a Bak-dependent mechanism [39, 40]. Endoplasmatic reticulum calcium homeostasis and endoplasmatic reticulum functions are believed to be impaired in various degenerative diseases of the brain including Alzheimer's disease, PD, Huntington's disease, and amyotrophic lateral sclerosis. Activation of inositol-requiring enzyme 1, apoptosis signal regulating kinase (ASK1), C/EBP homologous protein, and stressed-activated kinases lead to the activation/induction of pro-apoptotic Bcl-2 family members, which promotes the crosstalk between endoplasmatic reticulum and the mitochondria-triggered apoptotic pathway, including release of cytochrome *c* from mitochondria and activation of caspase-3 [41]. c-Jun N-terminal kinase (JNKs) are mitogen-activated protein (MAP) kinases that are preferentially activated by cellular stress including environmental stress and toxic chemical insults. It has been suggested that JNK and p38 kinase, play a critical role in the PQ-induced neuro-

degeneration process [42, 43]. It seems that ASK1 acts upstream of JNK and p38 kinases throughout the phosphorylation of MKK3/6 and MKK4/7 [44]. Sequential phosphorylation of JNK leads to the activation of caspase-3, and p53 transcription factors [45, 46] culminating in apoptosis. More recently, it was suggested that PQ-induced apoptosis is mediated through the Nrf-2-regulated mitochondrial dysfunction and endoplasmic reticulum stress [47]. Moreover, Niso-Santano et al. investigated the role of the transcription factor Nrf2, a master regulator of cytoprotective genes, and its target thioredoxin, which binds and inhibits ASK1. PQ induced a dose-dependent decrease in thioredoxin levels correlated with a major increase in phosphorylated ASK1 inhuman dopaminergic neuroblastoma SH-SY5Y cells, suggesting that Nrf2/thioredoxin is crucial in PQ-induced apoptosis [48, 49].

Autophagy

Progressive loss of dopaminergic neurons in the nigrostriatal system and deposition of filamentous α-synuclein aggregates are the main characteristics of PD [50]. α-Synuclein is a natively unfolded protein that plays a central role in the control of synaptic membrane processes and biogenesis, but when it becomes misfolded, aggregates, and accumulates in neuronal inclusion bodies, the Lewy bodies [51]. PQ markedly induces the *in vitro* conformational changes in α-synuclein, and accelerates the rate of aggregation of α-synuclein [52, 53]. α-synuclein overexpression induces the formation of membrane pore-like structures that increase membrane conductance [54]. Co-exposure with PQ and dopamine enhances α-synuclein -induced leak channel conductivity, leading to a disruption of ionic imbalance, and eventually dopaminergic cell death [55]. *In vivo* experiments have corroborated these results, showing that PQ treatment induces the accumulation of α-synuclein and hyperphosphorylation of Tau within the specific sites of phosphorylation found in PD *post mortem* striata [56, 57]. PQ, 1-methyl-4-phenylpyridine (MPP^+), and rotenone, but not maneb are known to induce synucleinopathy and tauopathy in striata of mice [57–61]. In addition to abnormal expression of -synuclein, alterations in protein degradation pathways have been implicated in PD by both genetic and pathological studies in PD patients as well as experimental studies in disease models [50, 51, 62].

The ubiquitin-proteasome system and autophagy are the major degradation pathways that neurons depend on to maintain protein homeostasis, responsible for the degradation of misfolded, oxidized, and aggregated proteins. While the ubiquitin-proteasome system degrades most short-lived, soluble proteins, autophagy is the bulk degradation process by which longer-lived macromolecules, damaged and aged organelles are directed for lysossomal-mediated degradation by acid hydrolases [63]. Relevant to PD, knockout mouse models with either genetic depletion of 26S proteasomes or macroautophagy function exhibit profound neurodegeneration and formation of inclusion bodies [64, 65, 66]. In PD, ubiquitin-proteasome system is impaired possibly due to depletion of ATP levels caused by mitochondrial dysfunction, oxidative stress or in cases of familial PD, mutations in *parkin* and *UCHL1* genes [67].

In 2003, Bonifati and colleagues found a large deletion and missense mutation in the *DJ-1* gene in Italian and Dutch PD patients, leading to identification of the DJ-1 gene as a causative gene for familial autosomal-recessive early-onset PD [68]. DJ-1 is a multifunctional protein that participates in transcriptional regulation, antioxidative stress reaction, and chaperone, protease, and mitochondrial regulation. Importantly, DJ-1 acts as a scavenger of ROS by self-oxidation of its cysteine residues, leading to the formation of oxidized DJ-1. In the case of sporadic PD, DJ-1 expression is induced in cells upon oxidative stress to prevent cell death. During the course of continuous oxidative stress, DJ-1 is oxidized giving rise to inactive DJ-1 that ultimately causes PD [see review [69]]. Interestingly, highly oxidized DJ-1 has been observed in patients with PD and Alzheimer's disease [70]. The role of DJ-1 in autophagy is still in debate, and almost all of the studies are focused on mitochondria-specific autophagy, mitophagy or dedicated to investigate the role of other proteins involved in the etiology of autosomal-recessive PD such as PINK1 and Parkin. Loss of DJ-1 leads to impaired autophagy and accumulation of dysfunctional mitochondria that under physiological conditions would be compensated via lysosomal clearance [71]. Further investigations suggested that DJ-1 works in parallel with PINK1/parkin pathway to maintain mitochondrial function in the presence of an oxidative environment [72]. In this context, Gonzalez-Polo and colleagues attempted to investigate the possible link between genetic and environmental factors in the pathogenesis of PD by exposing DJ-1 siRNA human neuroblastoma SH-SY5Y cells to PQ. Both insults acted synergistically with an enhanced apoptosis process, mitochondrial dysfunction and increase in mTOR phosphorylation [73]. The downregulation of DJ-1 inhibited the autophagic events induced by the herbicide and exacerbated cell death in the presence of the autophagy inhibitor 3-methyladenine. Interestingly, PQ significantly decreased the DJ-1 protein levels in non-transfected SH-SY5Y cells. This reduction was accompanied by mitochondrial cytochrome c release and typical apoptotic chromatin condensation [73]. DJ-1deficient mice exposed to PQ showed impaired proteasome activity and increased ubiquitinated protein levels. Nevertheless, the authors claimed that PQ exposure or deficient in *DJ-1* gene alone did not stimulate a decrease in proteasome activity showing a relationship between an environmental toxin and genes. The same study also showed that 19S ATPase Rpt6 and 20S 5 subunits and a transcription factor Nrf2 were decreased in *DJ-1*-deficient mice treated with PQ [74]. On opposition, more recent studies have shown that administrationof PQ (10 mg/kg i.p), twice per week for 6 weeks, significantly reduce the 26S proteolytic activity without loss of either 19S or 20S components or changes in the assembly of the 26S proteasome leading to the reduction of the proteasomal activity [57]. In view of the fact that PQ induces overexpression of α-synuclein and p-tau, it is expected that proteasomal activity would be reduced, due in part to the direct inhibitory effects of α-synuclein and p-Tau [57]. In addition it seems that PQ-induced increases in α-synuclein aggregate levels and disruption of ubiquitin-proteasome system are likely to interfere with autophagy.

Wills and colleagues have also shown that the inhibition of ubiquitin-proteasome system was accompanied by dysregulation of autophagy. PQ treatment (10 mg/kg i.p, twice per week for 6 weeks),increased levels of the autophagy inhibitor, the

mammalian target of rapamycin, mTOR, suggesting impaired axonal autophagy, despite increases in the autophagic proteins beclin 1 and Agt12. The ratios of LC3 II to LC3 Iwere reduced in treated animals, suggesting an impairment of the autophagic flux while the heat shock protein levels were either increased or unchanged upon PQ-treatment, implying that chaperone-mediated autophagy is not hampered by PQ [57]. On the other hand,*in vitro* results have shown different results. Exposure to low concentrations of PQ produces a ROS burst, followed by the characteristic autophagic events in human neuroblastoma SH-SY5Y cells, such as the accumulation of autophagic vacuoles in the cytoplasm and the recruitment of the LC3-GFP fusion protein to autophagic vacuoles, protein degradation, and a marked decrease in the phosphorylation (Ser2448) status of mTOR [75]. In this study, it seems that PQ triggers autophagy as a defense mechanism against the deleterious effects of oxidative stress since when autophagy is inhibited, the apoptotic cell death process is accelerated. Therefore, contradictory results have been reported, and both autophagy-dependent cell death and survival mechanisms have been suggested to regulate dopaminergic cell death induced by MPP^+ [76], whereas both stimulation and inhibition of autophagy have been reported to be induced by PQ [57, 75]. Autophagy acts as a protective mechanism promoting cell survival in response to a variety of toxic stimuli, whereas, only in very few cases, autophagy participates as a programmed cell death mechanism. In order to clarify the role of autophagy in dopaminergic cell death induced by environmental toxins, Garcia-Garcia and colleagues focused in the autophagic flux and the role of autophagy protein 5 (Atg5)-dependent autophagy in dopaminergic cell death induced by parkinsonian toxins [77]. Accumulation of autophagosomes might be associated with either an increase in or an impairment of autophagic flux. Inhibition of Atg5-dependent autophagy potentiates PQ and MPP^+-induced cell death and thus autophagy seems to exert a protective effect against the toxicity of these neurotoxins. The authors have shown that these results demonstrate that PQ and the complex I inhibitors, $MPP^+$and rotenone, induce an accumulation of autophagosomes by impairment of autophagy flux, whereas6-hydroxydopamine does it by increasing autophagy rate [77].

Concluding Remarks

Several pathways have been proposed to be involved in the neurotoxicity of PQ, including the induction of oxidative stress, microglia activation, mitochondrial dysfunction, apoptosis, induction of synucleinopathy and tauopathy, and autophagy and inhibition of the ubiquitin-proteasome system. In this chapter, the main mechanisms of neurotoxicity of PQ were discussed, with an emphasis in the autophagic process and its possible relationship to PD.

Autophagy is a lysosomal degradative process used to recycle old cellular constituents and eliminate damaged organelles and protein aggregates [78]. Due to the large axons expansions, neurons accumulate dysfunctional organelles and cellular waste in the extremities and therefore the autophagic vacuoles produced need to

efficientlyreach lysosomes in the cell body. Any disturbance in this balance makes neurons extremely vulnerable and not surprisingly, mutations of genes regulating autophagy cause neurodegenerative diseases across the age spectrum with exceptional frequency. Autophagy can be disturbed at three major different stages—selection, sequestration and lysosomal digestion of substrates. Although is not clear yet where PQ disturbs the autophagic process it seems that it affects the first stages, substrate sequestration and autophagosome formation and substrate recognition. In addition it remains inconclusive the role of autophagy in PQ-induced neurotoxicity, whether is a compensatory mechanism of cell death in response to the oxidative insult, or if in circumstances of a continuous insult culminates in the impairment of the autophagic process.

References

1. Lee K, Park EK, Stoecklin-Marois M, Koivunen ME, Gee SJ, Hammock BD, et al. Occupational paraquat exposure of agricultural workers in large Costa Rican farms. Int Arch Occup Environ Health. 2009;82(4):455–62.
2. Bertolote JM, Fleischmann A, Eddleston M, Gunnell D. Deaths from pesticide poisoning: a global response. Br J Psychiatry. 2006;189:201–3.
3. Smith P, Heath D. The pathology of the lung in paraquat poisoning. J Clin Pathol Suppl (R Coll Pathol). 1975;9:81–93.
4. Dinis-Oliveira RJ, Remiao F, Carmo H, Duarte JA, Navarro AS, Bastos ML, et al. Paraquat exposure as an etiological factor of Parkinson's disease. Neurotoxicology. 2006;27(6):1110–22.
5. Dinis-Oliveira RJ, Sousa C, Remiao F, Duarte JA, Ferreira R, Sanchez Navarro A, et al. Sodium salicylate prevents paraquat-induced apoptosis in the rat lung. Free Radic Biol Med. 2007;43(1):48–61.
6. Dinis-Oliveira RJ, Sousa C, Remiao F, Duarte JA, Navarro AS, Bastos ML, et al. Full survival of paraquat-exposed rats after treatment with sodium salicylate. Free Radic Biol Med. 2007;42(7):1017–28.
7. Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, Tsatsakis AM, Duarte JA, Carvalho F. Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases-A mechanistic approach. Toxicol Lett. 2014;230:85–103.
8. van der Mark M Brouwer M Kromhout H Nijssen P Huss A Vermeulen R. Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. Environ Health Perspect. 2012;120(3):340–7.
9. Van Maele-Fabry G Hoet P Vilain F Lison D. Occupational exposure to pesticides and Parkinson's disease: a systematic review and meta-analysis of cohort studies. Environ Int. 2012;46:30–43.
10. Costello S, Cockburn M, Bronstein J, Zhang X, Ritz B. Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. Am J Epidemiol. 2009;169(8):919–26.
11. Firestone JA, Smith-Weller T, Franklin G, Swanson P, Longstreth WT, Checkoway H. Pesticides and risk of Parkinson disease a population-based case-control study. Arch Neurol-Chicago. 2005;62(1):91–5.
12. Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B. Well-water consumption and Parkinson's disease in rural California. Environ Health Perspect. 2009;117(12):1912–8.
13. Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, et al. Pesticide exposure and self-reported Parkinson's disease in the agricultural health study. Am J Epidemiol. 2007;165(4):364–74.

14. Mandel JS, Adami HO, Cole P. Paraquat and Parkinson's disease: An overview of the epidemiology and a review of two recent studies. Regul Toxicol Pharm. 2012;62(2):385–92.
15. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 2011;119(6):866–72.
16. Wang A, Costello S, Cockburn M, Zhang XB, Bronstein J, Ritz B. Parkinson's disease risk from ambient exposure to pesticides. Eur J Epidemiol. 2011;26(7):547–55.
17. Chester G, Gurunathan G, Jones N, Woollen BH. Occupational exposure of Sri Lankan tea plantation workers to paraquat. Bull World Health Organ. 1993;71(5):625–32.
18. Van Wendel de Joode BN De Graaf IAM Wesseling C Kromhout H. Paraquat exposure of Knapsack spray operators on banana plantations in Costa Rica. Int J Occup Environ Health. 1996;2(4):294–304.
19. Dalvie MA, White N, Raine R, Myers JE, London L, Thompson M, et al. Long-term respiratory health effects of the herbicide, paraquat, among workers in the Western Cape. Occup Environ Med. 1999;56(6):391–6.
20. Machado-Neto JG, Matuo T, Matuo YK. Efficiency of safety measures applied to a manual knapsack sprayer for paraquat application to maize (Zea mays L.). Arch Environ Contam Toxicol. 1998;35(4):698–701.
21. Brent J, Schaeffer TH. Systematic review of parkinsonian syndromes in short- and long-term survivors of paraquat poisoning. J Occup Environ Med. 2011;53(11):1332–6.
22. Cicchetti F, Drouin-Ouellet J, Gross RE. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci. 2009;30(9):475–83.
23. Dinis-Oliveira RJ, Duarte JA, Sanchez-Navarro A, Remiao F, Bastos ML, Carvalho F. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit Rev Toxicol. 2008;38(1):13–71.
24. Cristovao AC, Choi DH, Baltazar G, Beal MF, Kim YS. The role of NADPH oxidase 1-derived reactive oxygen species in paraquat-mediated dopaminergic cell death. Antioxid Redox Sign. 2009;11(9):2105–18.
25. Yang WS, Tiffany-Castiglioni E. The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: Relevance to the dopaminergic pathogenesis. J Toxicol Env Heal A. 2005;68(22):1939–61.
26. Kang MJ, Gil SJ, Koh HC. Paraquat induces alternation of the dopamine catabolic pathways and glutathione levels in the substantia nigra of mice. Toxicol Lett. 2009;188(2):148–52.
27. McCormack AL, Atienza JG, Johnston LC, Andersen JK, Vu S, Di Monte DA. Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. J Neurochem. 2005;93(4):1030–7.
28. Van Remmen H, Qi W, Sabia M, Freeman G, Estlack L, Yang H, et al. Multiple deficiencies in antioxidant enzymes in mice result in a compound increase in sensitivity to oxidative stress. Free Radical Bio Med. 2004;36(12):1625–34.
29. Mockett RJ, Bayne ACV, Kwong LK, Orr WC, Sohal RS. Ectopic expression of catalase in Drosophila mitochondria increases stress resistance but not longevity. Free Radical Bio Med. 2003;34(2):207–17.
30. Tien Nguyen-nhu N, Knoops B. Mitochondrial and cytosolic expression of human peroxiredoxin 5 in Saccharomyces cerevisiae protect yeast cells from oxidative stress induced by paraquat. Febs Lett. 2003;544(1–3):148–52.
31. Czerniczyniec A, Lores-Arnaiz S, Bustamante J. Mitochondrial susceptibility in a model of paraquat neurotoxicity. Free Radic Res. 2013;47(8):614–23.
32. Czerniczyniec A, Karadayian AG, Bustamante J, Cutrera RA, Lores-Arnaiz S. Paraquat induces behavioral changes and cortical and striatal mitochondrial dysfunction. Free Radic Biol Med. 2011;51(7):1428–36.
33. Cocheme HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. J Biol Chem. 2008;283(4):1786–98.
34. Castello PR, Drechsel DA, Patel M. Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. J Biol Chem. 2007;282(19):14186–93.

35. Cantu D, Fulton RE, Drechsel DA, Patel M. Mitochondrial aconitase knockdown attenuates paraquat-induced dopaminergic cell death via decreased cellular metabolism and release of iron and H_2O_2. J Neurochem. 2011;118(1):79–92.
36. Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA. Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. Neurobiol Dis. 2007;25(2):392–400.
37. Mangano EN, Hayley S. Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: neuroimmune sensitization of neurodegeneration. Neurobiol Aging. 2009;30(9):1361–78.
38. Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA. Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. Neurobiol Dis. 2007;25(2):392–400.
39. Fei Q, McCormack AL, Di Monte DA, Ethell DW. Paraquat neurotoxicity is mediated by a Bak-dependent mechanism. J Biol Chem. 2008;283(6):3357–64.
40. Fei QY, Ethell DW. Maneb potentiates paraquat neurotoxicity by inducing key Bcl-2 family members. J Neurochem. 2008;105(6):2091–7.
41. Paschen W, Mengesdorf T. Endoplasmic reticulum stress response and neurodegeneration. Cell Calcium. 2005;38(3–4):409–15.
42. Klintworth H, Newhouse K, Li TT, Choi WS, Faigle R, Xia ZG. Activation of c-jun N-terminal protein kinase is a common mechanism underlying paraquat- and rotenone-induced dopaminergic cell apoptosis. Toxicol Sci. 2007;97(1):149–62.
43. Choi WS, Abel G, Klintworth H, Flavell RA, Xia Z. JNK3 mediates paraquat- and rotenone-induced dopaminergic neuron death. J Neuropathol Exp Neurol. 2010;69(5):511–20.
44. Yang W, Tiffany-Castiglioni E, Koh HC, Son IH. Paraquat activates the IRE1/ASK1/JNK cascade associated with apoptosis in human neuroblastoma SH-SY5Y cells. Toxicol Lett. 2009;191(2–3):203–10.
45. Yang W, Tiffany-Castiglioni E. Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria. J Toxicol Env Heal A. 2008;71(4):289–99.
46. Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK. The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. J Biol Chem. 2004;279(31):32626–32.
47. Chen YW, Yang YT, Hung DZ, Su CC, Chen KL. Paraquat induces lung alveolar epithelial cell apoptosis via Nrf-2-regulated mitochondrial dysfunction and ER stress. Arch Toxicol. 2012;86(10):1547–58.
48. Niso-Santano M, Bravo-San Pedro JM, Gomez-Sanchez R, Climent V, Soler G, Fuentes JM, et al. ASK1 overexpression accelerates paraquat-induced autophagy via endoplasmic reticulum stress. Toxicol Sci. 2011;119(1):156–68.
49. Niso-Santano M, Gonzalez-Polo RA, Bravo-San Pedro JM, Gomez-Sanchez R, Lastres-Becker I, Ortiz-Ortiz MA, et al. Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis. Free Radic Biol Med. 2010;48(10):1370–81.
50. Puschmann A, Bhidayasiri R, Weiner WJ. Synucleinopathies from bench to bedside. Parkinsonism Relat Disord. 2012;18(Suppl 1):S24–7.
51. Bellucci A, Navarria L, Zaltieri M, Missale C, Spano P. Alpha-synuclein synaptic pathology and its implications in the development of novel therapeutic approaches to cure Parkinson's disease. Brain Research. 2012;1432:95–113.
52. Uversky VN, Li J, Fink AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. Febs Lett. 2001;500(3):105–8.
53. Uversky VN, Li J, Bower K, Fink AL. Synergistic effects of pesticides and metals on the fibrillation of alpha-synuclein: implications for Parkinson's disease. Neurotoxicology. 2002;23(4–5):527–36.
54. Feng LR, Federoff HJ, Vicini S, Maguire-Zeiss KA. alpha-Synuclein mediates alterations in membrane conductance: a potential role for alpha-synuclein oligomers in cell vulnerability. Eur J Neurosci. 2010;32(1):10–7.

55. Feng LR, Maguire-Zeiss KA. Dopamine and paraquat enhance alpha-synuclein-induced alterations in membrane conductance. Neurotox Res. 2011;20(4):387–401.
56. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice—paraquat and alpha-synuclein. J Biol Chem. 2002;277(3):1641–4.
57. Wills J, Credle J, Oaks AW, Duka V, Lee JH, Jones J, et al. Paraquat, but not maneb, induces synucleinopathy and tauopathy in striata of mice through inhibition of proteasomal and autophagic pathways. Plos One. 2012;7(1):e30745.
58. Mitra S, Chakrabarti N, Bhattacharyya A. Differential regional expression patterns of alpha-synuclein, TNF-alpha, and IL-1 beta; and variable status of dopaminergic neurotoxicity in mouse brain after Paraquat treatment. J Neuroinflamm. 2011;8:163.
59. Hoglinger GU, Lannuzel A, Khondiker ME, Michel PP, Duyckaerts C, Feger J, et al. The mitochondrial complex I inhibitor rotenone triggers a cerebral tauopathy. J Neurochem. 2005;95(4):930–9.
60. Duka T, Duka V, Joyce JN, Sidhu A. Alpha-Synuclein contributes to GSK-3 beta-catalyzed Tau phosphorylation in Parkinson's disease models. Faseb J. 2009;23(9):2820–30.
61. Duka T, Rusnak M, Drolet RE, Duka V, Wersinger C, Goudreau JL, et al. Alpha-synuclein induces hyperphosphorylation of Tau in the MPTP model of parkinsonism. Faseb J. 2006;20(13):2302–12.
62. Coppede F. Genetics and epigenetics of Parkinson's disease. ScientificWorldJournal. 2012;2012:489830.
63. Ebrahimi-Fakhari D, Cantuti-Castelvetri I, Fan Z, Rockenstein E, Masliah E, Hyman BT, et al. Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of alpha-synuclein. J Neurosci. 2011;31(41):14508–20.
64. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 2006;441(7095):885–89.
65. Komatsu M1, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880–84.
66. Bedford L1, Hay D, Devoy A, Paine S, Powe DG, Seth R, Gray T, Topham I, Fone K, Rezvani N, Mee M, Soane T, Layfield R, Sheppard PW, Ebendal T, Usoskin D, Lowe J, Mayer RJ. Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J Neurosci. 2008;28(33):8189–98.
67. Valente EM, Arena G, Torosantucci L, Gelmetti V. Molecular pathways in sporadic PD. Parkinsonism Relat Disord. 2012;18(Suppl 1):S71–3.
68. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. 2003;299(5604):256–9.
69. Ariga H, Takahashi-Niki K, Kato I, Maita H, Niki T, Iguchi-Ariga SM. Neuroprotective function of DJ-1 in Parkinson's disease. Oxid Med Cell Longev. 2013;2013:683920.
70. Bandopadhyay R, Kingsbury AE, Cookson MR, Reid AR, Evans IM, Hope AD, et al. The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain. 2004;127(Pt 2):420–30.
71. Krebiehl G, Ruckerbauer S, Burbulla LF, Kieper N, Maurer B, Waak J, et al. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1. Plos One. 2010;5(2):e9367.
72. Thomas KJ, McCoy MK, Blackinton J, Beilina A, van der Brug M, Sandebring A, et al. DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Hum Mol Genet. 2011;20(1):40–50.
73. Gonzalez-Polo R, Niso-Santano M, Moran JM, Ortiz-Ortiz MA, Bravo-San Pedro JM, Soler G, et al. Silencing DJ-1 reveals its contribution in paraquat-induced autophagy. J Neurochem. 2009;109(3):889–98.
74. Yang W, Chen L, Ding Y, Zhuang X, Kang UJ. Paraquat induces dopaminergic dysfunction and proteasome impairment in DJ-1-deficient mice. Hum Mol Genet. 2007;16(23):2900–10.

75. Gonzalez-Polo RA, Niso-Santano M, Ortiz-Ortiz MA, Gomez-Martin A, Moran JM, Garcia-Rubio L, et al. Inhibition of paraquat-induced autophagy accelerates the apoptotic cell death in neuroblastoma SH-SY5Y cells. Toxicol Sci. 2007;97(2):448–58.
76. Dehay B, Bove J, Rodriguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci. 2010;30(37):12535–44.
77. Garcia-Garcia A, Anandhan A, Burns M, Chen H, Zhou Y, Franco R. Impairment of Atg5-dependent autophagic flux promotes paraquat- and MPP(+)-induced apoptosis but not rotenone or 6-hydroxydopamine toxicity. Toxicol Sci. 2013;136(1):166–82.
78. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013;19(8):983–97.

Chapter 10
Agrochemicals-Induced Dopaminergic Neurotoxicity: Role of Mitochondria-Mediated Oxidative Stress and Protein Clearance Mechanisms

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Abstract Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder that is characterized by the progressive loss of substantia nigral dopaminergic neurons resulting in the pronounced depletion of striatal DA levels which subsequently leads to the expression of cardinal features of PD including tremor, bradykinesia, rigidity and postural instability. The mechanisms underlying the selective loss of dopaminergic neurons remain poorly understood; however, studies conducted in post mortem PD brains and experimental PD models have implicated oxidative stress and mitochondrial dysfunction in the mechanism of dopaminergic neurodegeneration. In recent years, the etiology of several neurodegenerative diseases including PD has been linked to low dose and chronic exposure to a variety of agrochemicals including paraquat, rotenone and dieldrin. Here we discuss how several of these pesticides share common mechanistic events, including oxidative stress, mitochondrial impairment/complex I inhibition, abnormal protein aggregation and post translational modifications (PTMs) of proteins including α-synuclein, as well as dopaminergic cell death. Furthermore, intersecting and parallel effects of environmental neurotoxicants on protein clearance mechanisms and mitochondrial function are addressed and hence provide novel insights that might be beneficial in the development of targeted therapies for PD.

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Abbreviations

ALP	autophagolysosomal pathway
ARE	antioxidant response element
ASK1	apoptosis signal kinase 1
ATG	autophagy related gene
AV	autophagic vacuoles
BBB	blood-brain barrier
CI	confidence interval
CMA	chaperone-mediated autophagy
CNS	central nervous system
COX	cycloxygenase
DA	dopamine
DAT	dopamine transporter
L-DOPA	L-3,4-dihydroxyphenylalanine
ENS	enteric neuron system
ER	endoplasmic reticulum
ETC	electron transfer chain modification
GSK-3β	glycogen synthase kinase-3 beta
GSTT1	glutathione-S-transferase theta 1
HIF	hypoxia inducible factor
4-HNE	4-hydroxynonenal
HSC	heat shock conjugate
LAMP2	lysosome-associated membrane protein type-2
LB	Lewy body
LC3	microtubule-associated protein 1 light chain 3
LRRK2	leucine-rich repeat kinase 2

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LUHMES	Lund human mesencephalic
3-MA	3-methyladenine
MA	methamphetamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	mammalian target of rapamycin
NDI1	NADH dehydrogenase
Ndufs4	NADH dehydrogenase (ubiquinone) Fe-S protein 4
NOX	NADPH oxidase
OR	odds ratio
PD	Parkinson's disease
PINK-1	PTEN-induced putative kinase 1
PKCδ	protein kinase C delta
PQ	paraquat
PTM	posttranslational modification
UCH-L1	ubiquitin carboxyl terminal hydrolase isoenzyme L1
PTEN	phosphatase and tensin homolog
POLG	polymerase gamma
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCF	SKP1-Cullin1-F-box
SKP1	s-phase kinase-associated protein 1
SN	substantia nigra
TFAM	mitochondrial transcription factor A
UPS	ubiquitin proteasomal system
VMAT2	vesicular monoamine transporter 2
WT	wild-type

Introduction

Parkinson's disease is a devastating and progressing disorder that affects at least 1% of individuals over the age of 60 [1]. Monogenic mutations contribute to less than 10% of all PD cases while the majority of the cases are sporadic [2]. Aging has been identified as a major factor in PD, with a peak age of onset of 65 years; however, both young onset PD (defined when the disease onset is <40 years) and rare juvenile form have also been shown to occur [3]. The cardinal clinical features of PD including rigidity, resting tremor, bradykinesia and postural instability have been shown to occur when approximately 70% of nigrostriatal dopaminergic neurons are lost. The neuropathological hallmark of the disease includes the accumulation of intraneuronal inclusions known as Lewy bodies (LBs), which are predominantly composed of α-synuclein protein [4] in addition to ubiquitin, ubiquitin carboxyl terminal hydrolase isoenzyme L1 (UCH-L1), synphilin and parkin protein, among others [5], in the surviving substantia nigra (SN) dopaminergic neurons. At this

time whether Lewy bodies are toxic or neuroprotective remains elusive. In addition to the nigrostriatal neurons, the neuropathological correlates of PD are also observed in the cortex, amygdala, locus coeruleus and peripheral autonomic system [6]. Similarly, in addition to motor disturbance, a wide variety of non-motor symptoms including autonomic, sleep, olfactory dysfunctions and cognitive dysfunction have been shown to precede the onset of motor symptoms [7].

Although the role of approximately 18 genes in familial PD has been elucidated and numerous risk factors have been identified using genome-wide association studies, they still constitute only a small percentage of PD cases [8]. The identification of PD-causing gene mutations, namely, α-synuclein (SNCA), Parkin, PTEN-induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2) and UCH-L1, has created a paradigm shift in the manner research questions are addressed in PD pathogenesis [8–10]. The continued research into the cellular role of these PD-related genes indicate that mitochondrial dysfunction, impairment in protein degradation machinery and protein misfolding may play a key role in the cascade of deleterious events implicated in PD pathogenesis. However, the remaining majority of PD cases are most likely to stem from genetic susceptibility variants, environmental factors and complex gene-environment interactions [11].

Several environmental factors that are associated with the risk of development of PD have been identified, namely herbicides and pesticides (e.g., paraquat, rotenone, dieldrin and maneb), metals (e.g., manganese and lead), head trauma, well water consumption, and rural living [2, 12–17]. In this context, epidemiological studies have supported the hypothesis that occupational exposure to pesticides increases the risk of developing PD [18–24]; although, several other studies have disputed such an association [25, 26]. Despite the contradictory nature of the data, a meta-analysis of 19 studies showed a significant association between exposure to pesticides and PD with a combined odds ratio (OR) of 1.9 [27]. Thus, it is highly unlikely that exposure to a single agent might explain for a large number of cases, instead chronic exposure to multiple agents in association with the background of genetic susceptibility might account for majority of the cases [28].

The link between environmental factor and PD pathogenesis was triggered by the discovery in the early 1980s, when it was reported that a group of designer drug users exhibited clinical features reminiscent of Parkinsonism following intravenous administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a byproduct of synthetic opioid biosynthesis, which has been shown to cause dopaminergic neurodegeneration via inhibitory effects on complex I of the mitochondrial respiratory chain in animals [29–32]. Furthermore, the critical role of mitochondria in PD also comes from studies involving pesticides such as rotenone and paraquat (PQ) that bears close structural and toxicological similarity to MPTP [33, 34]. These studies illustrate that exogenous agrochemicals can target mitochondria; however, the functional interplay between mitochondrial dysfunction and oxidative stress in nigrostriatal dopaminergic neuronal loss and associated neurobehavioral deficits remain poorly understood [17, 35–39].

Despite studies demonstrating that pesticide exposure is epidemiologically linked with an increased risk for PD, interpretations of human exposure studies

have been challenging due to exposure to a combination of chemicals and interindividual differences in the variability of exposure levels [28]. Further investigations in experimental models of PD have provided novel insights into the mechanisms underlying the pathogenesis of PD. For example, *in vivo* models utilizing rodents or primates offer the system necessary to evaluate the neurodegenerative effects of individual environmental neurotoxic agent exposures. In contrast *in vitro* studies offer specific mechanistic insight into the cellular and molecular basis of neurotoxicant-induced cytotoxicity, although these studies fail to recapitulate several complex integrated functions of the human brain. In this context, the environmental risk factors, namely the putative contribution of select agrochemicals to dopaminergic neurodegeneration and its relevance to PD, are discussed using *in vitro* and *in vivo* models of pesticide exposures. In particular, the commonly shared toxicity mechanisms, including mitochondria dysfunction, oxidative stress, protein aggregation and impaired clearance mechanisms that may account for the disruption of dopaminergic neuronal function, are reviewed here.

Mitochondria and PD

Mitochondria serve as the primary energy source in neurons that heavily rely on aerobic respiration and oxidative phosphorylation for ATP generation. During oxidative phosphorylation (OXPHOS), the flow of electrons across the electron transport chain (complexes I–IV) is coupled to the pumping of protons in order to generate a mitochondrial membrane potential that drives the synthesis of ATP. Mitochondrial reactive oxygen species (ROS) and excessive generation of ROS appear to be critically linked to aging because they lead to organelle dysfunction, mutations in the nuclear genome and cell dysfunction [32, 40–43]. In addition to their role in the chronic process of aging, they also contribute to apoptotic cell death [44].

The pivotal role of mitochondrial dysfunction in the etiology of PD was recognized following the discovery of MPTP-induced Parkinsonism that was responsive to L-DOPA [45, 46]. This model has been further improved in lab animals whereby chronic infusion of rotenone, complex I inhibitor or MPTP was found to cause Parkinson phenotype and pathological changes associated with degeneration of nigral dopaminergic neurons that was accompanied by cytoplasmic inclusions that were immunoreactive for ubiquitin and synuclein [47]. Oxidative stress has been implicated as a pivotal factor in the mechanism of mitochondrial dysfunction associated neuronal injury. Decreased complex I activity or altered protein levels in the substantia nigra in postmortem PD brains [40, 48] has been well established. Mild reductions in complex I activity were also identified in the striatum [49], fibroblast [50], skeletal muscle [51], and blood platelets [52]. Interestingly, oxidized proteins were found in the catalytic subunits of complex I in PD patients, suggesting that oxidative damage to complex I subunit(s) may underlie complex I impairment. The deficits in the complex I activity have also been demonstrated in cybrid cell lines [53]. This generation of ROS subsequent to mitochondrial dysfunction may in turn further

impair the respiratory chain, thereby resulting in the production of exaggerated levels of ROS and pronounced neuronal injury [54]. Mitochondria-mediated bioenergetic deficits may interrupt the vesicular storage of DA leading to increased accumulation of the oxidation-prone DA. In fact, SN dopaminergic neurons have been shown to be highly sensitive to complex I dysfunction as compared to other neuronal phenotypes [55, 56].

Increased levels of mitochondrial DNA (mtDNA) deletions have been found in dopaminergic neurons in the substantia nigra of postmortem PD brains [57–59]. For example, the inactivation of the genes involved in the maintenance of mtDNA, namely mitochondrial transcription factor A (TFAM) and polymerase gamma (POLG), caused the disruption of complex I activity [55, 60], and thereby mitochondrial dysfunction in the mechanism of PD pathogenesis. The genetic deficiency of Ndufs4, one among the 49 subunits of complex I, demonstrated a decrease in complex I oxygen consumption, although the complex I levels were intact, suggesting that Ndufs4 may be pivotal in the assembly and stabilization of the complex. Interestingly, the oxygen consumption and ATP levels remained unchanged as compared to wild-type (WT) animals [61, 62]. Together, complex I inhibition and subsequent generation of ROS and RNS may contribute to PD pathogenesis.

Oxidative Stress and PD

Oxidative stress is defined as a mechanism, whereby the dysfunction of the cellular antioxidant system leads to the excessive buildup of ROS above a toxic threshold [54, 63]. The production of ROS is inexorably linked to mitochondrial respiration [64]. During aerobic respiration, electrons leak from the electron transfer chain (ETC) (in particular complex I) leading to the partial reduction of molecular oxygen to superoxide anion ($O_2^{\bullet}$), and subsequently to hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) production [63]. The superoxide ($O_2^{\bullet}$) is converted via an iron catalyzed reaction known as Fenton reaction to the highly reactive hydroxyl radical ($^{\bullet}OH$), which can further react with nitric oxide (NO) to form peroxynitrite. Both hydroxyl radical and peroxynitrite have been shown to cause extensive cellular damage by interacting with protein [65], lipids [66] and nucleic acids [66]. The oxidative damaged macromolecules, via a vicious feed forward cycle, might further accentuate the process of oxidative stress and neuronal injury.

Interestingly, SN dopaminergic neurons are highly susceptible to oxidative stress because of the increased energy demand necessary for the maintenance of long axonal projections. The presence of high levels of dopamine whose oxidative products are highly toxic, relative abundance of redox active iron (Fe) levels favoring hydroxyl radical formation, weaker antioxidant defense, and higher microglial population make post mitotic dopaminergic neurons prime target of aging/environmental toxicant-induced degeneration [67–70]. Markers of oxidative stress, namely products of lipid peroxidation, protein oxidation, oxidation of DNA and cytoplasmic RNA, are increased in the SN of postmortem PD brains [71]. These events are accompanied by altered iron metabolism and reduced glutathione levels

[72, 73]. In fact, mitochondria-targeted neurotoxins, which reproduce several features of PD in humans, cell and animal models of PD, frequently target complex I and leads to the formation of highly reactive free radicals that promote nigrostriatal dopaminergic neuronal injury [61]. A growing body of evidence supports a pivotal role for oxidative stress and alteration of synuclein conformation in the mechanism of dopaminergic neuronal loss evidenced in PD [74, 75]. In fact, oxidative stress has been shown to be a trigger in the posttranslational modifications (PTMs) of α-synuclein-associated neurodegeneration. PTMs of α-synuclein, such as modification by 4-HNE (HNE-α-Syn), nitration (n-α-Syn) and oxidation (o-α-Syn), have been shown to promote α-synuclein aggregation [76]. Interestingly, nitration, phosphorylation, and truncation have been observed in postmortem PD brains or in animal models of PD [77, 78]. In a recent report, exposure of differentiated LUHMES cells to HNE-α-Syn was found to trigger dopaminergic neurotoxicity via an ROS-dependent mechanism [78, 79].

Besides the ROS generated from intrinsic sources within the neuron, the components of the extracellular milieu that may be generated following neuronal injury can activate neighboring glial cells [80–84]. Importantly, glia-derived ROS has also been documented to exert deleterious effects in the brain regions that are highly susceptible to PD [85]. In fact, it has been proposed that glia-derived ROS may aggravate or promote a self-perpetuating vicious cycle of delayed and progressive loss of dopaminergic neurons.

Oxidative Stress and Agrochemicals

Rotenone

Rotenone has been used as an insecticide or fish poison during the past 50–150 years and has been identified as a potential risk factor for PD. Due to rotenone's relatively short environmental half-life and limited bioavailability, its role in PD is strongly debated [86, 87]. The half-life of rotenone is 3–5 days based on the levels of exposure to sunlight, also it is rapidly degraded in soil and water [reviewed in [88]]. As a result, rotenone is less likely to pollute the ground water. The rotenone model has garnered significant attention in the field of experimental PD and has provided novel insights into mitochondria-mediated mechanisms in dopaminergic neurodegeneration in PD. Rotenone has been shown to exhibit lipophilicity, BBB permeability, distribution throughout the body, and mitochondrial localization where it evokes inhibitory effects on the mitochondrial complex I activity [89]. In fact, chronic inhibition of mitochondrial complex I can cause selective loss of dopaminergic neurons despite its wide distribution throughout the brain [47]. It is widely accepted that rotenone elicits ROS generation via inhibition of the mitochondrial ETC complex. Rotenone has been shown to inhibit the transfer of electrons from iron-sulfur centers in complex I to ubiquinone, which interrupts the conversion of NADH into ATP [34]. In this context, rotenone-induced neuronal death is believed to

be a consequence of mitochondrial complex I inhibition, which leads to the marked reduction in ATP levels and subsequent loss of mitochondrial membrane potential [90, 91]. Consistent with its role in oxidative stress, antioxidants have been shown to confer protection against rotenone-induced cell death. Intriguingly, Arnold et al., 2011 demonstrated that chronic exposure to rotenone led to an increase in the mitochondrial fission and fusion initially, although it was reversed at later time points [92]. Also, inhibition of fission was found to attenuate neuritic loss, suggesting that aberrant mitochondrial dynamics may underlie rotenone-induced PD-like symptoms. In another study, Choi et al. [93] suggested that inhibition of mitochondrial complex I alone may not be sufficient to explain the mechanism of dopaminergic degeneration, but that depolarization of the microtubules and accumulation of dopamine-derived ROS might also be involved. However these studies are not compatible with studies from Greenamyre and colleagues [94], whereby exposure of SK-N-MC human neuroblastoma cells to rotenone (10 nM to 1 μM) was found to produce dose-dependent ATP depletion, oxidative damage and death. Interestingly, transfection with a single-subunit NADH dehydrogenase of *Saccharomyces cerevisiae* (NDI1) (which integrates into the mammalian ETC and serves as a "replacement" for endogenous complex), was found to ameliorate mitochondria-mediated oxidative stress events, suggesting that these effects were mediated by selective binding at complex I [94, 95]. Similar results were generated in *in vivo* systems, further establishing that rotenone effects may be related to inhibition of complex I [96, 97]. Intriguingly, other studies have demonstrated that rotenone-induced inhibition of mitochondrial complex I activity may lead to α-synuclein aggregation, LB deposition, motor deficits, and ultimately nigrostriatal dopaminergic neuronal loss. Also, it has been suggested that stimulation of microglial cells by rotenone leads to the generation of superoxide that is selectively toxic to dopaminergic neurons [89]. Interestingly, genetic depletion of NOX2 afforded neuroprotection against rotenone-associated dopaminergic neurodegenerative effects, indicating that aberrant NOX2-mediated microglial activation contributed to rotenone-induced dopaminergic neurotoxicity [98]. Therefore, it is most likely that rotenone-mediated complex I inhibition can cause excessive buildup of oxygen-free radicals, thereby leading to chronic oxidative stress and ultimately loss of dopaminergic neurons. Alternatively, it is also possible that chronic oxidative stress in this model may influence complex downstream signaling events that result in dopaminergic neuronal death.

Paraquat

Paraquat (N, N'-dimethyl-4-4-4'-bypiridinium) has been used as an herbicide for decades to control the growth of weeds and exposure to it has been suggested to increase the risk of developing PD [99, 100]. Also, as compared to separate exposure, those people who were exposed to PQ in combination with either ziram or maneb, demonstrated the greatest risk for developing PD [100]. PQ shares close structural similarity to MPP^+, a metabolite of MPTP. Due to this similarity, it was assumed

that PQ and MPP^+might share similar common neurotoxicity mechanisms, e.g., complex I inhibitory effects [101], although it is becoming increasingly clear that they might elicit dopaminergic neurotoxicity via disparate mechanisms [102]. PQ demonstrated a long half-life following systemic administration and has been shown to cause lipid peroxidation in rodent brain [103]. In addition, PQ administration also caused selective and dose-dependent loss of nigral dopaminergic neurons and mild reduction in striatal dopaminergic nerve terminals that were accompanied by modest motor deficits [74, 104].

PQ has been found to cross the blood-brain barrier (BBB) via the neutral amino acid transporter and to accumulate in select regions of the mouse brain, thereby inducing PD-like neuropathology [105]. In contrast to MPP^+, PQ has been shown to exert weak inhibitory effects on the mitochondrial respiratory complex I and it appears that PQ-induced dopaminergic neurotoxicity may occur independent of complex I inhibition and dopamine transporter (DAT)-mediated mechanism [106]. Alternatively, in another study, Castello et al. [107] demonstrated that inhibitory effects of PQ on mitochondrial respiratory complexes both I and III may be involved in ROS generation. Notably, autooxidative products of DA and monoamine oxidase (MAO)-mediated production of oxidative metabolites have been identified as important sources of ROS.

Despite numerous studies demonstrating the critical contribution of mitochondria mediated ROS generation in PQ-induced dopaminergic neurotoxicity, the underlying cellular mechanisms are still elusive. Interestingly, it appears that PQ is compartmentalized within the mitochondria [101] and functions as a potent redox cycler that converts molecular oxygen to superoxide and subsequently other free radicals [108, 109]. Numerous studies have highlighted the role of microglial NADPH oxidase (particularly NADPH-cytochrome P450 reductase) in catalyzing NADPH-dependent one electron reduction of PQ [107, 110, 111]. The PQ radical generated in turn donates one electron/reduces molecular oxygen in its vicinity, thereby generating superoxide and itself converts into parental compound, which depending on the electron supply, undergoes further redox cycling, thereby generating more superoxide oxide in subsequent cycles [112, 113]. The redox cycling of paraquat is further supported by studies showing that mice deficient in two mitochondrial antioxidant enzymes, namely manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx), demonstrated increased vulnerability to PQ-associated neurotoxicity as compared to WT mice [114]. Conversely, mitochondria-targeted overexpression of antioxidant enzymes, including catalase and peroxiredoxin 5, conferred better protection against PD-like neuropathology as compared to its cytoplasmic expression [115, 116]. Moreover, PQ-induced increase in H_2O_2, Fe^{2+}, and cell death were attenuated by siRNA-mediated silencing of aconitase expression, thus highlighting a critical role for the mitochondria-mediated ROS generation in the mechanism of PQ-induced dopaminergic neurotoxicity [117]. More recently, PQ has been shown to mediate oxidative stress via xanthine oxidase [118]. Finally, NF-E2-related factor-2 (Nrf2)-dependent regulation of antioxidant responsive element (ARE)-mediated gene expression has been shown to elicit neuroprotective effects against paraquat-induced neurotoxicity by induction of heme-oxygenase,

which prevents the buildup of heme-catalyzed production of hydroxyl radicals from H_2O_2 [119]. Although PQ-mediated ROS generation might originate from several sources, it is becoming increasingly clear that oxidative stress is a pivotal step in the mechanism of PQ-induced neuronal cell death. Collectively, these studies suggest that PQ can induce oxidative stress by a multitude of mechanisms involving mitochondrial complex I inhibition, redox cycling, DA turnover, and modulation of antioxidant status, among others.

Dieldrin

Dieldrin is an organochlorine pesticide that ranks one among the most environmentally persistent insecticide. Despite its ban in the 1970s, its low volatility and lipophilicity has led to its persistence in the environment and therefore demonstrates extensive bioaccumulation and biomagnification in the soil and food supply (for a review see [11, 86, 120]). Significantly increased levels of dieldrin were found in the caudate nucleus of PD patients as compared to age matched controls [121–123]. In another study, dieldrin was found in 6 out of 20 PD brains and in none of the control patients, suggesting a positive association between dieldrin exposure and risk of PD [124]. Previously, oxidative stress has been implicated in dieldrin-induced apoptosis. Treatment with dieldrin induced ROS production in a number of cell culture models, including neuronal cell lines, mouse lung fibroblasts, and in mouse liver [125–127]. Moreover, Chun et al. [126] reported that dieldrin exposure induced ROS production in SN4741, a mouse nigral dopaminergic cell line, and studies from our lab showed that dieldrin-induced ROS generation in two cell lines, namely PC12 cells [128] and N27 dopaminergic cells [126, 129]. Additionally, we demonstrated that pretreatment with SOD, an enzyme that scavenges superoxide anions attenuated dieldrin-induced ROS generation. Similarly, scavenging of superoxide anions using an SOD mimetic, Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), in PC12 cells blocked dieldrin-induced ROS generation [128]. In a recent study using N27 cells, we demonstrated that dieldrin activates the non-receptor tyrosine kinase Fyn to promote PKC delta (a redox sensitive kinase)-mediated dopaminergic cell death [130]. In order to evaluate the influence of dieldrin on dopamine turnover and associated generation of ROS, PC12 cells were exposed to 100–500 μM -methyl para tyrosine (-MPT), a TH inhibitor, prior to dieldrin treatment. -MPT effectively inhibited dieldrin-induced ROS generation, suggesting a role for dopamine turnover in the mechanism of dieldrin-induced elevated ROS production. In another study, heptachlor, an organochlorine pesticide related to dieldrin, inhibited vesicular monoamine transporter 2 (VMAT2) function, suggesting the failure of the vesicular sequestration mechanism may be conducive for the generation of ROS and ultimately dopaminergic neuronal demise [131]. Some more studies show that exposure of mice to low levels of dieldrin for 30 days caused changes in the levels of DAT and VMAT2, decreased levels of DA metabolites, and elevation of cysteinyl-catechol adducts in the striatum, suggesting

a role for oxidative stress mechanism in the increased vulnerability of nigrostriatal dopaminergic neurons to dieldrin-induced neurotoxicity [132, 133]. In order to better understand the association between dieldrin and increased risk of developing PD, detailed mechanistic studies aimed at understanding the cell signaling events underlying dieldrin-induced dopaminergic neuronal loss are needed.

Dysregulation of Protein Clearance Mechanisms by Agrochemicals

Agrochemicals and UPS

Protein clearance mechanism is critical for numerous cellular functions because it facilitates the elimination of toxic protein species and damaged organelles in order to prevent toxicity in post mitotic neurons. Eukaryotic system is endowed with two major mechanisms to prevent the buildup of toxic proteins, namely the ubiquitin proteasomal system (UPS) and autophagolysosomal pathway (ALP) [134]. The UPS is present in the cytoplasm, nucleus, and other subcellular regions, whereas autophagy is primarily localized to the cytoplasm [135, 136]. The UPS function remains elevated, while autophagy activity is relatively low in quiescent cells for the maintenance of cellular homeostasis. The UPS system breaks down proteins in a highly regulated manner [137, 138]. The molecular mechanisms regulating the UPS complex is a tightly regulated event which involves sequential ubiquitination of protein and its subsequent degradation by 26 S proteasome. In brief, the initial step of ubiquitin activation is governed by ubiquitin activating enzymes (E1s). These enzymes bind with ubiquitin C-terminus by forming a thiol ester bond. During the second step, the activated ubiquitin is transferred from E1s to E2s (ubiquitin conjugating enzymes) where ubiquitin conjugates with C-terminus of E2s. In the last step, E2s then coordinates with E3s (ubiquitin protein ligase), transferring the ubiquitin to a lysine residue of substrate protein. The substrate protein is further poly-ubiquitinated with four or more ubiquitin molecules (K48-linked poly ubiquitinating chains). These ubiquitinated proteins are then subjected to 26 S proteasomal degradation [139–143]. Moreover, ubiquitination is a reversible process that is often regulated by deubiquitinating enzymes, which participates in recycling ubiquitin.

In a healthy cell, UPS regulates physiological processes including neurotransmission, protein trafficking, cell cycle progression and DNA repair, by maintaining the dynamic equilibrium between the generation and degradation of short lived or abnormal proteins [144–146]. Biochemical and genetic studies using PD models have implicated the central role of dysfunction of ubiquitin-proteasome system in the pathogenesis of PD [147]. In particular, mutations in several genetically inherited genes associated with early onset PD, such as those regulating mitochondrial function, namely PINK1, DJ-1, Parkin and LRRK2, as well as those directly

regulating UPS functioning, namely UCH-L1, Parkin and α-synuclein, have been reported to be linked to PD [143, 148–151]. In fact, numerous studies have reported decreased UPS activity in postmortem PD brains [152]. Accumulation of protein aggregates evidenced in PD pathogenesis is attributed to the failure of cellular clearance machinery to remove the damaged and misfolded proteins [153]. The formation of protein aggregates known as LBs has been critically linked to the selective dopaminergic neuronal death associated with PD [148]. α-Synuclein (α-syn) is a major component of LBs [154, 155], and has been shown to misfold and attain an abnormal *β*-sheet rich structure ultimately leading to its aggregation [156]. This misfolding has been shown to be triggered by several factors, including oxidative stress, gene mutations and environmental factors [157]. The accumulation and aggregation of misfolded proteins, including α-synuclein, can promote further inhibition of UPS [143]. Interestingly, recent studies show that neurons can release the α-synuclein-positive aggregates into the synaptic space, which are then taken up by neighboring glial cells [158]. Due to the immunogenic nature of the misfolded proteins, it is probable that a vicious and progressive feed forward cycle via glial activation may promote rotenone-induced dopaminergic neurodegeneration. Thus, these protein aggregates not only induce cytotoxicity but also influence other processes, including proteasomal function and neuroinflammation. In summary, it appears that impaired protein clearance mechanisms and mitochondria-derived oxidative stress response might act cooperatively to promote the accumulation of protein aggregates in PD brains.

While pesticide exposure has been shown to be associated with increased risk of PD, epidemiological studies fail to investigate individual pesticides based on their biological mechanism of action. In recent years, a few studies have investigated the link between pesticide exposure and risk of developing PD based on their mechanism of action. For example, Tanner et al. [99] demonstrated that exposure to paraquat and rotenone in rural farming communities showed a 2.5-fold increased risk of developing PD. These studies suggested agrochemicals with different mechanisms of action, namely those that impair mitochondrial function and those that induce oxidative stress response, might represent a potential risk factor for PD, thereby implicating these mechanisms in the pathophysiology of PD. In another study linking gene-environment interaction in PD, Rhodes et al. [159] screened 28 pesticides for inhibition of 26 S proteasomal activity at 10 μM. Benomyl, cyanizine, dieldrin, endosulfan and ziram were found to be associated with increased risk of PD. An OR of 2.14 (95% CI: 1.42, 3.22) was estimated for subjects (453 controls, 287 cases) who had been exposed to any UPS-inhibiting pesticide at both residential and occupational areas. Notably, this association was found to be modified by genetic variant of s-phase kinase-associated protein 1 (SKP1) gene. SKP1 protein is a component of the SKP1-cullin 1-fbox (SCF) protein complexes involved in target identification for ubiquitination and cell cycle regulation [160]. The study suggests a role for UPS-inhibiting pesticides in the etiology of PD and raises the possibility that gene variants in the candidate genes involved in the UPS pathway might exacerbate the toxic effects of pesticide exposures. Taken together, these studies suggest that interaction between agrochemicals and genetic susceptibility may modulate the risk of developing PD.

Rotenone

Accumulating evidence suggests that rotenone induces the selective loss of nigrostriatal neurons leading to the accumulation of α-synuclein-positive aggregates, presumably via inhibition of complex I of the respiratory chain and associated elevation of oxidative stress response [for a review see [94]]. The BBB permeability and hydrophobicity of rotenone may account at least in part for the increased susceptibility of dopaminergic neurons to complex I inhibitory effects [29]. It is well accepted that chronic exposure to low doses of rotenone leads to an inhibition of mitochondrial electron transport chain in the rodent brain [47]. Moreover, in lab animals rotenone has been administered via different routes. For example, Betarbet et al. [47] demonstrated that in Lewis rats, chronic systemic administration of rotenone (3 mg/kg) via osmotic mini pump caused highly selective nigrostriatal dopaminergic neurodegeneration and concomitant accumulation of α-synuclein-positive cytoplasmic inclusions similar to LBs, in surviving dopaminergic neurons. Intraperitoneal administration of rotenone has been shown to cause extensive aggregation of α-synuclein and accumulation of poly-ubiquitin positive aggregates in nigral dopaminergic neurons [161]. In a similar fashion, IV administration of rotenone has been shown to cause nigrostriatal dopaminergic damage that was accompanied by α-synuclein aggregation, Lewy-like body formation, oxidative stress and GI dysfunction [47]. In another study, rotenone-induced nigrostriatal damage was found to be accompanied by the accumulation of α-synuclein positive aggregates in different parts of the brain [162]. Remarkably, intragastric administration of rotenone (5 mg/kg per day for a period of 10 weeks) in rodents induced the accumulation of α-synuclein-positive in the ENS, the dorsal motor nucleus of the vagus, intermediolateral nucleus of the spinal cord and the substantia nigra, regions that are also affected in human PD [163]. This study suggested that local effects in the ENS may be sufficient to induce PD-like progression and to reproduce neuroanatomical and neurochemical features of PD staging. Furthermore, it provided novel insight into how environmental factors could trigger PD and suggested a trans-synaptic mechanism by which PD might elicit wide spread distribution throughout the CNS. Interestingly, the intraperitoneal (IP) administration of rotenone in rats also induced the formation of α-synuclein inclusions in the enteric nervous system [164]. Taken together, these studies highlight the critical role of oxidative stress response in rotenone-induced α-synuclein aggregation and its impact on the susceptibility of nigrostriatal dopaminergic neurons.

Paraquat

A growing body of evidence suggests that the selective vulnerability of dopaminergic neurons to PQ-induced neurotoxicity is attributed to elevated oxidative stress that is mediated at least in part via increased uptake of dopamine into the dopaminergic neurons [24, 165]. In this context, oxidative stress has been established as a key factor influencing aggregation of α-syn [165]. In fact, PQ-mediated oxidative

stress has been shown to increase the levels of α-syn expression and aggregation in the SN of mice [166]. Additionally, other factors, including nitration, hyperphosphorylation at serine 129, and the presence of DA adducts, have also been shown to promote PQ-induced α-syn aggregation [167, 168]. Also, paraquat at micromolar concentrations has been shown to accelerate fibril formation [168] and this phenomena has also been demonstrated *in vivo* in mice (10 mg/kg i.p. once per week for 3 weeks) whereby a reversible increase in the level of intraneuronal aggregates and α-syn levels in the ventral midbrain and frontal cortex were evidenced [166]. Furthermore, PQ administration in transgenic mice overexpressing α-syn produced an increase in the number of insoluble α-synuclein (proteinase K-resistant) inclusions in the substantia nigra that bore close resemblance to the neuropathology of PD [169]. Conversely, attenuating the expression levels of α-syn in a mouse model of PD was found to exert beneficial effects by limiting the progression of neurodegeneration [170]. More recently, Cristovao et al. [171] demonstrated that using PQ-based *in vitro* and *in vivo* PD models, NADPH oxidase 1 (NOX1)-mediated α-syn expression and aggregation in dopaminergic neurons positively correlated with its neurodegenerative process, suggesting a role for NOX1-dervied ROS in the mechanism of excessive accumulation of α-synuclein aggregates. Similarly, studies conducted in A53T transgenic mice showed that combined exposure to maneb (manganese ethylene(bis)dithiocarbamate) and PQ exacerbated the aberrant accumulation of α-synuclein-positive aggregates, thus increasing the vulnerability of nigral dopaminergic neurons to their neurotoxic effects [172–174]. In summary, these studies suggest that oxidative stress may serve as a critical trigger for paraquat-induced increased expression and aggregation of α-synculein at least in the nigral dopaminergic neurons.

Impairment of UPS has been shown to cause α-syn aggregation, which in turn promotes further impairment of mitochondrial function [11, 175]. In fact, PQ treatment leads to an impairment in proteasomal function in human neuroblastoma SH-SY5Y cells, whereby a reduction in the protein levels of 19 S subunit of proteasome was found to parallel drug-induced cytotoxicity [176]. In another study, DJ-1 deficient mice exposed to PQ demonstrated proteasomal dysfunction, increased ubiquitinated protein levels, and a decline in ATP and subunit protein levels of 19 S ATPase Rpt6 and 20 Sβ5. These studies implicated that both environmental insults and genetic factors might act cooperatively to induce proteasomal dysfunction in PD pathogenesis [176]. On the contrary, a recent study demonstrated that PQ (10 mg/kg, twice per week for 6 weeks) treatment led to a significant decrease in 26 S proteosomal activity, although the levels of 19 S or 20 S components and assembly of the 26 S proteasome remained unchanged [177]. The authors postulated that PQ might exert its effects via direct interaction with the 20 S component of the proteasome. Taken together, these studies suggest that proteasomal dysfunction renders dopaminergic neurons more vulnerable to paraquat-induced neurodegenerative processes. Thus understanding the mechanism by which PQ induces the expression and aggregation of α-syn may not only lead to the identification of novel targets that halt the progression of this insidious disorder but may also shed light on the significant contribution of environmental insults in the etiopathogenesis of PD.

Dieldrin

It is becoming increasingly clear that pesticides are involved in the PTMs of several proteins, including α-synuclein, in oxidative stressed neurons [for a review see [178]]. Previously, Uversky et al. [168] demonstrated that pesticides, including dieldrin, can induce a conformational change in α-synuclein, thereby accelerating the formation of α-synuclein fibrils *in vitro* [168]. Moreover, studies conducted in our lab showed that dieldrin can inhibit proteasomal activity in N27 immortalized dopaminergic cells, therefore leading to the accumulation of ubiquitin-positive aggregates. Interestingly, the magnitude of inhibition of the proteasomal system and apoptotic cell death were exacerbated in α-synuclein overexpressing cells, suggesting that dieldrin acts synergistically with α-synuclein to enhance the vulnerability of dopaminergic neurons to apoptotic cell death [179]. In another study performed in SK-N-MC cells overexpressing a GFP-conjugated proteasome degradation signal, several pesticides, including dieldrin, were screened for their ability to alter UPS. Dieldrin was found to inhibit proteasomal activity and the cytotoxic effects were found to closely correlate with proteasomal inhibition [159]. Taken together, these studies suggest that a potential interaction between α-synuclein and exposure to dieldrin is most likely to accelerate the susceptibility of dopaminergic neurons to oxidative stress-induced neuronal injury.

Agrochemicals and Autophagy

Autophagy is a dynamic clearance mechanism that is involved in the degradation of proteins, protein complexes, and damaged organelles via association with lysosomal degradative machinery [180]. In fact, post mitotic neurons are highly reliant on basal autophagy because they maintain the balance between synthesis, degradation, recycling of intracellular components as compared to non-neuronal cells; however, impairment in autophagy leads to the accumulation of damaged organelles and misfolded and aggregated proteins, which in turn exerts deleterious effects on normal cellular homeostasis [181]. Aberrant proteolytic degradation machinery has been identified as a key component contributing to neurodegeneration. In fact, dysfunctional autophagic activity is frequently observed in distinct neuronal populations that are selectively targeted in neurodegenerative diseases, including Alzheimer's disease (AD), PD, Huntington's disease (HD), and amyotrophic lateral schlerosis (ALS) [182, 183]. The exact role of autophagy is unclear; however, emerging evidence favors a neuroprotective role for autophagy, whereby insufficient or dysfunctional autophagy rather than excessive autophagy leads to neuronal cell death in neurodegenerative conditions. Autophagy is particularly important when UPS function is impaired because of the formation of large protein aggregates or oxidative stress [184–186]. Macroautophagy is an essential component of the autophagy-lysosomal pathway (ALP). During autophagy, soluble and aggregated proteins are sequestered within double membrane vesicles termed autophagosomes,

which then fuse with lysosomes to promote degradation via the lysosomal hydrolases. Accumulating evidence demonstrates that autophagy related genes (ATGs) and their proteins regulate the formation of autophagic vacuoles (AV) in eukaryotic cells [187, 188]. In fact, covalently bound complex of Atg12-Atg5 has been shown to regulate the elongation of AV isolation membrane, also known as phagophore [189, 190]. Additionally, Atg12-Atg5 exhibits ubiquitin E3-like enzyme activity that facilitates the lipidation of LC3 family members. The lipidated form of LC3, namely LC3-II, is subsequently incorporated into the autophagosomal membrane. The serine/threonine kinase mTOR (mammalian target of rapamycin) is a key regulator of autophagy during starvation conditions. It has other functions, including regulation of cell cycle progression, protein synthesis and cell growth. Activation of mTOR promotes protein synthesis and inhibits macroautophagy via an Atg13 phosphorylation-dependent mechanism that modulates Atg1 activity. Under conditions of physiological stress, namely starvation, mTOR is inactivated leading to the formation of Atg1-Atg13 complexes required for the activation of autophagy [191]. Microautophagy, on the other hand, is a relatively simpler process and involves the engulfment of cytoplasm by lysosomes via a membrane involution [192]. Finally, chaperone-mediated autophagy (CMA) as compared to macroautophagy and microautophagy differs in regards to its selectivity and mechanism of degradation. CMA is involved in the selective degradation of 30% of all cytosolic proteins that contain a consensus sequence, Lys-Phe-Glu-Arg-Gln (KFERQ). This amino acid sequence is recognized by a cytosolic chaperone, heat-shock cognate 70 (HSC70) and its co-chaperones, which targets these proteins to the lysosomal membrane, where the complex is recognized by the CMA receptor lysosome-associated membrane protein type-2A (LAMP2A), internalized, and then subsequently unfolded and degraded in the lysosomal lumen [181, 193].

Rotenone

Rotenone model has been shown to reproduce delayed and specific loss of dopaminergic neurons and aggregation of α-synuclein, which effectively captures several aspects of sporadic PD [89, 161, 194–196]. In fact, rotenone-based *in vitro* and *in vivo* models of PD have been routinely used to explore the role of autophagy in PD [197]. Additionally, rotenone has been shown to exert suppressive effects on cytoprotective proteins namely Parkin and DJ-1 that are critically linked to organelle quality control program-mitophagy, antioxidant response and mitochondrial function [198]. Moreover, rotenone has also been shown to exhibit inhibitory effects on autophagy, whereby treatment with rapamycin, an inducer of autophagy, afforded protection against rotenone-induced neurotoxicity [199, 200]. Consistently, treatment of SH-SY5Y cells with rotenone (high dose) has been shown to induce the mitochondrial release of cytochrome C, reduced COX IV activity, accumulation of ubiquitinated aggregates and ROS production. Importantly, siRNA-mediated knockdown of ATG5 partially blocked the neuroprotective effects of rapamycin in rotenone-treated cells, suggesting a neuroprotective role for autophagy in the

clearance of oxidatively damaged molecules [199]. In a similar fashion, enhancement of autophagy via rapamycin attenuated rotenone-induced ROS production, restored mitochondrial membrane potential and decreased HIF expression in mutant α-synuclein expressing neuronal cells as compared to the cells overexpressing WT α-synuclein. These studies suggest that mutant α-synuclein aggravates rotenone-induced cytotoxicity and that autophagy induction affords significant protection against cytotoxicity in α-synuclein overexpressing cells [201]. In another study, Giordano et al., [202] reported that autophagy can elicit a protective response in primary neurons exposed to rotenone and that the surviving neurons exhibit bioenergetic adaptations to this metabolic stressor. In contrast, an increase in autophagic flux has been observed in primary cortical neurons treated with sublethal concentration of rotenone as demonstrated by a decrease in p62 levels and a rapid loss of mitochondria that can be reversed by co-treatment with bafilomycin A2, a vacuolar ATPase inhibitor, or by siRNA-mediated gene silencing of ATG7 and ATG8. Mechanistically, rotenone-induced mitophagy involved the translocation of mitochondrial inner membrane-bound phospholipid, cardiolipin, to the outer mitochondrial membrane. This externalized cardiolipin was found to contain cargo recognition signals necessary for the engulfment of damaged mitochondria by autophagy [203]. The authors proposed that externalization of cardiolipin serves as a degradation signal for the clearance of damage mitochondria. Thus, it appears that rotenone exerts both stimulatory and inhibitory effects on on autophagic flux; the differential effects might be attributed to the dose, duration of exposure and cell type, whereby the magnitude of oxidative damage to the mitochondria and subsequent effects on cellular bioenergetics might impact autophagic flux.

Paraquat

Despite numerous studies demonstrating the role of oxidative stress in paraquat-induced dopaminergic toxicity, the exact contribution of autophagy in dopaminergic neurotoxicity remains poorly understood. Paraquat induced oxidative stress response presumably via mitochondrial respiratory chain disruption has been shown to be a critical trigger for the induction of autophagy and apoptotic cell death [204]. In fact, a close parallelism between autophagy and apoptotic cell dell death in PQ-induced cytotoxicity has been consistently demonstrated in SH-SY5Y cells. For example, exposure of human neuroblastoma SH-SY5Y cells to PQ displayed hallmarks of autophagy, including accumulation of AVs in the cytoplasm and the recruitment of LC3-GFP fusion proteins to AVs [205]. While inhibition of caspase attenuated cell death, inhibition of autophagy via 3-methyladenine (3-MA) accelerated apoptotic cell death induced by PQ, suggesting a neuroprotective role for autophagy in the mechanism of PQ-induced dopaminergic neurotoxicity [205]. In another study, impairment of ATG5-dependent autophagic flux via overexpression of a dominant negative form of Atg5 has been shown to promote PQ-induced dopaminergic toxicity, suggesting a protective role for ATG5-dependent autophagic pathways during PQ-induced dopaminergic apoptotic cell death [206]. Intriguingly,

induction of apoptosis following exposure of SH-SY5Y cells to paraquat has been shown to be mediated via an endoplasmic reticular (ER) stress-dependent mechanism [207–209]. Notably, in PQ-treated SH-SY5Y cells autophagy and apoptotic cell death were found to be mediated via an ER stress dependent apoptosis signal kinase 1 (ASK1) activation process. Niso-Santano et al., [210] reported that paraquat exposure induced an early ER stress response that was correlated with the induction of an adaptive response, autophagy, as evidenced by accumulation of LC3II, p62 degradation, and mTOR dephosphorylation. In fact, the autophagic response was enhanced in cells that overexpressed WT ASK1. In this cell culture model, the inhibition of autophagy was found to exaggerate apoptosis induced by ASK1 WT overexpression in the presence or absence of PQ, suggesting that ASK1 is a critical intermediate in PQ-induced neurodegeneration and that autophagy may play a crucial role in the cell survival/cell death mechanisms [210]. In a recent report, chronic exposure of mice to PQ has been shown to cause an increase in p-Tau levels, accumulation of α-synuclein, and elevated p-GSK-3β levels in the striata. Furthermore, mTOR levels were also increased following paraquat treatment, suggestive of impaired autophagy. Interestingly, following PQ exposure chaperone mediated autophagy was not disrupted; however, proteasomal and macroautophagy mechanisms were inhibited, thereby leading to accumulation of α-synuclein and p-Tau [177]. These studies suggest that induction of macroautophagy may elicit neuroprotective effects during PQ-induced disruption of protein clearance machinery.

PD is likely to be a multifactorial neurodegenerative disorder, whereby both genes and environment might contribute to increased disease risk [211]. In a recent study, Goldman et al. [212] suggested that PD risk following PQ exposure might be particularly high in individuals lacking glutathione S transferase T1 (GSTT1) and that homozygous deletion of GSTT1 may be an important genetic marker in identifying subpopulation at high risk of developing PD following exposure to PQ. Consistent with this, Gonzalez-Polo et al. [213] using SH-SY5Y cells, demonstrated that transfection with DJ-1 specific siRNA and subsequent exposure to PQ caused an inhibition of the autophagic response, as well as additive sensitization of SH-SY5Y neuronal cells to PQ-induced apoptotic cell death and exacerbation of cell death in the presence of 3-MA, an autophagy inhibitor. These results support an active role for DJ-1 in the autophagic response elicited by paraquat. Other proposed mechanism by which PQ induces autophagy has been shown to involve Parkin, an ubiquitin ligase [214]. Interestingly, USP30, a deubiquitinase localized to mitochondria antagonized Parkin- and PINK-1-induced mitophagy. Notably, knock down of USP30 in dopaminergic neurons protected flies against PQ-induced dopaminergic toxicity in vivo, as evidenced by amelioration of deficits in DA levels, motor dysfunction and organismal survival. Thus, this study suggests that inhibition of USP30 may promote clearance of damaged mitochondria via mitophagy, ultimately improving quality control mechanisms within the cell. Collectively, these studies suggests a possible link between pesticide exposure and genetic factors in PD pathogenesis and raises the possibility that autophagy may exert neuroprotective effects following PQ exposure.

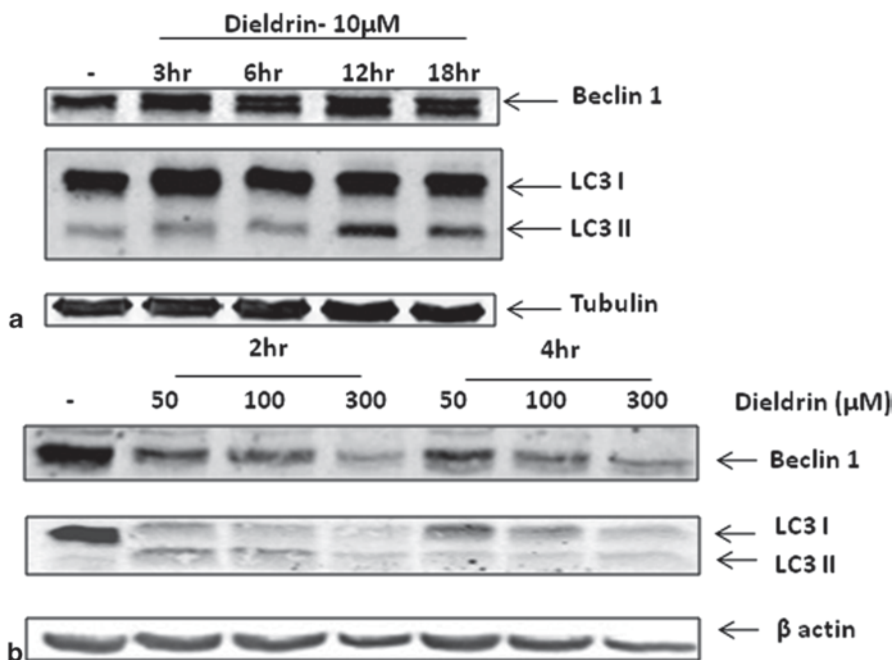

Fig. 10.1 Dose and time-dependent regulation of autophagy by dieldrin. a N27 cells were treated with either 10 µM dieldrin or DMSO for upto 18 h. A time-dependent increase in the induction of autophagic markers, namely Beclin-1 and LC3-II, were evidenced via WB analyses. Cells were harvested at the indicated time points and lysates were prepared using RIPA buffer and then subjected to immunoblotting analysis with antibodies against Beclin 1 and LC3-II. b A time-dependent reduction in autophagy was evidenced in cells treated with either DMSO or higher concentrations (50–300 µM) of dieldrin. Cells were treated with increasing concentrations (50–300 µM) of dieldrin for 2 and 4 h respectively. Subsequently, cells were harvested at the indicated time periods following drug treatment and the extent of LC3 and Beclin 1 levels were determined by WB analysis as mentioned previously. Equal protein loading was confirmed by using β-actin as loading control

Dieldrin

Based on previous studies from our lab demonstrating that overexpression of human α-synuclein in N27 dopaminergic cells accelerated dieldrin-induced accumulation of intracellular inclusion bodies and dopaminergic neurotoxicity [179], we hypothesized that cell degradation machinery, especially autophagy, may have gone awry following treatment of N27 cells with dieldrin. Therefore in the present study we sought to investigate the role of autophagy in dieldrin-induced neurotoxicity. Treatment of N27 dopaminergic cells a low dose of dieldrin (10 µM) (Fig. 10.1a) for upto 18 h resulted in a time-dependent induction of autophagy. Conversely, exposure of N27 cells to higher concentrations (50–300 µM) of dieldrin for 2–4 h produced a marked reduction in autophagic markers (Fig. 10.1b). In order to further confirm dieldrin-induced autophagy, we performed double immunolabeling studies

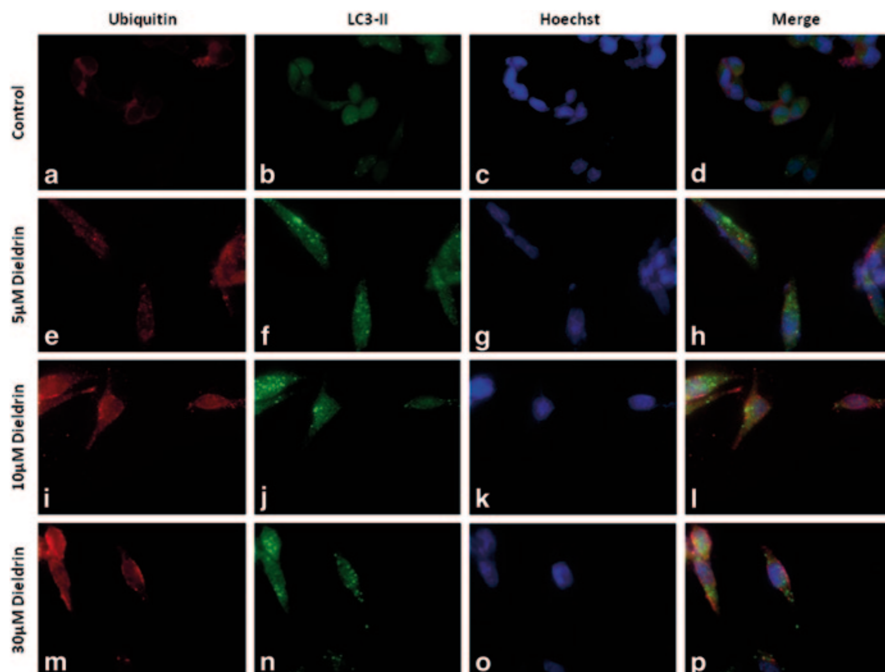

Fig. 10.2 Alteration of UPS and ALP by dieldrin in N27 cells. N27 cells were treated with increasing concentrations of dieldrin (5, 10 or 30 μM) or DMSO for 9 h and then the formation of ubiquitin positive aggregates and LC3-II positive structures in dieldrin-treated N27 cells were determined via immunofluorescence analysis. Representative microscopic images of LC3-II (*green*), UB (*red*) proteins, or colocalization of LC3-II/UB proteins (*merge*) in N27 dopaminergic cells exposed to either DMSO or dieldrin are shown. DMSO-treated cells show a diffused and faint staining pattern for LC3-II and ubiquitin (**a**–**d**), whereas dieldrin-treated cells display co-localization of both proteins within punctate-like structures at 5 (**e**–**h**), 10 (**i**–**l**), and 30 μM (**m**–**p**), respectively. Hoechst staining was used to counter stain nuclei (*blue*)

for LC3-II and ubiquitin. Our studies (Fig. 10.2) showed a gradual concentration-dependent increase in LC3-II staining that exhibited marked co-localization with ubiquitin, suggesting an impairment of protein clearance machinery. Consistent with this observation, we recently demonstrated that in N27 cells, methamphetamine (MA)-induced dopaminergic neurotoxicity was associated with the impairment of protein clearance mechanisms and that induction of autophagy may in fact represent a cytoprotective response that confers resistance against neurotoxic effects of MA [215, 216]. Taken together, to the best of our knowledge, this is the first study demonstrating that in addition to UPS dysfunction, impairment in autophagy might also contribute to dieldrin-induced apoptotic cell death in dopaminergic neuronal cells. Further studies are currently underway to evaluate the cell signaling mechanisms underlying dieldrin-induced alteration of autophagy and their impact on cell death in dopaminergic neuronal cells.

Concluding Remarks

PD is a progressive neurodegenerative disorder that affects ~2.0 million Americans. As the median age of the population increases, PD incidence will increase, and therefore, it is critical to obtain a better understanding of the causative factors to develop disease modifying strategies for the treatment of PD. Collectively, convergence of chronic low dose exposure to agrochemicals, genetic susceptibility and aging can increase the risk of developing PD [217–219]. The pathophysiological basis of the proposed agrochemicals include potential interaction between mitochondrial impairment, dysregulation of protein clearance mechanisms and elevated ROS levels, although the cellular mechanisms underlying ROS generation may be diverse (mitochondria dependent and independent sources) [34, 220–222]. Studies showing mitochondrial damage and accumulation of oxidatively damaged macromolecules in PD brains further confirm the essential role of mitochondrial dysfunction in the increased vulnerability of substantia nigral dopaminergic neurons to PD pathogenesis [61, 223]. Moreover, the existence of α-synuclein aggregates in the vicinity of oxidatively damaged macromolecules in PD brains further highlights the essential role of oxidative stress in the deregulation of protein clearance machinery [94, 224]. Given that PD is a multifactorial disease and that deregulation of mitochondria function and protein clearance machinery may mediate, at least in part, agrochemical-induced dopaminergic neurotoxicity, mechanistic studies aimed at dissecting the overlapping signaling cascades involving mitochondria dysfunction and protein clearance machinery may lead to the elucidation of novel intermediates that are critical for the cross talk between mitochondria and protein clearance mechanisms and ultimately neurotoxicity.

Several of the environmental neurotoxicants discussed here, namely dieldrin, paraquat, and rotenone, fail to recapitulate several cardinal features of human PD [28]. Also, there is a growing problem, whereby several studies utilized high dose and acute exposure to a single environmental neurotoxicant, usually over a short period of time (days-weeks), and the behavioral studies were also performed over a short period. However, human exposure cases involve low dose, chronic exposure over a protracted period (several years) to a combination of environmental neurotoxicants [11]. Therefore, it is essential to develop more relevant animal models that better recapitulate human exposure levels and enable the evaluation of the neurobehavioral deficits, which will be pivotal for the identification of novel therapeutic interventions for PD treatment. Although these toxicants target nigrostriatal dopaminergic system, it would be beneficial to study the potential damaging effects of agrochemicals in other brain regions, namely olfactory bulb, cortex, and brain stem, among others, which has been critically linked to the expression of non-motor symptoms in PD patients.

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References

1. Dexter DT, Jenner P. Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med. 2013;62:132–44 (PubMed PMID: 23380027).
2. Braak H, Braak E. Pathoanatomy of Parkinson's disease. J Neurol. 2000;247(Suppl 2):II3–10 (PubMed PMID: 10991663).
3. Dick FD, De Palma G, Ahmadi A, Scott NW, Prescott GJ, Bennett J, et al. Environmental risk factors for Parkinson's disease and parkinsonism: the Geoparkinson study. Occup Environ Med. 2007;64(10):666–72 (PubMed PMID: 17332139. Pubmed Central PMCID: 2078401).
4. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839–40 (PubMed PMID: 9278044).
5. Licker V, Kovari E, Hochstrasser DF, Burkhard PR. Proteomics in human Parkinson's disease research. J Proteomics. 2009;73(1):10–29 (PubMed PMID: 19632367).
6. Venda LL, Cragg SJ, Buchman VL, Wade-Martins R. α-Synuclein and dopamine at the crossroads of Parkinson's disease. Trends Neurosci. 2010;33(12):559–68 (PubMed PMID: 20961626. Pubmed Central PMCID: 3631137).
7. Ubhi K, Low P, Masliah E. Multiple system atrophy: a clinical and neuropathological perspective. Trends Neurosci. 2011;34(11):581–90 (PubMed PMID: 21962754. Pubmed Central PMCID: 3200496).
8. Wood-Kaczmar A, Gandhi S, Wood NW. Understanding the molecular causes of Parkinson's disease. Trends Mol Med. 2006;12(11):521–8 (PubMed PMID: 17027339).
9. Coppede F, Mancuso M, Siciliano G, Migliore L, Murri L. Genes and the environment in neurodegeneration. Biosci Rep. 2006;26(5):341–67 (PubMed PMID: 17029001).
10. Thomas B, Beal MF. Parkinson's disease. Hum Mol Genet. 2007;16(Spec No. 2):R183–94 (PubMed PMID: 17911161).
11. Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, Tsatsakis AM, Duarte JA, Carvalho F. Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases—a mechanistic approach. Toxicol Lett. 2014;230:85–103 (PubMed PMID: 24503016).
12. Davie CA. A review of Parkinson's disease. British Med Bull. 2008;86:109–27 (PubMed PMID: 18398010).
13. Foltynie T, Brayne C, Barker RA. The heterogeneity of idiopathic Parkinson's disease. J Neurol. 2002;249(2):138–45 (PubMed PMID: 11985378).
14. Foltynie T, Sawcer S, Brayne C, Barker RA. The genetic basis of Parkinson's disease. J Neurol Neurosurg Psychiatry. 2002;73(4):363–70 (PubMed PMID: 12235301. Pubmed Central PMCID: 1738059).
15. Gorell JM, Peterson EL, Rybicki BA, Johnson CC. Multiple risk factors for Parkinson's disease. J Neurol Sci. 2004;217(2):169–74 (PubMed PMID: 14706220).
16. Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, et al. Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. BMC Neurol. 2008;8:6 (PubMed PMID: 18373838. Pubmed Central PMCID: 2323015).
17. Tufekci KU, Meuwissen R, Genc S, Genc K. Inflammation in Parkinson's disease. Adv Protein Chem Struct Biol. 2012;88:69–132 (PubMed PMID: 22814707).
18. Androutsopoulos VP, Kanavouras K, Tsatsakis AM. Role of paraoxonase 1 (PON1) in organophosphate metabolism: implications in neurodegenerative diseases. Toxicol Appl Pharmacol. 2011;256(3):418–24 (PubMed PMID: 21864557).
19. Freire C, Koifman S. Pesticide exposure and Parkinson's disease: epidemiological evidence of association. Neurotoxicology. 2012;33(5):947–71 (PubMed PMID: 22627180).
20. Tanner CM. Advances in environmental epidemiology. Mov Disord. 2010;25(Suppl 1):S58–62 (PubMed PMID: 20187243).
21. Rugbjerg K, Harris MA, Shen H, Marion SA, Tsui JK, Teschke K. Pesticide exposure and risk of Parkinson's disease—a population-based case-control study evaluating the potential for recall bias. Scand J Work Environ Health. 2011;37(5):427–36 (PubMed PMID: 21240453).

22. Vlajinac HD, Sipetic SB, Maksimovic JM, Marinkovic JM, Dzoljic ED, Ratkov IS, et al. Environmental factors and Parkinson's disease: a case-control study in Belgrade, Serbia. Int J Neurosci. 2010;120(5):361–7 (PubMed PMID: 20402575).
23. Weisskopf MG, Knekt P, O'Reilly EJ, Lyytinen J, Reunanen A, Laden F, et al. Persistent organochlorine pesticides in serum and risk of Parkinson disease. Neurol. 2010;74(13):1055–61 (PubMed PMID: 20350979. Pubmed Central PMCID: 2848105).
24. Brown TP, Rumsby PC, Capleton AC, Rushton L, Levy LS. Pesticides and Parkinson's disease—is there a link? Environ Health Perspect. 2006;114(2):156–64 (PubMed PMID: 16451848. Pubmed Central PMCID: 1367825).
25. Behari M, Srivastava AK, Das RR, Pandey RM. Risk factors of Parkinson's disease in Indian patients. J Neurol Sci. 2001;190(1–2):49–55 (PubMed PMID: 11574106).
26. Firestone JA, Lundin JI, Powers KM, Smith-Weller T, Franklin GM, Swanson PD, et al. Occupational factors and risk of Parkinson's disease: a population-based case-control study. Am J Ind Med. 2010;53(3):217–23 (PubMed PMID: 20025075. Pubmed Central PMCID: 3299410).
27. Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology. 2000;21(4):435–40 (PubMed PMID: 11022853).
28. Cannon JR, Greenamyre JT. The role of environmental exposures in neurodegeneration and neurodegenerative diseases. Toxicol Sci. 2011;124(2):225–50 (PubMed PMID: 21914720. Pubmed Central PMCID: 3216414).
29. Bove J, Prou D, Perier C, Przedborski S. Toxin-induced models of Parkinson's disease. NeuroRx. 2005;2(3):484–94 (PubMed PMID: 16389312. Pubmed Central PMCID: 1144492).
30. Langston JW, Ballard PA, Jr. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983;309(5):310 (PubMed PMID: 6602944).
31. Richardson JR, Caudle WM, Guillot TS, Watson JL, Nakamaru-Ogiso E, Seo BB, et al. Obligatory role for complex I inhibition in the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Toxicol Sci. 2007;95(1):196–204 (PubMed PMID: 17038483).
32. Exner N, Lutz AK, Haass C, Winklhofer KF. Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. EMBO J. 2012;31(14):3038–62 (PubMed PMID: 22735187. Pubmed Central PMCID: 3400019).
33. Moretto A, Colosio C. Biochemical and toxicological evidence of neurological effects of pesticides: the example of Parkinson's disease. Neurotoxicology. 2011;32(4):383–91 (PubMed PMID: 21402100).
34. Franco R, Li S, Rodriguez-Rocha H, Burns M, Panayiotidis MI. Molecular mechanisms of pesticide-induced neurotoxicity: relevance to Parkinson's disease. Chem Biol Interact. 2010;188(2):289–300 (PubMed PMID: 20542017. Pubmed Central PMCID: 2942983).
35. Ghosh A, Chandran K, Kalivendi SV, Joseph J, Antholine WE, Hillard CJ, et al. Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model. Free Radic Biol Med. 2010;49(11):1674–84 (PubMed PMID: 20828611. Pubmed Central PMCID: 4020411).
36. Jin H, Kanthasamy A, Ghosh A, Anantharam V, Kalyanaraman B, Kanthasamy AG. Mitochondria-targeted antioxidants for treatment of Parkinson's disease: preclinical and clinical outcomes. Biochim Biophys Acta. 2014;1842(8):1282–94 (PubMed PMID: 24060637. Pubmed Central PMCID: 3961561).
37. Lin M, Chandramani-Shivalingappa P, Jin H, Ghosh A, Anantharam V, Ali S, et al. Methamphetamine-induced neurotoxicity linked to ubiquitin-proteasome system dysfunction and autophagy-related changes that can be modulated by protein kinase C delta in dopaminergic neuronal cells. Neuroscience. 2012;210:308–32 (PubMed PMID: 22445524. Pubmed Central PMCID: 3358550).
38. Shen YF, Tang Y, Zhang XJ, Huang KX, Le WD. Adaptive changes in autophagy after UPS impairment in Parkinson's disease. Acta Pharmacol Sin. 2013;34(5):667–73 (PubMed PMID: 23503475).

39. Simon-Sanchez J, Schulte C, Bras JM, Sharma M, Gibbs JR, Berg D, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet. 2009;41(12):1308–12 (PubMed PMID: 19915575. Pubmed Central PMCID: 2787725).
40. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem. 1990;54(3):823–7 (PubMed PMID: 2154550).
41. Coskun P, Wyrembak J, Schriner SE, Chen HW, Marciniack C, Laferla F, et al. A mitochondrial etiology of Alzheimer and Parkinson disease. Biochim Biophys Acta. 2012;1820(5):553–64 (PubMed PMID: 21871538. Pubmed Central PMCID: 3270155).
42. Hauser DN, Hastings TG. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. Neurobiol Dis. 2013;51:35–42 (PubMed PMID: 23064436. Pubmed Central PMCID: 3565564).
43. Celardo I, Martins LM, Gandhi S. Unravelling mitochondrial pathways to Parkinson's disease. Br J Pharmacol. 2014;171(8):1943–57 (PubMed PMID: 24117181. Pubmed Central PMCID: 3976614).
44. Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science. 2011;333(6046):1109–12 (PubMed PMID: WOS:000294244300037. English).
45. Shin JY, Park HJ, Ahn YH, Lee PH. Neuroprotective effect of L-dopa on dopaminergic neurons is comparable to pramipexol in MPTP-treated animal model of Parkinson's disease: a direct comparison study. J Neurochem. 2009;111(4):1042–50 (PubMed PMID: 19765187).
46. Ogawa N, Asanuma M, Miyazaki I, Diaz-Corrales FJ, Miyoshi K. L-DOPA treatment from the viewpoint of neuroprotection. Possible mechanism of specific and progressive dopaminergic neuronal death in Parkinson's disease. J Neurol. 2005;252(Suppl 4):IV23–31 (PubMed PMID: 16222434).
47. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 2000;3(12):1301–6 (PubMed PMID: 11100151).
48. Hattori N, Tanaka M, Ozawa T, Mizuno Y. Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. Ann Neurol. 1991;30(4):563–71 (PubMed PMID: 1665052).
49. Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, et al. Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. Biochem Biophys Res Commun. 1989;163(3):1450–5 (PubMed PMID: 2551290).
50. Mytilineou C, Werner P, Molinari S, Di Rocco A, Cohen G, Yahr MD. Impaired oxidative decarboxylation of pyruvate in fibroblasts from patients with Parkinson's disease. J Neural Transm Park Dis Dement Sect. 1994;8(3):223–8 (PubMed PMID: 7748465).
51. Blin O, Desnuelle C, Rascol O, Borg M, Peyro Saint Paul H, Azulay JP, et al. Mitochondrial respiratory failure in skeletal muscle from patients with Parkinson's disease and multiple system atrophy. J Neurol Sci. 1994;125(1):95–101 (PubMed PMID: 7964895).
52. Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, et al. Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. Ann Neurol. 1995;37(6):714–22 (PubMed PMID: 7778844).
53. Swerdlow RH. Mitochondria in cybrids containing mtDNA from persons with mitochondriopathies. J Neurosci Res. 2007;85(15):3416–28 (PubMed PMID: 17243174).
54. Drechsel DA, Patel M. Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radic Biol Med. 2008;44(11):1873–86 (PubMed PMID: 18342017. Pubmed Central PMCID: 2723777).
55. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006;443(7113):787–95 (PubMed PMID: 17051205).
56. Schapira AH. Mitochondrial dysfunction in Parkinson's disease. Cell Death Differ. 2007;14(7):1261–6 (PubMed PMID: 17464321).
57. Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet. 2006;38(5):515–7 (PubMed PMID: 16604074).

58. Guo X, Kudryavtseva E, Bodyak N, Nicholas A, Dombrovsky I, Yang D, et al. Mitochondrial DNA deletions in mice in men: substantia nigra is much less affected in the mouse. Biochim Biophys Acta. 2010;1797(6–7):1159–62 (PubMed PMID: 20388490. Pubmed Central PMCID: 2891141).
59. Reeve A, Meagher M, Lax N, Simcox E, Hepplewhite P, Jaros E, et al. The impact of pathogenic mitochondrial DNA mutations on substantia nigra neurons. J Neurosci. 2013;33(26):10790–801 (PubMed PMID: 23804100).
60. Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum Mol Genet. 2004;13(9):935–44 (PubMed PMID: 15016765).
61. Subramaniam SR, Chesselet MF. Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Prog Neurobiol. 2013;106–107:17–32 (PubMed PMID: 23643800. Pubmed Central PMCID: 3742021).
62. Kruse SE, Watt WC, Marcinek DJ, Kapur RP, Schenkman KA, Palmiter RD. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. Cell Metab. 2008;7(4):312–20 (PubMed PMID: 18396137. Pubmed Central PMCID: 2593686).
63. Varcin M, Bentea E, Michotte Y, Sarre S. Oxidative stress in genetic mouse models of Parkinson's disease. Oxid Med Cell Longev. 2012;2012:624925 (PubMed PMID: 22829959. Pubmed Central PMCID: 3399377).
64. Lim KL, Zhang CW. Molecular events underlying Parkinson's disease—an interwoven tapestry. Front Neurol. 2013;4:33 (PubMed PMID: 23580245. Pubmed Central PMCID: 3619247).
65. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 2003;25(3–4):207–18 (PubMed PMID: 14661084).
66. Floriano-Sanchez E, Villanueva C, Medina-Campos ON, Rocha D, Sanchez-Gonzalez DJ, Cardenas-Rodriguez N, et al. Nordihydroguaiaretic acid is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents in vivo ozone-induced tyrosine nitration in lungs. Free Radic Res. 2006;40(5):523–33 (PubMed PMID: 16551579).
67. Lotharius J, Brundin P. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nat Rev Neurosci. 2002;3(12):932–42 (PubMed PMID: 12461550).
68. Cohen G. Oxidative stress, mitochondrial respiration, and Parkinson's disease. Ann N Y Acad Sci. 2000;899:112–20 (PubMed PMID: 10863533).
69. Lawson LJ, Perry VH, Dri P, Gordon S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience. 1990;39(1):151–70 (PubMed PMID: 2089275).
70. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell. 2010;140(6):918–34 (PubMed PMID: 20303880. Pubmed Central PMCID: 2873093).
71. de Oliveira MR, da Rocha RF, Stertz L, Fries GR, de Oliveira DL, Kapczinski F, et al. Total and mitochondrial nitrosative stress, decreased brain-derived neurotrophic factor (BDNF) levels and glutamate uptake, and evidence of endoplasmic reticulum stress in the hippocampus of vitamin A-treated rats. Neurochem Res. 2011;36(3):506–17 (PubMed PMID: 21188516).
72. Kumar C, Igbaria A, D'Autreaux B, Planson AG, Junot C, Godat E, et al. Glutathione revisited: a vital function in iron metabolism and ancillary role in thiol-redox control. EMBO J. 2011;30(10):2044–56 (PubMed PMID: 21478822. Pubmed Central PMCID: 3098478).
73. Martin HL, Teismann P. Glutathione—a review on its role and significance in Parkinson's disease. FASEB J. 2009;23(10):3263–72 (PubMed PMID: 19542204).
74. McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, et al. Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis. 2002;10(2):119–27 (PubMed PMID: 12127150).

75. Peng J, Stevenson FF, Doctrow SR, Andersen JK. Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantial nigra: implications for Parkinson disease. J Biol Chem. 2005;280(32):29194–8 (PubMed PMID: 15946937).
76. Danielson SR, Andersen JK. Oxidative and nitrative protein modifications in Parkinson's disease. Free Radic Biol Med. 2008;44(10):1787–94 (PubMed PMID: 18395015. Pubmed Central PMCID: 2422863).
77. Oueslati A, Fournier M, Lashuel HA. Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. Prog Brain Res. 2010;183:115–45 (PubMed PMID: 20696318).
78. Xiang W, Schlachetzki JC, Helling S, Bussmann JC, Berlinghof M, Schaffer TE, et al. Oxidative stress-induced posttranslational modifications of alpha-synuclein: specific modification of alpha-synuclein by 4-hydroxy-2-nonenal increases dopaminergic toxicity. Mol Cell Neurosci. 2013;54:71–83 (PubMed PMID: 23369945).
79. Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, et al. In vivo demonstration that alpha-synuclein oligomers are toxic. Proc Natl Acad Sci USA. 2011;108(10):4194–9 (PubMed PMID: 21325059. Pubmed Central PMCID: 3053976).
80. Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci. 1998;18(5):1633–41 (PubMed PMID: 9464988).
81. Bianco F, Fumagalli M, Pravettoni E, D'Ambrosi N, Volonte C, Matteoli M, et al. Pathophysiological roles of extracellular nucleotides in glial cells: differential expression of purinergic receptors in resting and activated microglia. Brain ResBrain Res Rev. 2005;48(2):144–56 (PubMed PMID: 15850653).
82. Levesque S, Wilson B, Gregoria V, Thorpe LB, Dallas S, Polikov VS, et al. Reactive microgliosis: extracellular micro-calpain and microglia-mediated dopaminergic neurotoxicity. Brain. 2010;133(Pt 3):808–21 (PubMed PMID: 20123724. Pubmed Central PMCID: 2860703).
83. Verkhratsky A, Kettenmann H. Calcium signalling in glial cells. Trends Neurosci. 1996;19(8):346–52 (PubMed PMID: 8843604).
84. Zhang W, Phillips K, Wielgus AR, Liu J, Albertini A, Zucca FA, et al. Neuromelanin activates microglia and induces degeneration of dopaminergic neurons: implications for progression of Parkinson's disease. Neurotox Res. 2011;19(1):63–72 (PubMed PMID: 19957214. Pubmed Central PMCID: 3603276).
85. Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? Exp Neurol. 2005;193(2):279–90 (PubMed PMID: 15869932).
86. Hatcher JM, Pennell KD, Miller GW. Parkinson's disease and pesticides: a toxicological perspective. Trends Pharmacol Sci. 2008;29(6):322–9 (PubMed PMID: 18453001).
87. Li AA, Mink PJ, McIntosh LJ, Teta MJ, Finley B. Evaluation of epidemiologic and animal data associating pesticides with Parkinson's disease. J Occup Environ Med. 2005;47(10):1059–87 (PubMed PMID: 16217247).
88. Jackson-Lewis V, Blesa J, Przedborski S. Animal models of Parkinson's disease. Parkinsonism Relat Disord. 2012;18(Suppl 1):S183–5 (PubMed PMID: 22166429).
89. Greenamyre JT, Cannon JR, Drolet R, Mastroberardino PG. Lessons from the rotenone model of Parkinson's disease. Trends Pharmacol Sci. 2010;31(4):141–2 (author reply 2–3. PubMed PMID: 20096940. Pubmed Central PMCID: 2846992).
90. Cabeza-Arvelaiz Y, Schiestl RH. Transcriptome analysis of a rotenone model of parkinsonism reveals complex I-tied and -untied toxicity mechanisms common to neurodegenerative diseases. PLoS One. 2012;7(9):e44700 (PubMed PMID: 22970289. Pubmed Central PMCID: 3436760).
91. Gyulkhandanyan AV, Feeney CJ, Pennefather PS. Modulation of mitochondrial membrane potential and reactive oxygen species production by copper in astrocytes. J Neurochem. 2003;87(2):448–60 (PubMed PMID: 14511122).

92. Arnold B, Cassady SJ, VanLaar VS, Berman SB. Integrating multiple aspects of mitochondrial dynamics in neurons: age-related differences and dynamic changes in a chronic rotenone model. Neurobiol Dis. 2011;41(1):189–200 (PubMed PMID: 20850532. Pubmed Central PMCID: 3021420).
93. Choi WS, Palmiter RD, Xia Z. Loss of mitochondrial complex I activity potentiates dopamine neuron death induced by microtubule dysfunction in a Parkinson's disease model. J Cell Biol. 2011;192(5):873–82 (PubMed PMID: 21383081. Pubmed Central PMCID: 3051820).
94. Sanders LH, Greenamyre JT. Oxidative damage to macromolecules in human Parkinson disease and the rotenone model. Free Radic Biol Med. 2013;62:111–20 (PubMed PMID: 23328732. Pubmed Central PMCID: 3677955).
95. Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, et al. Mechanism of toxicity in rotenone models of Parkinson's disease. J Neurosci. 2003;23(34):10756–64 (PubMed PMID: 14645467).
96. Marella M, Seo BB, Nakamaru-Ogiso E, Greenamyre JT, Matsuno-Yagi A, Yagi T. Protection by the NDI1 gene against neurodegeneration in a rotenone rat model of Parkinson's disease. PLoS One. 2008;3(1):e1433 (PubMed PMID: 18197244. Pubmed Central PMCID: 2175531).
97. Sterky FH, Hoffman AF, Milenkovic D, Bao B, Paganelli A, Edgar D, et al. Altered dopamine metabolism and increased vulnerability to MPTP in mice with partial deficiency of mitochondrial complex I in dopamine neurons. Hum Mol Genet. 2012;21(5):1078–89 (PubMed PMID: 22090423. Pubmed Central PMCID: 3277308).
98. Gao HM, Liu B, Hong JS. Critical role for microglial NADPH oxidase in rotenone-induced degeneration of dopaminergic neurons. J Neurosci. 2003;23(15):6181–7 (PubMed PMID: 12867501).
99. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 2011;119(6):866–72 (PubMed PMID: 21269927. Pubmed Central PMCID: 3114824).
100. Wang A, Costello S, Cockburn M, Zhang X, Bronstein J, Ritz B. Parkinson's disease risk from ambient exposure to pesticides. Eur J Epidemiol. 2011;26(7):547–55 (PubMed PMID: 21505849. Pubmed Central PMCID: 3643971).
101. Cocheme HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. J Biol Chem. 2008;283(4):1786–98 (PubMed PMID: 18039652).
102. Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. Toxicol Sci. 2005;88(1):193–201 (PubMed PMID: 16141438).
103. Prasad K, Winnik B, Thiruchelvam MJ, Buckley B, Mirochnitchenko O, Richfield EK. Prolonged toxicokinetics and toxicodynamics of paraquat in mouse brain. Environ Health Perspect. 2007;115(10):1448–53 (PubMed PMID: 17938734. Pubmed Central PMCID: 2022643).
104. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. Brain Res. 1999;823(1–2):1–10 (PubMed PMID: 10095006).
105. McCormack AL, Di Monte DA. Effects of L-dopa and other amino acids against paraquat-induced nigrostriatal degeneration. J Neurochem. 2003;85(1):82–6 (PubMed PMID: 12641729).
106. Ramachandiran S, Hansen JM, Jones DP, Richardson JR, Miller GW. Divergent mechanisms of paraquat, MPP+, and rotenone toxicity: oxidation of thioredoxin and caspase-3 activation. Toxicol Sci. 2007;95(1):163–71 (PubMed PMID: 17018646).
107. Castello PR, Drechsel DA, Patel M. Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. J Biol Chem. 2007;282(19):14186–93 (PubMed PMID: 17389593. Pubmed Central PMCID: 3088512).
108. Jones GM, Vale JA. Mechanisms of toxicity, clinical features, and management of diquat poisoning: a review. J Toxicol Clin Toxicol. 2000;38(2):123–8 (PubMed PMID: 10778908).

109. Yumino K, Kawakami I, Tamura M, Hayashi T, Nakamura M. Paraquat- and diquat-induced oxygen radical generation and lipid peroxidation in rat brain microsomes. J Biochem. 2002;131(4):565–70 (PubMed PMID: 11926994).
110. Miller RL, Sun GY, Sun AY. Cytotoxicity of paraquat in microglial cells: involvement of PKCdelta- and ERK1/2-dependent NADPH oxidase. Brain Res. 2007;1167:129–39 (PubMed PMID: 17662968. Pubmed Central PMCID: 2084263).
111. Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA. Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. Neurobiol Dis. 2007;25(2):392–400 (PubMed PMID: 17166727. Pubmed Central PMCID: 2001246).
112. Bonneh-Barkay D, Reaney SH, Langston WJ, Di Monte DA. Redox cycling of the herbicide paraquat in microglial cultures. Brain Res Mol Brain Res. 2005;134(1):52–6 (PubMed PMID: 15790529).
113. Fussell KC, Udasin RG, Gray JP, Mishin V, Smith PJ, Heck DE, et al. Redox cycling and increased oxygen utilization contribute to diquat-induced oxidative stress and cytotoxicity in Chinese hamster ovary cells overexpressing NADPH-cytochrome P450 reductase. Free Radic Biol Med. 2011;50(7):874–82 (PubMed PMID: 21215309. Pubmed Central PMCID: 3647689).
114. Van Remmen H, Qi W, Sabia M, Freeman G, Estlack L, Yang H, et al. Multiple deficiencies in antioxidant enzymes in mice result in a compound increase in sensitivity to oxidative stress. Free Radic Biol Med. 2004;36(12):1625–34 (PubMed PMID: 15182862).
115. Mockett RJ, Bayne AC, Kwong LK, Orr WC, Sohal RS. Ectopic expression of catalase in Drosophila mitochondria increases stress resistance but not longevity. Free Radic Biol Med. 2003;34(2):207–17 (PubMed PMID: 12521602).
116. Tien Nguyen-nhu N, Knoops B. Mitochondrial and cytosolic expression of human peroxiredoxin 5 in Saccharomyces cerevisiae protect yeast cells from oxidative stress induced by paraquat. FEBS Lett. 2003;544(1–3):148–52 (PubMed PMID: 12782306).
117. Cantu D, Fulton RE, Drechsel DA, Patel M. Mitochondrial aconitase knockdown attenuates paraquat-induced dopaminergic cell death via decreased cellular metabolism and release of iron and H(2)O(2). J Neurochem. 2011;118(1):79–92 (PubMed PMID: 21517855. Pubmed Central PMCID: 3182850).
118. Gonzalez-Polo RA, Rodriguez-Martin A, Moran JM, Niso M, Soler G, Fuentes JM. Paraquat-induced apoptotic cell death in cerebellar granule cells. Brain Res. 2004;1011(2):170–6 (PubMed PMID: 15157803).
119. Minelli A, Conte C, Grottelli S, Bellezza I, Emiliani C, Bolanos JP. Cyclo(His-Pro) up-regulates heme oxygenase 1 via activation of Nrf2-ARE signalling. J Neurochem. 2009;111(4):956–66 (PubMed PMID: 19735445).
120. Kanthasamy AG, Kitazawa M, Kanthasamy A, Anantharam V. Dieldrin-induced neurotoxicity: relevance to Parkinson’s disease pathogenesis. Neurotoxicology. 2005;26(4):701–19 (PubMed PMID: 16112328).
121. Corrigan FM, French M, Murray L. Organochlorine compounds in human brain. Hum Exp Toxicol. 1996;15(3):262–4 (PubMed PMID: 8839217).
122. Corrigan FM, Murray L, Wyatt CL, Shore RF. Diorthosubstituted polychlorinated biphenyls in caudate nucleus in Parkinson’s disease. Exp Neurol. 1998;150(2):339–42 (PubMed PMID: 9527905).
123. Corrigan FM, Wienburg CL, Shore RF, Daniel SE, Mann D. Organochlorine insecticides in substantia nigra in Parkinson’s disease. J Toxicol Environ Health A. 2000;59(4):229–34 (PubMed PMID: 10706031).
124. Fleming L, Mann JB, Bean J, Briggle T, Sanchez-Ramos JR. Parkinson’s disease and brain levels of organochlorine pesticides. Ann Neurol. 1994;36(1):100–3 (PubMed PMID: 7517654).
125. Bachowski S, Kolaja KL, Xu Y, Ketcham CA, Stevenson DE, Walborg EF, Jr., et al. Role of oxidative stress in the mechanism of dieldrin’s hepatotoxicity. Ann Clin Lab Sci. 1997;27(3):196–209 (PubMed PMID: 9142372).

126. Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH. Dopaminergic cell death induced by MPP(+), oxidant and specific neurotoxicants shares the common molecular mechanism. J Neurochem. 2001;76(4):1010–21 (PubMed PMID: 11181820).
127. Kannan K, Holcombe RF, Jain SK, Alvarez-Hernandez X, Chervenak R, Wolf RE, et al. Evidence for the induction of apoptosis by endosulfan in a human T-cell leukemic line. Mol Cell Biochem. 2000;205(1–2):53–66 (PubMed PMID: 10821422).
128. Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. Free Radic Biol Med. 2001;31(11):1473–85 (PubMed PMID: 11728820).
129. Kanthasamy AG, Kitazawa M, Yang Y, Anantharam V, Kanthasamy A. Environmental neurotoxin dieldrin induces apoptosis via caspase-3-dependent proteolytic activation of protein kinase C delta (PKCdelta): implications for neurodegeneration in Parkinson's disease. Mol Brain. 2008;1:12 (PubMed PMID: 18945348. Pubmed Central PMCID: 2584097).
130. Saminathan H, Asaithambi A, Anantharam V, Kanthasamy AG, Kanthasamy A. Environmental neurotoxic pesticide dieldrin activates a non receptor tyrosine kinase to promote PKCdelta-mediated dopaminergic apoptosis in a dopaminergic neuronal cell model. Neurotoxicology. 2011;32(5):567–77 (PubMed PMID: 21801747. Pubmed Central PMCID: 3328137).
131. Miller GW, Kirby ML, Levey AI, Bloomquist JR. Heptachlor alters expression and function of dopamine transporters. Neurotoxicology. 1999;20(4):631–7 (PubMed PMID: 10499361).
132. Miller GW, Erickson JD, Perez JT, Penland SN, Mash DC, Rye DB, et al. Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. Exp Neurol. 1999;156(1):138–48 (PubMed PMID: 10192785).
133. Hatcher JM, Richardson JR, Guillot TS, McCormack AL, Di Monte DA, Jones DP, et al. Dieldrin exposure induces oxidative damage in the mouse nigrostriatal dopamine system. Exp Neurol. 2007;204(2):619–30 (PubMed PMID: 17291500. Pubmed Central PMCID: 1896133).
134. Ebrahimi-Fakhari D, Cantuti-Castelvetri I, Fan Z, Rockenstein E, Masliah E, Hyman BT, et al. Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of alpha-synuclein. J Neurosci. 2011;31(41):14508–20 (PubMed PMID: 21994367. Pubmed Central PMCID: 3587176).
135. Nedelsky NB, Todd PK, Taylor JP. Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. Biochim Biophys Acta. 2008;1782(12):691–9 (PubMed PMID: 18930136. Pubmed Central PMCID: 2621359).
136. Korolchuk VI, Menzies FM, Rubinsztein DC. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS Lett. 2010;584(7):1393–8 (PubMed PMID: 20040365).
137. Lilienbaum A. Relationship between the proteasomal system and autophagy. Int J Biochem Mol Biol. 2013;4(1):1–26 (PubMed PMID: 23638318. Pubmed Central PMCID: 3627065).
138. Korolchuk VI, Menzies FM, Rubinsztein DC. A novel link between autophagy and the ubiquitin-proteasome system. Autophagy. 2009;5(6):862–3 (PubMed PMID: 19458478).
139. van Wijk SJ Timmers HT. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. FASEB J. 2010;24(4):981–93 (PubMed PMID: 19940261).
140. Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem. 2001;70:503–33 (PubMed PMID: 11395416).
141. Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. J Biosci. 2006;31(1):137–55 (PubMed PMID: 16595883).
142. Tanaka K. The proteasome: overview of structure and functions. Proc Jpn Acad Ser B Phys Biol Sci. 2009;85(1):12–36 (PubMed PMID: 19145068. Pubmed Central PMCID: 3524306).

143. Cook C, Petrucelli L. A critical evaluation of the ubiquitin-proteasome system in Parkinson's disease. Biochim Biophys Acta. 2009;1792(7):664–75 (PubMed PMID: 19419700. Pubmed Central PMCID: 2828612).
144. Rinetti GV, Schweizer FE. Ubiquitination acutely regulates presynaptic neurotransmitter release in mammalian neurons. J Neurosci. 2010;30(9):3157–66 (PubMed PMID: 20203175. Pubmed Central PMCID: 2905680).
145. d'Azzo A, Bongiovanni A, Nastasi T. E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. Traffic. 2005;6(6):429–41 (PubMed PMID: 15882441).
146. Ulrich HD, Walden H. Ubiquitin signalling in DNA replication and repair. Nat Rev Mol Cell Biol. 2010;11(7):479–89 (PubMed PMID: 20551964).
147. McNaught KS, Jackson T, JnoBaptiste R, Kapustin A, Olanow CW. Proteasomal dysfunction in sporadic Parkinson's disease. Neurology. 2006;66(10 Suppl 4):S37–49 (PubMed PMID: 16717251).
148. Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci. 2005;28:57–87 (PubMed PMID: 16022590).
149. Ying Z, Wang H, Wang G. The ubiquitin proteasome system as a potential target for the treatment of neurodegenerative diseases. Curr Pharm Des. 2013;19(18):3305–14 (PubMed PMID: 23151138).
150. Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI, et al. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell. 2002;10(1):55–67 (PubMed PMID: 12150907).
151. Fishman-Jacob T, Reznichenko L, Youdim MB, Mandel SA. A sporadic Parkinson disease model via silencing of the ubiquitin-proteasome/E3 ligase component SKP1A. J Biol Chem. 2009;284(47):32835–45 (PubMed PMID: 19748892. Pubmed Central PMCID: 2781700).
152. McNaught KS, Olanow CW, Halliwell B, Isacson O, Jenner P. Failure of the ubiquitin-proteasome system in Parkinson's disease. Nat Rev Neurosci. 2001;2(8):589–94 (PubMed PMID: 11484002).
153. Haigis MC, Yankner BA. The aging stress response. Mol Cell. 2010;40(2):333–44 (PubMed PMID: 20965426. Pubmed Central PMCID: 2987618).
154. Surguchov A. Molecular and cellular biology of synucleins. Int Rev Cell Mol Biol. 2008;270:225–317 (PubMed PMID: 19081538).
155. Surguchov A. Synucleins: are they two-edged swords? J Neurosci Res. 2013;91(2):161–6 (PubMed PMID: 23150342).
156. Cookson MR. alpha-Synuclein and neuronal cell death. Mol Neurodegener. 2009;4:9 (PubMed PMID: 19193223. Pubmed Central PMCID: 2646729).
157. Takalo M, Salminen A, Soininen H, Hiltunen M, Haapasalo A. Protein aggregation and degradation mechanisms in neurodegenerative diseases. Am J Neurodegener Dis. 2013;2(1):1–14 (PubMed PMID: 23516262. Pubmed Central PMCID: 3601466).
158. Zhang W, Wang T, Pei Z, Miller DS, Wu X, Block ML, et al. Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. FASEB J. 2005;19(6):533–42 (PubMed PMID: 15791003).
159. Rhodes SL, Fitzmaurice AG, Cockburn M, Bronstein JM, Sinsheimer JS, Ritz B. Pesticides that inhibit the ubiquitin-proteasome system: effect measure modification by genetic variation in SKP1 in Parkinsons disease. Environ Res. 2013;126:1–8 (PubMed PMID: 23988235. Pubmed Central PMCID: 3832349).
160. Liao EH, Hung W, Abrams B, Zhen M. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. Nature. 2004;430(6997):345–50 (PubMed PMID: 15208641).
161. Cannon JR, Tapias V, Na HM, Honick AS, Drolet RE, Greenamyre JT. A highly reproducible rotenone model of Parkinson's disease. Neurobiol Dis. 2009;34(2):279–90 (PubMed PMID: 19385059. Pubmed Central PMCID: 2757935).
162. Fornai F, Schluter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, et al. Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. Proc Natl Acad Sci USA. 2005;102(9):3413–8 (PubMed PMID: 15716361. Pubmed Central PMCID: 552938).

163. Pan-Montojo FJ, Funk RH. Oral administration of rotenone using a gavage and image analysis of alpha-synuclein inclusions in the enteric nervous system. J Vis Exp. 2010 (44). PubMed PMID: 21085094. Pubmed Central PMCID: 3280635.
164. Drolet RE, Cannon JR, Montero L, Greenamyre JT. Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. Neurobiol Dis. 2009;36(1):96–102 (PubMed PMID: 19595768).
165. Krishnan S, Chi EY, Wood SJ, Kendrick BS, Li C, Garzon-Rodriguez W, et al. Oxidative dimer formation is the critical rate-limiting step for Parkinson's disease alpha-synuclein fibrillogenesis. Biochemistry. 2003;42(3):829–37 (PubMed PMID: 12534296).
166. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem. 2002;277(3):1641–4 (PubMed PMID: 11707429).
167. Valente EM, Arena G, Torosantucci L, Gelmetti V. Molecular pathways in sporadic PD. Parkinsonism Relat Disord. 2012;18(Suppl 1):S71–3 (PubMed PMID: 22166460).
168. Uversky VN, Li J, Fink AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. FEBS Lett. 2001;500(3):105–8 (PubMed PMID: 11445065).
169. Fernagut PO, Hutson CB, Fleming SM, Tetreaut NA, Salcedo J, Masliah E, et al. Behavioral and histopathological consequences of paraquat intoxication in mice: effects of alpha-synuclein over-expression. Synapse. 2007;61(12):991–1001 (PubMed PMID: 17879265. Pubmed Central PMCID: 3097512).
170. Nuber S, Petrasch-Parwez E, Winner B, Winkler J, von Horsten S, Schmidt T, et al. Neurodegeneration and motor dysfunction in a conditional model of Parkinson's disease. J Neurosci. 2008;28(10):2471–84 (PubMed PMID: 18322092).
171. Cristovao AC, Guhathakurta S, Bok E, Je G, Yoo SD, Choi DH, et al. NADPH oxidase 1 mediates alpha-synucleinopathy in Parkinson's disease. J Neurosci. 2012;32(42):14465–77 (PubMed PMID: 23077033. Pubmed Central PMCID: 3501265).
172. Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci. 2000;20(24):9207–14 (PubMed PMID: 11124998).
173. Thiruchelvam MJ, Powers JM, Cory-Slechta DA, Richfield EK. Risk factors for dopaminergic neuron loss in human alpha-synuclein transgenic mice. Eur J Neurosci. 2004;19(4):845–54 (PubMed PMID: 15009131).
174. Norris EH, Uryu K, Leight S, Giasson BI, Trojanowski JQ, Lee VM. Pesticide exposure exacerbates alpha-synucleinopathy in an A53T transgenic mouse model. Am J Pathol. 2007;170(2):658–66 (PubMed PMID: 17255333. Pubmed Central PMCID: 1851868).
175. Branco DM, Arduino DM, Esteves AR, Silva DF, Cardoso SM, Oliveira CR. Cross-talk between mitochondria and proteasome in Parkinson's disease pathogenesis. Front Aging Neurosci. 2010;2:17 (PubMed PMID: 20577640. Pubmed Central PMCID: 2890153).
176. Yang W, Tiffany-Castiglioni E. The bipyridyl herbicide paraquat induces proteasome dysfunction in human neuroblastoma SH-SY5Y cells. J Toxicol Environ Health A. 2007;70(21):1849–57 (PubMed PMID: 17934957).
177. Wills J, Credle J, Oaks AW, Duka V, Lee JH, Jones J, et al. Paraquat, but not maneb, induces synucleinopathy and tauopathy in striata of mice through inhibition of proteasomal and autophagic pathways. PloS one. 2012;7(1):e30745 (PubMed PMID: 22292029. Pubmed Central PMCID: 3264632).
178. Perfeito R, Cunha-Oliveira T, Rego AC. Reprint of: revisiting oxidative stress and mitochondrial dysfunction in the pathogenesis of Parkinson disease-resemblance to the effect of amphetamine drugs of abuse. Free Radic Biol Med. 2013;62:186–201 (PubMed PMID: 23743292).
179. Sun F, Anantharam V, Latchoumycandane C, Kanthasamy A, Kanthasamy AG. Dieldrin induces ubiquitin-proteasome dysfunction in alpha-synuclein overexpressing dopaminergic

neuronal cells and enhances susceptibility to apoptotic cell death. J Pharmacol Exp Ther. 2005;315(1):69–79 (PubMed PMID: 15987830).

180. Son JH, Shim JH, Kim KH, Ha JY, Han JY. Neuronal autophagy and neurodegenerative diseases. Exp Mol Med. 2012;44(2):89–98 (PubMed PMID: 22257884. Pubmed Central PMCID: 3296817).
181. Banerjee R, Beal MF, Thomas B. Autophagy in neurodegenerative disorders: pathogenic roles and therapeutic implications. Trends Neurosci. 2010;33(12):541–9 (PubMed PMID: 20947179. Pubmed Central PMCID: 2981680).
182. Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med. 2004;10(Suppl):S10–7 (PubMed PMID: 15272267).
183. Taylor JP, Hardy J, Fischbeck KH. Toxic proteins in neurodegenerative disease. Science. 2002;296(5575):1991–5 (PubMed PMID: 12065827).
184. Boland B, Nixon RA. Neuronal macroautophagy: from development to degeneration. Mol Aspects Med. 2006;27(5–6):503–19 (PubMed PMID: 16999991).
185. Rideout HJ, Lang-Rollin I, Stefanis L. Involvement of macroautophagy in the dissolution of neuronal inclusions. Int J Biochem Cell Biol. 2004;36(12):2551–62 (PubMed PMID: 15325592).
186. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. Nat Rev Drug Discov. 2007;6(4):304–12 (PubMed PMID: 17396135).
187. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004;6(4):463–77 (PubMed PMID: 15068787).
188. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. Nat Rev Mol Cell Biol. 2001;2(3):211–6 (PubMed PMID: 11265251).
189. Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem. 1998;273(51):33889–92 (PubMed PMID: 9852036).
190. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol. 2001;152(4):657–68 (PubMed PMID: 11266458. Pubmed Central PMCID: 2195787).
191. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013;19(8):983–97 (PubMed PMID: 23921753).
192. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. Cell Mol Life Sci. 2012;69(7):1125–36 (PubMed PMID: 22080117).
193. Bejarano E, Cuervo AM. Chaperone-mediated autophagy. Proc Am Thorac Soc. 2010;7(1):29–39 (PubMed PMID: 20160146. Pubmed Central PMCID: 3137147).
194. Xiong N, Zhang Z, Huang J, Chen C, Zhang Z, Jia M, et al. VEGF-expressing human umbilical cord mesenchymal stem cells, an improved therapy strategy for Parkinson's disease. Gene Ther. 2011;18(4):394–402 (PubMed PMID: 21107440).
195. Xiong N, Cao X, Zhang Z, Huang J, Chen C, Zhang Z, et al. Long-term efficacy and safety of human umbilical cord mesenchymal stromal cells in rotenone-induced hemiparkinsonian rats. Biol Blood Marrow Transplant. 2010;16(11):1519–29 (PubMed PMID: 20542126).
196. Pan-Montojo F, Anichtchik O, Dening Y, Knels L, Pursche S, Jung R, et al. Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. PLoS One. 2010;5(1):e8762 (PubMed PMID: 20098733. Pubmed Central PMCID: 2808242).
197. Xiong N, Xiong J, Jia M, Liu L, Zhang X, Chen Z, et al. The role of autophagy in Parkinson's disease: rotenone-based modeling. Behav Brain Funct. 2013;9:13 (PubMed PMID: 23497442. Pubmed Central PMCID: 3606411).
198. Mili A, Ben Charfeddine I, Mamai O, Abdelhak S, Adala L, Amara A, et al. Molecular and biochemical characterization of Tunisian patients with glycogen storage disease type III. J Hum Genet. 2012;57(3):170–5 (PubMed PMID: 22089644).
199. Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience. 2009;164(2):541–51 (PubMed PMID: 19682553).

200. Filomeni G, Graziani I, De Zio D, Dini L, Centonze D, Rotilio G, et al. Neuroprotection of kaempferol by autophagy in models of rotenone-mediated acute toxicity: possible implications for Parkinson's disease. Neurobiol Aging. 2012;33(4):767–85 (PubMed PMID: 20594614).
201. Dadakhujaev S, Noh HS, Jung EJ, Cha JY, Baek SM, Ha JH, et al. Autophagy protects the rotenone-induced cell death in alpha-synuclein overexpressing SH-SY5Y cells. Neurosci Lett. 2010;472(1):47–52 (PubMed PMID: 20117172).
202. Giordano S, Dodson M, Ravi S, Redmann M, Ouyang X, Darley Usmar VM, et al. Bioenergetic adaptation in response to autophagy regulators during rotenone exposure. J Neurochem. 2014;131:625–33 (PubMed PMID: 25081478).
203. Chu CT, Ji J, Dagda RK, Jiang JF, Tyurina YY, Kapralov AA, et al. Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. Nat Cell Biol. 2013;15(10):1197–205 (PubMed PMID: 24036476. Pubmed Central PMCID: 3806088).
204. Gonzalez-Polo RA, Niso-Santano M, Ortiz-Ortiz MA, Gomez-Martin A, Moran JM, Garcia-Rubio L, et al. Relationship between autophagy and apoptotic cell death in human neuroblastoma cells treated with paraquat: could autophagy be a "brake" in paraquat-induced apoptotic death? Autophagy. 2007;3(4):366–7 (PubMed PMID: 17438367).
205. Gonzalez-Polo RA, Niso-Santano M, Ortiz-Ortiz MA, Gomez-Martin A, Moran JM, Garcia-Rubio L, et al. Inhibition of paraquat-induced autophagy accelerates the apoptotic cell death in neuroblastoma SH-SY5Y cells. Toxicol Sci. 2007;97(2):448–58 (PubMed PMID: 17341480).
206. Garcia-Garcia A, Anandhan A, Burns M, Chen H, Zhou Y, Franco R. Impairment of Atg5-dependent autophagic flux promotes paraquat- and MPP(+)-induced apoptosis but not rotenone or 6-hydroxydopamine toxicity. Toxicol Sci. 2013;136(1):166–82 (PubMed PMID: 23997112. Pubmed Central PMCID: 3829573).
207. Chen YW, Yang YT, Hung DZ, Su CC, Chen KL. Paraquat induces lung alveolar epithelial cell apoptosis via Nrf-2-regulated mitochondrial dysfunction and ER stress. Arch Toxicol. 2012;86(10):1547–58 (PubMed PMID: 22678742).
208. Omura T, Asari M, Yamamoto J, Oka K, Hoshina C, Maseda C, et al. Sodium tauroursodeoxycholate prevents paraquat-induced cell death by suppressing endoplasmic reticulum stress responses in human lung epithelial A549 cells. Biochem Biophys Res Commun. 2013;432(4):689–94 (PubMed PMID: 23416354).
209. Yang W, Tiffany-Castiglioni E, Koh HC, Son IH. Paraquat activates the IRE1/ASK1/JNK cascade associated with apoptosis in human neuroblastoma SH-SY5Y cells. Toxicol Lett. 2009;191(2–3):203–10 (PubMed PMID: 19735704).
210. Niso-Santano M, Bravo-San Pedro JM, Gomez-Sanchez R, Climent V, Soler G, Fuentes JM, et al. ASK1 overexpression accelerates paraquat-induced autophagy via endoplasmic reticulum stress. Toxicol Sci. 2011;119(1):156–68 (PubMed PMID: 20929985).
211. Tsuboi Y. Environmental-genetic interactions in the pathogenesis of Parkinson's disease. Exp Neurobiol. 2012;21(3):123–8 (PubMed PMID: 23055790. Pubmed Central PMCID: 3454809).
212. Goldman SM, Kamel F, Ross GW, Bhudhikanok GS, Hoppin JA, Korell M, et al. Genetic modification of the association of paraquat and Parkinson's disease. Mov Disord. 2012;27(13):1652–8 (PubMed PMID: 23045187. Pubmed Central PMCID: 3572192).
213. Gonzalez-Polo R, Niso-Santano M, Moran JM, Ortiz-Ortiz MA, Bravo-San Pedro JM, Soler G, et al. Silencing DJ-1 reveals its contribution in paraquat-induced autophagy. J Neurochem. 2009;109(3):889–98 (PubMed PMID: 19425177).
214. Bingol B, Tea JS, Phu L, Reichelt M, Bakalarski CE, Song Q, et al. The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. Nature. 2014;510(7505):370–5 (PubMed PMID: 24896179).
215. Kanthasamy A, Jin H, Anantharam V, Sondarva G, Rangasamy V, Rana A, et al. Emerging neurotoxic mechanisms in environmental factors-induced neurodegeneration. Neurotoxicology. 2012;33(4):833–7 (PubMed PMID: 22342404. Pubmed Central PMCID: 3377824).

216. Kanthasamy A, Anantharam V, Ali SF, Kanthasamy AG. Methamphetamine induces autophagy and apoptosis in a mesencephalic dopaminergic neuronal culture model: role of cathepsin-D in methamphetamine-induced apoptotic cell death. Ann N Y Acad Sci. 2006;1074:234–44 (PubMed PMID: 17105920).
217. Abbott RD, Ross GW, White LR, Sanderson WT, Burchfiel CM, Kashon M, et al. Environmental, life-style, and physical precursors of clinical Parkinson's disease: recent findings from the Honolulu-Asia Aging Study. J Neurol. 2003;250(Suppl 3):III30–9 (PubMed PMID: 14579122).
218. Le Couteur DG Muller M Yang MC Mellick GD McLean AJ. Age-environment and gene-environment interactions in the pathogenesis of Parkinson's disease. Rev Environ Health. 2002;17(1):51–64 (PubMed PMID: 12088093).
219. Zorzon M, Capus L, Pellegrino A, Cazzato G, Zivadinov R. Familial and environmental risk factors in Parkinson's disease: a case-control study in north-east Italy. Acta Neurol Scand. 2002;105(2):77–82 (PubMed PMID: 11903115).
220. Martinez TN, Greenamyre JT. Toxin models of mitochondrial dysfunction in Parkinson's disease. Antioxid Redox Signal. 2012;16(9):920–34 (PubMed PMID: 21554057. Pubmed Central PMCID: 3292753).
221. Ojha A, Yaduvanshi SK, Srivastava N. Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. Pestic Biochem Phys. 2011;99(2):148–56 (PubMed PMID: WOS:000286961500004. English).
222. Sharma H, Zhang P, Barber DS, Liu B. Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. Neurotoxicology. 2010;31(2):215–22 (PubMed PMID: 20036686).
223. Chinta SJ, Andersen JK. Redox imbalance in Parkinson's disease. Biochim Biophys Acta. 2008;1780(11):1362–7 (PubMed PMID: 18358848. Pubmed Central PMCID: 2547405).
224. Le W. Role of iron in UPS impairment model of Parkinson's disease. Parkinsonism Relat Disord. 2014;20(Suppl 1):S158–61 (PubMed PMID: 24262171).

Chapter 11
Molecular and Neurochemical Mechanisms Dopamine Oxidation To *O*-Quinones in Parkinson's Disease Pathogenesis

Patricia Muñoz, Catalina Melendez, Irmgard Paris and Juan Segura-Aguilar

Abstract Four decades after L-dopa was introduced as a therapy for Parkinson's disease, it is still the gold standard for Parkinson's pharmacological treatment because no new drug has been discovered. This is due to the ambiguity regarding the molecular mechanisms responsible for the degeneration of dopaminergic neurons containing neuromelanin in the substantia nigra, which induces the motor symptoms of Parkinson's disease. The question is to identify the mechanisms underlying the neurodegenerative process of Parkinson's disease initiated years before the motor symptoms are evident, through pre-motor symptoms. The possible role of an exogenous environmental neurotoxin has been proposed. However, MPTP generates severe Parkinsonism in just 3 days, in contrast with idiopathic Parkinson's disease, which occurs after years of slow degeneration. The discovery of proteins (such as alpha synuclein and parkin) associated with the familial form of the disease opened new lines of basic research, resulting in a general agreement that five mechanisms are involved in the degeneration of dopaminergic neurons containing neuromelanin: protein degradation dysfunction, mitochondrial dysfunction, alpha synuclein aggregation, oxidative stress and neuroinflammation. Dopamine oxidation sequentially generates dopamine *o*-quinone, aminochrome, 5,6-indolequinone and finally, neuromelanin. These *o*-quinones have been reported to be directly involved in four of

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the five above-mentioned mechanisms: protein degradation dysfunction, mitochondria dysfunction, alpha synuclein aggregation and oxidative stress. This suggests that dopamine oxidation to *o*-quinone may play a role in the degeneration of the dopaminergic neurons of the nigrostriatal system in Parkinson's disease.

Keywords Dopamine oxidation · Parkinson's disease · Neuromelanin · Aminochrome · Neurodegeneration · Neuroprotection

Abbreviations

AADC	Aromatic amino acid decarboxylase
TH	Tyrosine hydroxylase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
VMAT-2	Vesicular monoaminergic transporter-2
DAT	Dopamine transporter
MAO	Monoamine oxidase
COMT	Catechol ortho-methyltransferase
UCHL-1	Ubiquitin carboxyl-terminal hydrolase isozyme
GSTM2	Glutathione S-transferase M2-2
ATP13A2	ATPase type 13 A
PINK1	PTEN-induced kinase 1
LRRK-2	Leucine-rich repeat kinase 2
DJ-1	Parkinson protein 7

Dopamine Synthesis

Dopamine is the neurotransmitter in dopaminergic neurons, and striatal dopamine contributes to the regulation of motor activity. In the dopaminergic neurons, dopamine synthesis occurs in the cytosol and there is the re-uptake of dopamine released during neurotransmission. Tyrosine is the amino acid precursor of dopamine, and two enzymes participate in the synthesis de novo of dopamine. The enzyme tyrosine hydroxylase (TH) catalyzes the hydroxylation of tyrosine to L-dopa using tyrosine, oxygen and tetrahydrobiopterin as substrates to mediate tyrosine hydroxylation [1, 2]. The enzyme aromatic amino acid decarboxylase (AADC) catalyzes the decarboxylation of L-dopa to a molecule of dopamine and CO_2. The enzymes TH and AADC form a type of complex with the vesicular monoaminergic transporter-2 (VMAT-2) that is localized in the membrane of monoaminergic synaptic vesicles [3]. Both reactions are localized in the cytosol; thus, it is expected that dopamine accumulates in the cytosol. However, the formation of a complex consisting of TH, AADC and VAMT-2 prevents dopamine accumulation in the cytosol. Tyrosine enters into this complex, TH catalyzes the formation of L-dopa, and AADC immediately catalyzes the formation of dopamine, which is transported into the monoaminergic synaptic vesicles by VMAT-2. The TH-AADC-VMAT-2 complex prevents

dopamine oxidation to *o*-quinones, which can otherwise occur in the cytosol at a pH of 7.4 [4]. Protons are bound to the oxygen in the dopamine hydroxyl groups inside the monoaminergic synaptic vesicles; VMAT-2 accumulates monoamines inside the vesicles utilizing the electrochemical gradient across the membrane, established by vacuolar ATPase generating an approximate pH of 5.0 [5]. Therefore, VMAT-2 is important in preventing dopamine oxidation because a high concentration of dopamine can be stored in monoaminergic synaptic vesicles with low pH for neurotransmission, where it is stable and resistant to autoxidation in the presence of oxygen. Inhibition of VMAT-2 by reserpine increases dopamine oxidation and neuromelanin formation [6], and the over expression of VMAT-2 prevents neuromelanin formation [7]. Interestingly, midbrain dopaminergic neurons produce greater amounts of dopamine and have a higher vesicular storage capacity, compared with the ventral substantia nigra neurons, which produce the most neuromelanin, partly because they have the least VMAT2 protein [8].

Free dopamine can occur following the reuptake of dopamine released into the intersynaptic space that binds to dopamine receptors in the postsynaptic neurons. The reuptake of dopamine from the intersynaptic space is mediated by dopamine transporter (DAT) localized in the plasma membrane. Studies with animals deficient in DAT revealed that the animals presented a low level of dopamine and decreased vesicular storage of dopamine [9–11] (Fig. 11.1).

Dopamine Degradation

There are enzymes that can degrade dopamine to prevent the existence of free dopamine. One of them is the enzyme monoamine oxidase (MAO, E.C. 1.4.3.4) that plays a role in dopamine degradation by catalyzing the oxidative deamination of the dopamine amino group, using dopamine as the substrate, a molecule of water and oxygen to form 3,4-dihydroxyphenylacetaldehyde, hydrogen peroxide and ammonia. MAO isozymes (A and B) are flavoenzymes [12] possessing a 70 % primary structure identity and are localized in the mitochondrial outer membrane in neurons and glia cells [13, 14]. However, MAO-A is found in catecholaminergic neurons, whereas MAO-B is found in astrocytes and histaminergic and serotonergic neurons [15, 16]. MAO-B has a higher affinity for phenylethylamine and benzylamine, whereas MAO-A has a higher affinity for the substrates serotonin, norepinephrine and dopamine. However, dopamine is oxidized by MAO-B in humans and MAO-A in rodents [17]. The Km for serotonin is 295 ± 46 μM for human MAO-A and 2270 ± 310 μM for human MAO-B [18].

MPTP, which induces Parkinsonism in humans, is taken up into astrocytes where MAO-B catalyzes the activation of MPTP to MPP+. MPP+ is neurotoxic in dopaminergic neurons because it has a high affinity for dopamine transporter and accumulates in dopaminergic neurons (Fig. 11.1) [19].

The product of the dopamine deamination catalyzed by MAO-A, 3,4-dihydroxyphenylacetaldehyde (Fig. 11.2, reaction 1), is the substrate for aldehyde dehydroge-

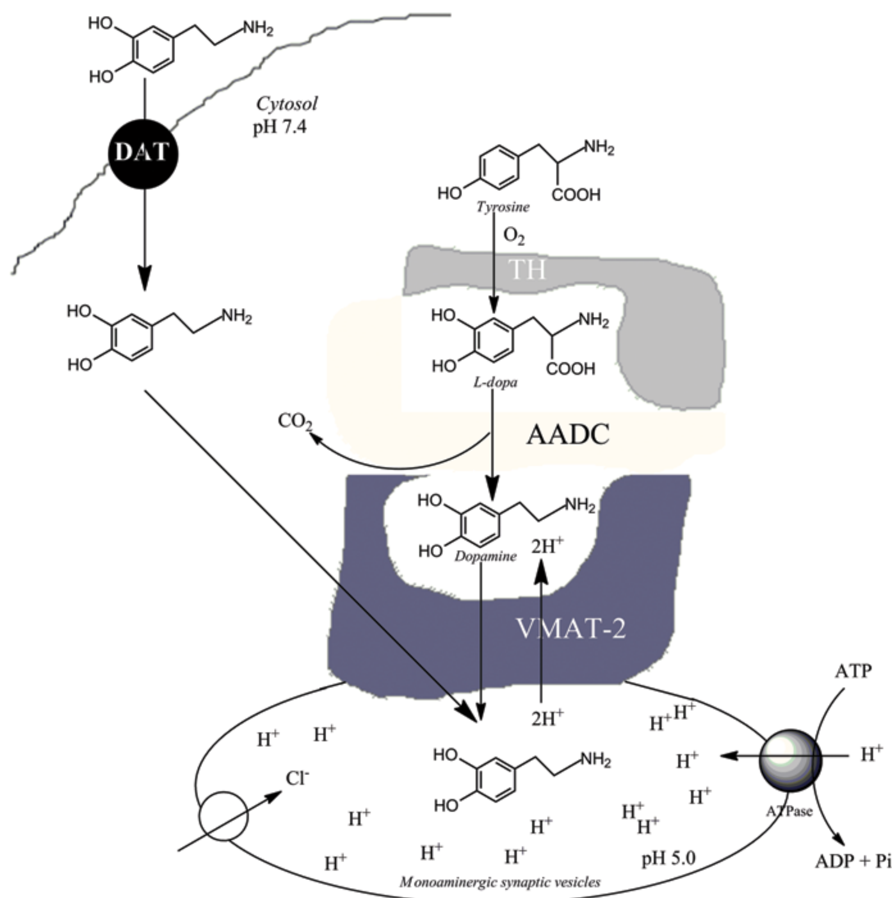

Fig. 11.1 Synthesis of dopamine from the amino acid tyrosine and storage in monoaminergic vesicles

nase, which uses a molecule of NAD^+ and water to oxidize 3,4-dihydroxyphenylacetaldehyde to 3,4-dihydroxyphenylacetic acid, one molecule of NADH and a proton (Fig. 11.2, reaction 2).

Dopamine can also be degraded by an ortho methylation reaction catalyzed by catechol ortho-methyltransferase (COMT; EC 2.1.1.6), which uses S-adenosylmethionine (SAM) as the cofactor to form 3-methoxytyramine and S-adenosylhomocysteine (SAH) Fig. 11.2, reaction 3). Two isoforms of COMT have been reported, the membrane-bound COMT and the soluble COMT isoforms, both in astroglia and microglia. COMT is also expressed in pyramidal neurons, granular cells, cerebellum Purkinje cells, and striatum spiny neurons. Membrane bound COMT has been reported to be found in the cell body, dendrites and axons of rat cortical neurons [20].

Additionally, MAO catalyzes the oxidative deamination of 3-methoxytyramine to 4-hydroxy-3-methoxyphenylacetaldehyde, with concomitant formation of ammonia and hydrogen peroxide under aerobic conditions (Fig. 11.2, reaction 4).

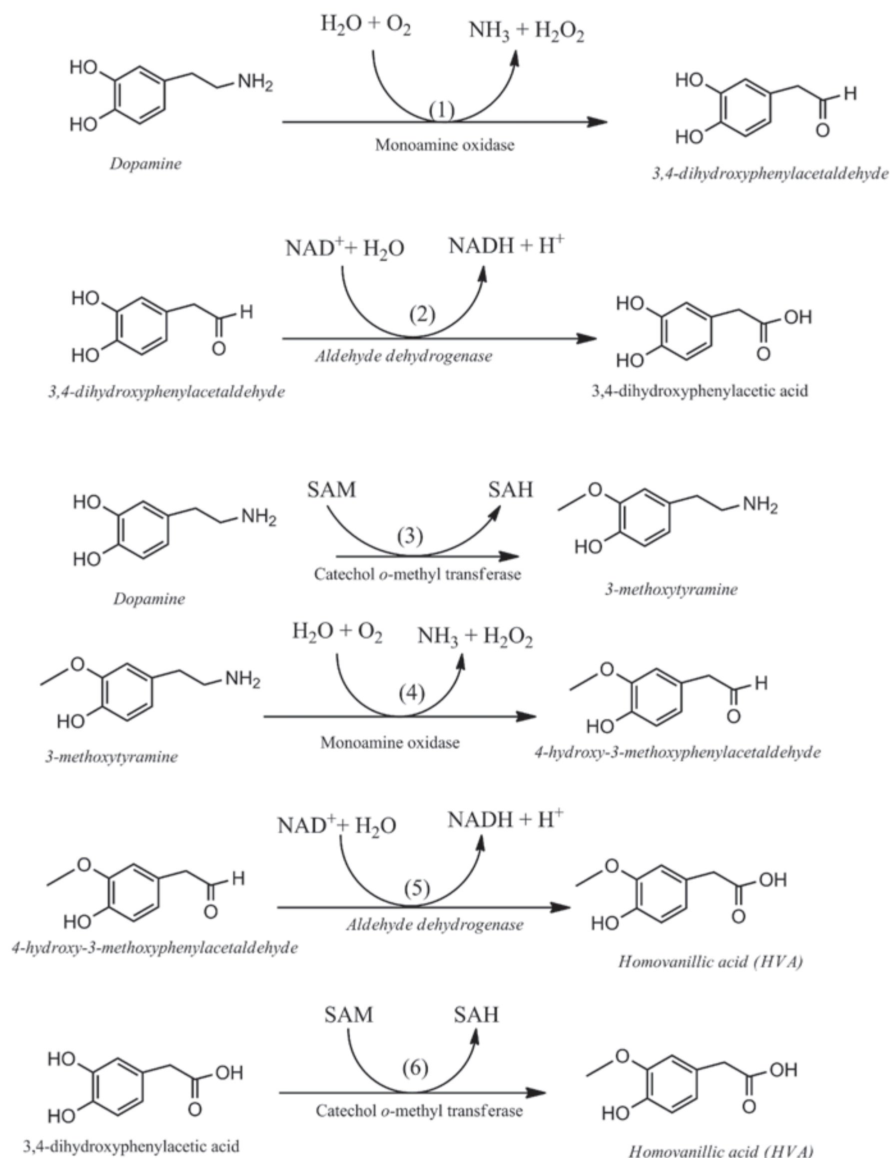

Fig. 11.2 MAO and COMT reactions with dopamine and metabolites

Finally, to complete the degradation of dopamine initiated by COMT followed by the enzyme MAO, the enzyme aldehyde dehydrogenase catalyzes the oxidation of 4-hydroxy-3-methoxyphenylacetaldehyde to homovanillic acid (Fig. 11.2, reaction 5).

The degradation of dopamine initiated by MAO followed by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid is completed by the enzyme COMT, which catalyzes the formation of homovanillic acid (Fig. 11.2, reaction 6).

Dopamine Oxidation to Ortho-Quinones

Dopamine oxidation to *o*-quinones and their polymerization to neuromelanin seems to be a natural process because normal individuals have this pigment that increases with age in the substantia nigra [21]. The dopamine oxidation is catalyzed by metals such as manganese(III) that catalyzes this reaction both under anaerobic and aerobic conditions [22]. Interestingly, high exposure to manganese has been related to a Parkinsonism syndrome observed in workers in the manganese mining industry and in workers exposed to welding [23]. Manganese neurotoxicity has been associated with the impairment of dopaminergic neurons as a consequence of manganese accumulation in the dopaminergic neurons of the basal ganglia, particularly in the globus pallidus, causing extrapyramidal motor dysfunction [24]. Studies performed with manganese-resistant cells revealed that the calcium-binding proteins S100A9 and S100A10 are linked with manganese resistance in these cell lines [25]. Copper sulfate has been reported to catalyze dopamine oxidation, and high exposure to this metal in the mining industry has been associated with a Parkinsonism syndrome [26]. Copper(II) forms a complex with dopamine that is taken up specifically by dopamine transporters into dopaminergic neurons, where dopamine is oxidized to an aminochrome that induces mitochondrial autophagy preceding caspase-independent apoptotic cell death [27, 28]. Interestingly, a study on Wilson's syndrome where 282 patients were evaluated for 3 decades revealed that Parkinsonism is the most common clinical symptom in this disease [29]. The high prevalence of Parkinsonism in Wilson's disease, where the patients are exposed to high concentrations of copper due to a mutation in ATP7A transporter responsible for copper excretion to the bile [30], can be explained by the formation of a copper-dopamine complex that specifically induces neurotoxicity in dopaminergic neurons. Additionally, dopamine can be oxidized by both free iron ions (trivalent or bivalent) and iron complexed with cyanide ions, such as $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$, although iron complexes with cyanide ions have been reported only in *in vitro* experiments [31]. Dopamine can also form a complex with iron(III) that can be taken up by cells via dopamine transporter and induce specific neurotoxicity [32, 33]. Sodium periodate has also been used to oxidize dopamine [34].

In addition, enzymes catalyze dopamine oxidation to *o*-quinones, including tyrosinase, prostaglandin H synthase, xanthine oxidase, cytochrome P450, dopamine *β*-monooxygenase, and lactoperoxidase and may play a role in the neurodegeneration of dopaminergic neurons [35–41].

Interestingly, dioxygen (O_2) is also able to oxidize dopamine to *o*-quinones in the absence of metal-ion at pH 7.4 [4]. Dioxygen catalyzes the one-electron oxidation of dopamine to the dopamine *o*-semiquinone radical (Fig. 11.3, reaction 1), which can reduce O_2 to superoxide and form a molecule of dopamine *o*-quinone (Fig. 11.3, reaction 2). Alternatively, two dopamine *o*-semiquinone radicals can react with each other in a disproportionation reaction generating one molecule of dopamine *o*-quinone and one molecule of dopamine (Fig. 11.3, reaction 3). Dopamine can also oxidize to dopamine *o*-quinone without the formation of the dopamine *o*-semiquinone radical (Fig. 11.3 reaction 4) [36, 42]. Dopamine *o*-quinone is extremely unstable at

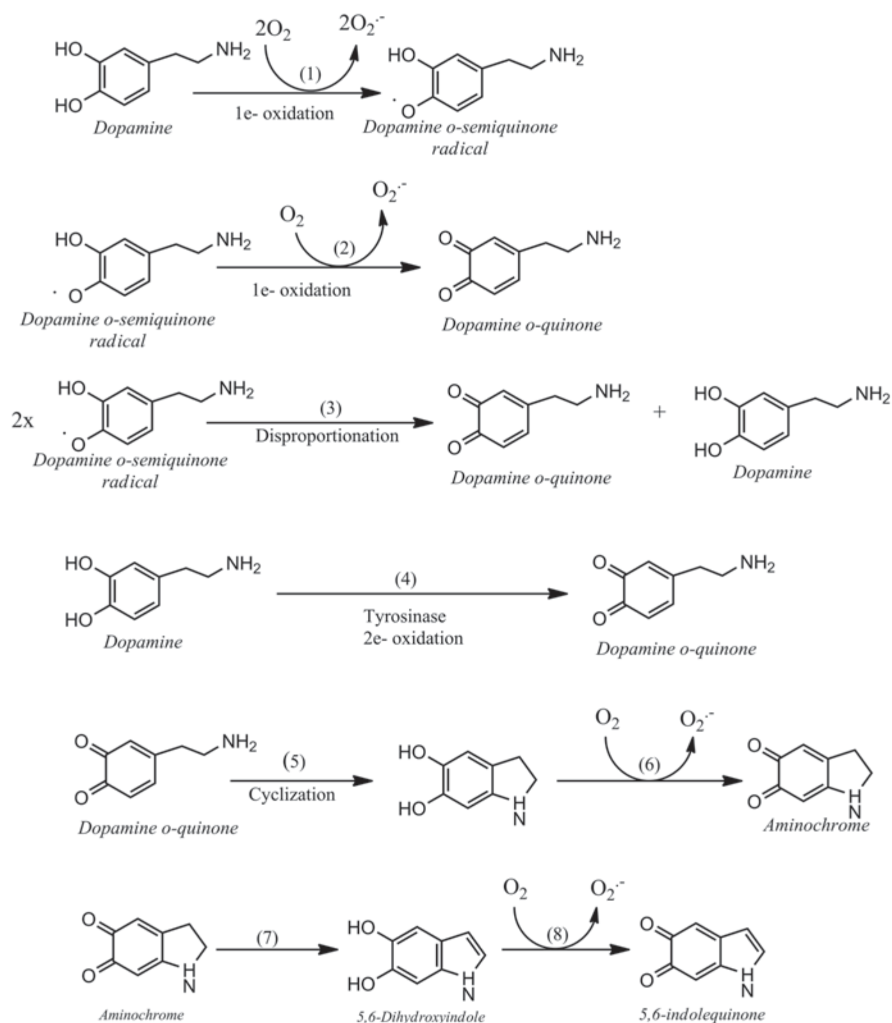

Fig. 11.3 Dopamine oxidation to the o-quinones dopaminer o-quinone, aminochrome and 5,6-indolequinone

pH 7.4, undergoing intramolecular cyclization to form leukoaminochrome that later oxidizes to aminochrome (Fig. 11.3 reactions 5 and 6) [22, 43]. The rate constant for the intramolecular cyclization of dopamine *o*-quinone to aminochrome is only 0.15 s^{-1}, whereas the rate for aminochrome rearrangement to 5,6-dihydroxyindole is much slower (0.06 min^{-1}), resulting in aminochrome accumulation [43, 44].

The aminochrome rearrangement to 5,6-indolequinone involves the rearrangement of aminochrome to 5,6-dihydroxyindole (Fig. 11.3 reaction 7), followed by the oxidation of 5,6-dihydroxyindole to 5,6-indolequinone (Fig. 11.3, reaction 8) [46]. The NMR spectra of dopamine oxidation showed dopamine, aminochrome

and 5,6-dihydroxyindole; however, dopamine *o*-quinone and 5,6-indolequinone were not detected because both intramolecular cyclization of dopamine *o*-quinone to aminochrome and neuromelanin formation from 5,6-indolequinone are too rapid [34].

Dopamine oxidation resulting in the formation of neuromelanin involves the formation of three ortho-quinone species, namely dopamine *o*-quinone, aminochrome and 5,6-indolequinone. These studies have been performed under different conditions where dopamine has been oxidized by tyrosinase [45] or metals, or pure aminochrome has been used. The *o*-quinone species responsible for these results are unknown. Dopamine *o*-quinone and 5,6-indolequinone have shorter lives than aminochrome; aminochrome rearrangement has a rate constant of 0.06 min^{-1}. However, the rate for the nucleophilic addition of GSH to dopamine *o*-quinone is faster than the rate of intramolecular cyclization [43], suggesting that dopamine *o*-quinone plays an important role in nucleophilic additions with proteins. However, NMR studies with dopamine *o*-quinone and alpha synuclein revealed that only aminochrome adducts with alpha synuclein are observed, after 40 min [47]. Therefore, we cannot be sure that during the tyrosinase-catalyzed dopamine oxidation, the measured effect represents dopamine *o*-quinone alone, excluding other *o*-quinones such as aminochrome and 5,6-indolequinone.

Several proteins have been reported to form adducts with dopamine *o*-quinone, such as mitochondrial glutathione peroxidase 4 [48], mitochondrial complex I, III and V, JC-1, UCHL-1 [49, 50], tyrosine hydroxylase [51], dopamine transporter [52] and parkin [53]. In other experiments, the treatment was the addition of dopamine that autoxidizes in the presence of oxygen [6, 54]. Enzymatic GSH conjugation of dopamine *o*-quinone to 5-S-glutathionyl dopamine was catalyzed by GSTM2, although a non-enzymatic reaction has also been reported [43, 55]. In contrast, other studies have involved aminochrome formation prior to the experiment [56] or the use of purified aminochrome [57–62]; thus, it is possible that 5,6-indolequinone had been formed in these experiments. However, the aminochrome conjugation with glutathione catalyzed by glutathione S-transferase M2 (GSTM2) generated only 4-*S*-glutathionyl-5,6-dihydroxyindoline and not 4-*S*-glutathionyl-5,6-dihydroxyindole, which should be the product of 5,6-indolequinone glutathione conjugation [63].

Dopamine Ortho-Quinones Metabolism

Melanin Formation

Neuromelanin is a dark pigment found in the substantia nigra and in the locus coeruleus of healthy individuals, which increases with age [21, 64], suggesting that the formation of neuromelanin is not a neurotoxic reaction. Neuromelanin biosynthesis may begin with the autoxidation of catecholamines to *o*-quinones, followed by the

addition of a thiol group, as observed in the brain [65, 66]. The synthesis of neuromelanin depends on the level of free dopamine in the cytosol that can be oxidized and polymerized to form neuromelanin [7]. Dopamine undergoes a sequential oxidation to (i) dopamine *o*-quinone that undergoes intramolecular cyclization to generate aminochrome and (ii) aminochrome rearrangement to 5,6-dihydroxyindole that oxidizes to 5,6-indolequinone, which is the molecule generating neuromelanin [44]. This pigment is composed of a mixture of both eumelanin and pheomelanin [65, 66] bound to both lipids and peptides. The participation of enzymes in neuromelamin formation is unclear; however, several enzymes catalyze dopamine oxidation, such as liver microsomal vitamin D 25-hydroxylase, cytochrome P450 1 A2, Dopamine *β*-monooxygenase, lactoperoxidase and xanthine oxidase [35–41, 45, 67]. Tyrosinase is an enzyme that catalyzes the hydroxylation of tyrosine to L-dopa and the oxidation of L-dopa or dopamine to *o*-quinone, in the formation of other melanins. However, immunohistochemical studies have not found tyrosinase in the substantia nigra, although its mRNA has been detected [68]. The presence of neuromelanin in albinos, who do not express an active tyrosinase, support the theory that tyrosinase does not play a role in neuromelanin formation [69]. It has been suggested that neuromelanin can promote toxic reactions as well because the injection of neuromelanin into rat substantia nigra induces microglial activation and degeneration of dopaminergic neurons [70]. Free neuromelanin has been associated with microglial activation because its phagocytosis and degradation is mediated by activated microglia [71–74]. Neuromelanin chelates iron ions at high affinity sites in a stable form, preventing its reactivity [75]. However, when iron is in excess and the high affinity sites are saturated, neuromelanin can bind iron ions with low affinity in a reactive form, generating redox cycling reactions [76–77].

Formation of Adducts with Proteins

Dopamine oxidation generates three *o*-quinones that form adducts with proteins, including mitochondrial complexes (I, III and V) and isocitrate dehydrogenase, suggesting mitochondrial dysfunction [49, 50]. Parkinson protein 7 (DJ-1) [78] and ubiquitin carboxyl-terminal hydrolase isozyme L1(UCHL-1) also form adducts with dopamine *o*-quinone [49].

Aminochrome forms adducts with the 125YEMPS129 motif of alpha synuclein, generating and stabilizing the neurotoxic protofibrils/oligomers of alpha synuclein [79]. Additionally, it forms adducts with α- and *β*-tubulin, disrupting the cytoskeleton architecture and generating aggregates that completely change the cell shape and disrupt the cytoskeleton architecture further [58]. 5,6-Indolequinone also forms adducts with alpha synuclein [34]. Parkin, a ubiquitin ligase 3 of the proteasome system, forms adducts with one of the *o*-quinones formed during dopamine oxidation, resulting in parkin inactivation [53]. It is unknown what happens *in vivo* when dopamine oxidizes to dopamine *o*-quinone, aminochrome and 5,6-indolequinone.

One-Electron Reduction

In general, quinones can undergo one-electron reduction by flavoenzymes that can use NADH or NADPH as the electron donator. However, no study reports the one-electron reduction of dopamine *o*-quinone or 5,6-indolequinone. Aminochrome can undergo one-electron reduction through flavoenzymes to the leukoaminochrome-*o*-semiquinone radical, which is highly reactive with oxygen and immediately autoxidizes to aminochrome with the concomitant formation of superoxide radicals, generating a redox cycling between aminochrome and leukoaminochrome-*o*-semiquinone radical [22, 80]. This redox cycling depletes both NADH/NADPH and oxygen levels, inducing (i) an energy collapse because NADH and oxygen are not available for ATP synthesis in the mitochondria; (ii) the formation of superoxide radicals, causing oxidative stress. The depletion of NADPH results in a decrease in reduced glutathione because NADPH is required for the enzymatic reduction of glutathione. Several reports demonstrate that the one-electron reduction of aminochrome is a neurotoxic reaction both in cell cultures and *in vivo* [27, 28, 32, 33, 54, 56–62, 81–86].

Two-Electron Reduction

Aminochrome has been reported to undergo two-electron reduction by DT-diaphorase (E.C.1.6.99.2; NQO1) to leukoaminochrome, which in the presence superoxide radicals autoxidizes to the leukoaminochrome *o*-semiquinone radical; however, superoxide dismutase prevents its autoxidation [22, 80]. There is no information about the role of dopamine *o*-quinone and 5,6-indolequinone in this process. DT-diaphorase is unique flavoenzyme that catalyzes the two-electron reduction of quinones, using NADH or NADPH as the electron donator. This enzyme is expressed in several regions of the brain, such as the substantia nigra, striatum, cerebellum, hypothalamus, hippocampus, and frontal cortex of the rat brain. This enzyme is expressed in astrocytes and dopaminergic neurons, and it is responsible for 97 % of the total quinone reductase activity in substantia nigra [83]. The two-electron reduction of aminochrome prevents aminochrome neurotoxic pathways because its inhibition or silencing with siRNA induces neurotoxicity both in cell cultures [56–58] or *in vivo* [81, 82].

Glutathione Conjugation

The short-lived dopamine *o*-quinone has been reported to be conjugated with GSH because the pseudo first order rate constant was found to be 208 s^{-1}; thus it is faster than the intramolecular cyclization of dopamine *o*-quinone [44]. Dopamine oxidation mediated by lipoperoxidase in the presence of H_2O_2 and N-acetyl-l-cysteine

resulted in the formation of 5-S-N-acetyl-l-cysteinyl–dopamine as the predominant conjugate [87]. However, 2-S-N-acetyl-l-cysteinyl–dopamine and 2-S-5-S-di-N-acetyl-l-cysteinyl–dopamine were detected as well [88]. The conjugation of dopamine *o*-quinone with GSH results in the formation of 5-glutathionyl dopamine, which is degraded to 5-S-cysteinyldopamine [89]. The oxidation of 5-S-cysteinyl-dopamine has been proposed as a possible neurotoxic mechanism, depending on the redox conditions [90]. Glutathione S-transferase (GST) M2-2 (GSTM2) catalyzes the GSH conjugation of dopamine *o*-quinone to 5-glutathionyl-dopamine [55].

Aminochrome is spontaneously conjugated with GSH to form 4-*S*-glutathionyl-5,6-dihydroxyindoline, but the rate of enzymatic conjugation catalyzed by GSTM2 was significantly higher than that for the non-enzymatic reaction. This conjugate was found to be stable under oxidative conditions in the presence of hydrogen peroxide, superoxide radicals and oxygen [63]. The GSH conjugation of aminochrome has been proposed to be a protective reaction because it prevents aminochrome-induced cell death in an astrocyte model cell line [59].

Role of Dopamine Ortho-Quinones in Parkinson's Disease

Parkinson's disease is diagnosed using the motors symptoms; however, these symptoms are preceded by earlier symptoms such as depression, sleep fragmentation and olfactory disturbances [91]. Majority of the people with Parkinsonian signs present the idiopathic form of Parkinson's disease (70 %), in which the cause for this disease is unknown. Thirty percent of the individuals with Parkinsonian signs present Parkinsonism, in which the disease is a consequence of exposure to metals [23], copper [26], contaminant in drugs (MPTP) [73], neuroleptics [101] and pesticides [102] or mutations in genes associated with the disease [92–100]. Parkinsonism induced by manganese has a long list of atypical symptoms, including neurobehavioral manifestations, psychosis and others [102]. The appearance of motor symptoms are related to the loss of dopaminergic neurons containing neuromelanin. Studies on the association of several genes with familial Parkinson's disease have a huge impact on basic research by aiming to understand the role of these genes in the sporadic form of the disease. These genes include alpha synuclein, parkin, ATP13A2, PINK1, LRRK-2 and DJ-1 [92–100]. However, to date, the molecular mechanism responsible for the loss of dopaminergic neurons containing neuromelanin is an open question, although the scientific community in this field generally agree that the molecular mechanism for the degeneration of dopaminergic neurons involve mitochondria dysfunction, protein degradation dysfunction, aggregation of alpha synuclein to neurotoxic oligomers, oxidative stress and neuroinflamamtion [103–107].

Interestingly, the *o*-quinones, dopamine *o*-quinone, aminochrome, and 5,6-indolequinone formed during dopamine oxidation are directly connected to four of these five mechanisms associated with the degenerative process of dopaminergic

neurons in Parkinson's disease: (i) The formation of neurotoxic oligomers in which aminochrome forms adducts with alpha synuclein generating neurotoxic oligomers, and 5,6-Indolequinone form adduct with alpha synuclein as well, although after 40 min [47]. (ii) Mitochondrial dysfunction in which dopamine *o*-quinone inactivates mitochondrial complex I, III and V and isocitrate dehydrogenase [49], and aminochrome inactivates complex I of mitochondria [60]. (iii) Protein degradation dysfunction in which dopamine oxidation inactivates the proteasome system, although it is not clear which of the *o*-quinones is responsible for this inactivation [53]. However, aminochrome has been reported to inactivate the proteasome [108, 109]. Aminochrome forms adducts with α- and β-tubulin preventing the formation of microtubules required for autophagosome fusion with lysosomes and induces lysosome dysfunction by increasing its internal pH [58, 59, 110]. (iv) Aminochrome induces oxidative stress via hydroxyl radical formation (Fig. 11.4) [54]. Therefore, dopamine oxidation to *o*-quinones seems to play a key role in the degeneration of dopaminergic neurons containing neuromelanin.

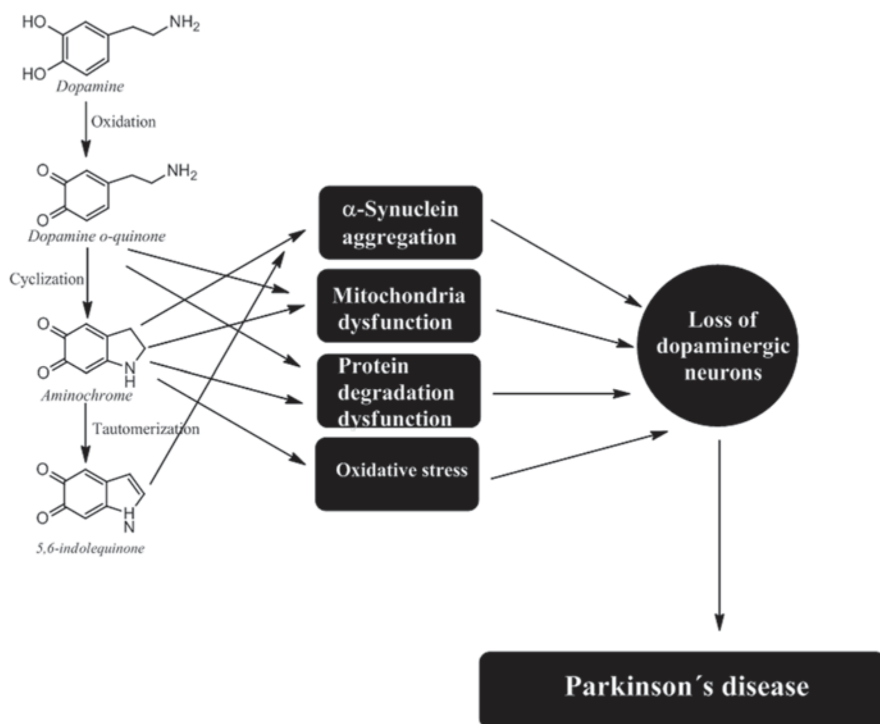

Fig. 11.4 The reactions of dopamine o-quinone, aminochrome and 5,6-indolequinone directly involved in mitochondria dysfunction, alpha synuclein aggregation, protein degradation dysfunction and oxidative stress

Conclusions

For several years, the possible role of environmental factors in the development of Parkinson's disease has been discussed; environmental factors were believed to result in the loss of dopaminergic neurons containing neuromelanin in Parkinson's disease. The entire experimental model for Parkinson's disease used exogenous neurotoxins such as 6-hydroxydopamine [111], MPTP [112], paraquat [113], rotenone [114] and dithiocarbamates [115]. Most likely, the degeneration of dopaminergic neurons containing neuromelanin is primarily dependent on an endogenous neurotoxin(s), such as (i) the o-quinones (dopamine o-quinone, aminochrome and 5,6-indolequinone) generated during dopamine oxidation; (ii) 3,4-dihydroxyphenylacetaldehyde generated by the oxidative deamination of dopamine catalyzed by MAO [116]; (iii) salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, the condensation product of dopamine with aldehyde [86]. It is plausible that certain genetic polymorphisms will potentiate the effect of endogenous neurotoxins. The specific loss of dopaminergic neurons in substantia nigra in Parkinson's disease seems to be related to the oxidation of dopamine to o-quinones, suggesting that the latter is an essential step because the neurons lost in Parkinson's disease contains neuromelanin. Dopamine o-quinone, aminochrome and 5,6-indole quinone are directly involved in four of five of the molecular mechanisms associated with Parkinson's disease, such as mitochondria dysfunction, aggregation of alpha synuclein to neurotoxic oligomers, protein degradation dysfunction and oxidative stress, strongly supporting the idea that dopamine oxidation to *o*-quinones is essential in the degeneration of dopaminergic neurons containing neuromelanin.

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References

1. Eser BE, Fitzpatrick PF. Measurement of intrinsic rate constants in the tyrosine hydroxylase reaction. BioChemistry. 2010;49:645–52.
2. Nagatsu T. Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology. Essays Biochem. 1995;30:15–35.
3. Cartier EA, Parra LA, Baust TB, Quiroz M, Salazar G, Faundez V, Egaña L, Torres GE. A biochemical and functional protein complex involving dopamine synthesis and transport into synaptic vesicles. J Biol Chem. 2010;151:957–66.
4. Linert W, Herlinger E, Jameson RF, Kienzl E, Jellinger K, Youdim MB. Dopamine, 6-hydroxydopamine, iron, and dioxygen–their mutual interactions and possible implication in the development of Parkinson's disease. Biochim Biophys Acta. 1996;1316:160–8.
5. Guillot TS, Miller GW. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. Mol Neurobiol. 2009;39:149–70.
6. Fuentes P, Paris I, Nassif M, Cavies P, Segura-Aguilar J. Inhibition of VMAT-2 and DT-diaphorase induce cell death in a substantia nigra-derived cell line–an experimental cell model for dopamine toxicity studies. Chem Res Toxicol. 2007;20:776–83.

7. Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA, Zecca L. Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc Natl Acad Sci U S A. 2000;97:11869–74.
8. Liang CL, Nelson O, Yazdani U, Pasbakhsh P, German DC. Inverse relationship between the contents of neuromelanin pigment and the vesicular monoamine transporter-2: human midbrain dopamine neurons. J Comp Neurol. 2004;473:97–106.
9. Eriksen J, Jørgensen TN, Gether U. Regulation of dopamine transporter function by protein-protein interactions: new discoveries and methodological challenges. J Neurochem. 2010;113:27–41.
10. Giros B, Jaber M, Jones SR, Wightman RM, Caron MG. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature. 1996;379: 606–12.
11. Jones SR, Gainetdinov RR, Wightman RM, Caron MG. Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. J Neurosci. 1998;18:1979–86.
12. Bach AWJ, Lan NC, Johnson DL, Abell CW, Bemkenek ME, Kwan SW, Seeburg PH, Shih JC. cDNA cloning of human liver monoamine oxidase A and B: molecular basis of differences in enzymatic properties. Proc Natl Acad Sci U S A. 1988;85:4934–8.
13. Weyler W, Hsu YP, Breakefield XO. Biochemistry and genetics of monoamine oxidase. Pharmacol Ther. 1990;47:391–417.
14. Shih JC, Grimsby J, Chen K. Molecular biology of monoamine oxidase A and B: their role in the degradation of serotonin. In: Baumgarten HG, Gothert M, editors. Handbook of experimental pharmacology. Serotoninergic neurons and 5-HT receptors in the CNS. Vol. 129. Berlin: Springer-Verlag; 1997. pp. 655–70.
15. Westlund KN, Denney RM, Rose RM, Abell CW. Localization of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem. Neuroscience. 1988;25:439–56.
16. Saura J, Luque JM, Cesura AM, Da Prada M, Chan-Palay V, Huber G, Loffler J, Richards JG. Increased monoamine oxidase B activity in plaque-associated astrocytes of Alzheimer brains revealed by quantitative enzyme radioautography. Neuroscience. 1994;62:15–30.
17. Shih JC. Molecular basis of human MAO A and B. Neuropsychopharmacology. 1991;4:1–7.
18. Strolin-Benedetti M, Dostert P, Tipton KF. Developmental aspects of the monoamine-degrading enzyme monoamine oxidase. Dev Pharmacol Ther. 1992;18:191–200.
19. Di Monte DA, Royland JE, Irwin I, Langston JW. Astrocytes as the site for bioactivation of neurotoxins. Neurotoxicol Fall-Winter. 1996;17:697–703.
20. Myöhänen TT, Schendzielorz N, Männistö PT. Distribution of catechol-O-methyltransferase (COMT) proteins and enzymatic activities in wild-type and soluble COMT deficient mice. J Neurochem. 2010;113:1632–43.
21. Zecca L, Bellei C, Costi P, Albertini A, Monzani E, Casella L, Gallorini M, Bergamaschi L, Moscatelli A, Turro NJ, Eisner M, Crippa PR, Ito S, Wakamatsu K, Bush WD, Ward WC, Simon JD, Zucca FA. New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals. Proc Natl Acad Sci U S A. 2008;105:17567–72.
22. Segura-Aguilar J, Lind C. On the mechanism of Mn3(+)-induced neurotoxicity of dopamine: prevention of quinone derived oxygen toxicity by DT-diaphorase and superoxide dismutase. Chem Biol Interact. 1989;72:309–24.
23. Park RM. Neurobehavioral deficits and parkinsonism in occupations with manganese exposure: a review of methodological issues in the epidemiological literature. Saf Health Work. 2013;4:123–35.
24. Tuschl K, Mills PB, Clayton PT. Manganese and the brain. Int Rev Neurobiol. 2013;110:277–312.
25. Fujishiro H, Ohashi T, Takuma M, Himeno S. Down-regulation of S100A9 and S100A10 in manganese-resistant RBL-2H3 cells. J Toxicol Sci. 2013;38:753–7.
26. Caviedes P, Segura-Aguilar J. The price of development in Chile: overcoming environmental hazards produced by heavy industrial exploitation. Neuroreport. 2001;12:A25.

27. Paris I, Dagnino–Subiabre A, Marcelain K, Bennett LB, Caviedes P, Caviedes R, Olea-Azar C, Segura-Aguilar J. Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line. J Neurochem. 2001;77:519–29.
28. Paris I, Perez-Pastene C, Couve E, Caviedes P, Ledoux S, Segura-Aguilar J. Copper dopamine complex induces mitochondrial autophagy preceding caspase-independent apoptotic cell death. J Biol Chem. 2009;284:13306–15.
29. Taly AB, Meenakshi-Sundaram S, Sinha S, Swamy HS, Arunodaya GR. Wilson disease: description of 282 patients evaluated over 3 decades. Medicine (Baltimore). 2007;86:112–21.
30. Przybyłkowski A, Gromadzka G, Członkowska A. Polymorphisms of metal transporter genes DMT1 and ATP7A in Wilson's disease. J Trace Elem Med Biol. 2014;28:8–12.
31. Zhou ZD, Lan YH, Tan EK, Lim TM. Iron species-mediated dopamine oxidation, proteasome inhibition, and dopaminergic cell demise: implications for iron-related dopaminergic neuron degeneration. Free Radic Biol Med. 2010;49:1856–71.
32. Paris I, Martinez-Alvarado P, Cardenas S, Perez-Pastene C, Graumann R, Fuentes P, Olea-Azar C, Caviedes P, Segura-Aguilar J. Dopamine-dependent iron toxicity in cells derived from rat hypothalamus. Chem Res Toxicol. 2005;18:415–9.
33. Paris I, Martinez-Alvarado P, Perez-Pastene C, Vieira MN, Olea-Azar C, Raisman-Vozari R, Cardenas S, Graumann R, Caviedes P, Segura-Aguilar J. Monoamine transporter inhibitors and norepinephrine reduce dopamine-dependent iron dependent iron toxicity in cells derived from the substantia nigra. J Neurochem. 2005;92:1021–32.
34. Bisaglia M, Mammi S, Bubacco L. Kinetic and structural analysis of the early oxidation products of dopamine: analysis of the interactions with alpha-synuclein. J Biol Chem. 2007;282:15597–605.
35. Jana S, Sinha M, Chanda D, Roy T, Banerjee K, Munshi S, Patro BS, Chakrabarti S. Mitochondrial dysfunction mediated by quinone oxidation products of dopamine: implications in dopamine cytotoxicity and pathogenesis of Parkinson's disease. Biochim Biophys Acta. 2011;1812:663–73.
36. Jimenez M, Garcia-Carmona F, Garcia-Canovas F, Iborra JL, Lozano JA, Martinez F. Chemical intermediates in dopamine oxidation by tyrosinase, and kinetic studies of the process. Arch Biochem Biophys. 1984;235:438–48.
37. Hastings TG. Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. J Neurochem. 1995;64:919–24.
38. Foppoli C, Coccia R, Cini C, Rosei MA. Catecholamines oxidation by xanthine oxidase. Biochim Biophys Acta. 1997;1334:200–6.
39. Segura-Aguilar J. Peroxidase activity of liver microsomal vitamin D 25-hydroxylase and cytochrome P450 1A2 catalyzes 25-hydroxylation of vitamin D3 and oxidation of dopamine to aminochrome. Biochem Mol Med. 1996;58:122–9.
40. Galzigna L, De Iuliis A, Zanatta L. Enzymatic dopamine peroxidation in substantia nigra of human brain. Clin Chim Acta. 2000;300:131–8.
41. Thompson M, Capdevila JH, Strobel HW. Recombinant cytochrome P450 2D18 metabolism of dopamine and arachidonic acid. J Pharmacol Exp Ther. 2000;294:1120–30.
42. Segura-Aguilar J, Metodiewa D, Welch C. Metabolic activation of dopamine o-quinones to o-semiquinones by NADPH cytochrome P450 reductase may play an important role in oxidative stress and apoptotic effects. Biochim Biophys Acta. 1998;1381:1–6.
43. Bisaglia M, Soriano ME, Arduini I, Mammi S, Bubacco L. Molecular characterization of dopamine-derived quinones reactivity toward NADH and glutathione: implications for mitochondrial dysfunction in Parkinson disease. Biochim Biophys Acta. 2010;1802:699–706.
44. Tse DC, McCreery RL, Adams RN. Potential oxidative pathways of brain catecholamines. J Med Chem. 1976;19:37–40.
45. Greggio E, Bergantino E, Carter D, Ahmad R, Costin GE, Hearing VJ, Clarimon J, Singleton A, Eerola J, Hellström O, Tienari PJ, Miller DW, Beilina A, Bubacco L, Cookson MR. Tyrosinase exacerbates dopamine toxicity but is not genetically associated with Parkinson's disease. J Neurochem. 2005;93:246–56.

46. Napolitano A, Manini P, d'Ischia M. Oxidation chemistry of catecholamines and neuronal degeneration: an update. Curr Med Chem. 2011;18:1832–45.
47. Bisaglia M, Tosatto L, Munari F, Tessari I, de Laureto PP, Mammi S, Bubacco L. Dopamine quinones interact with alpha-synuclein to form unstructured adducts. Biochem Biophys Res Commun. 2010;394:424–8.
48. Hauser DN, Hastings TG. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. Neurobiol Dis. 2013;51:35–42.
49. Van Laar VS, Mishizen AJ, Cascio M, Hastings TG. Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. Neurobiol Dis. 2009;34:487–500.
50. Van Laar VS, Dukes AA, Cascio M, Hastings TG. Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: implications for Parkinson disease. Neurobiol Dis. 2008;29:477–89.
51. Xu Y, Stokes AH, Roskoski R Jr, Vrana KE. Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. J Neurosci Res. 1998;54:691–7.
52. Whitehead RE, Ferrer JV, Javitch JA, Justice JB. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. J Neurochem. 2001;76:1242–51.
53. LaVoie MJ, Ostaszewski BL, Weihofen A, Schlossmacher MG, Selkoe DJ. Dopamine covalently modifies and functionally inactivates parkin. Nat Med. 2005;11:1159–61.
54. Arriagada A, Paris I, Sanchez de las Matas MJ, Martinez-Alvarado P, Cardenas S, Castaña P, Graumann R, Perez-Pastene C, Olea-Azar C, Couve E, Herrero MT, Caviedes P, Segura-Aguilar J. On the neurotoxicity of leukoaminochrome o-semiquinone radical derived of dopamine oxidation: mitochondria damage, necrosis and hydroxyl radical formation. Neurobiol Dis. 2004;16:468–77.
55. Dagnino-Subiabre A, Cassels BK, Baez S, Johansson AS, Mannervik B, Segura-Aguilar J. Glutathione transferase M2-2 catalyzes conjugation of dopamine and dopa o-quinones. Biochem Biophys Res Commun. 2000;274:32–6.
56. Lozano J, Muñoz P, Nore BF, Ledoux S, Segura-Aguilar J. Stable expression of short interfering RNA for DT-diaphorase induces neurotoxicity. Chem Res Toxicol. 2010;23:1492–6.
57. Paris I, Muñoz P, Huenchuguala S, Couve E, Sanders LH, Greenamyre JT, Caviedes P, Segura-Aguilar J. Autophagy protects against aminochrome-induced cell death in substantia nigra-derived cell line. Toxicol Sci. 2011;121:376–88.
58. Paris I, Perez-Pastene C, Cardenas S, Iturriaga-Vasquez P, Muñoz P, Couve E, Caviedes P, Segura-Aguilar J. Aminochrome induces disruption of actin, alpha-, and beta-tubulin cytoskeleton networks in substantia-nigra-derived cell line. Neurotox Res. 2010;18:82–92.
59. Huenchuguala S, Muñoz P, Zavala P, Villa M, Cuevas C, Ahumada U, Graumann R, Nore BF, Couve E, Mannervik B, Paris I, Segura-Aguilar J. Glutathione transferase mu 2 protects glioblastoma cells against aminochrome toxicity by preventing autophagy and lysosome dysfunction. Autophagy. 2014;10:618–30.
60. Aguirre P, Urrutia P, Tapia V, Villa M, Paris I, Segura-Aguilar J, Núñez MT. The dopamine metabolite aminochrome inhibits mitochondrial complex I and modifies the expression of iron transporters DMT1 and FPN1. Biometals. 2012;25:795–803.
61. Muñoz P, Paris I, Sanders LH, Greenamyre JT, Segura-Aguilar J. Overexpression of VMAT-2 and DT-diaphorase protects substantia nigra-deriv cells against aminochrome neurotoxicity. Biochim Biophys Acta. 2012;1822:1125–36.
62. Muñoz P, Huenchuguala S, Paris I, Cuevas C, Villa M, Cavies P, Segura-Aguilar J, Tizabi Y. Protective effects of nicotine against aminochrome-induced toxicity in substantia nigra derived cells: implications for Parkinson's disease. Neurotox Res. 2012;22:177–80.
63. Segura-Aguilar J, Baez S, Widersten M, Welch CJ, Mannervik B. Human class Mu glutathione transferases, in particular isoenzyme M2-2, catalyze detoxication of the dopamine metabolite aminochrome. J Biol Chem. 1997;272:5727–31.
64. Sulzer D, Mosharov E, Talloczy Z, Zucca FA, Simon JD, Zecca L. Neuronal pigmented autophagic vacuoles: lipofuscin, neuromelanin, and ceroid as macroautophagic responses during aging and disease. J Neurochem. 2008;106:24–36.

65. Wakamatsu K, Fujikawa K, Zucca FA, Zecca L, Ito S. The structure of neuromelanin as studied by chemical degradative methods. J Neurochem. 2003;86:1015–23.
66. Wakamatsu K, Murase T, Zucca FA, Zecca L, Ito S. Biosynthetic pathway to neuromelanin and its aging process. Pigment Cell Melanoma Res. 2012;25:792–803.
67. Mattammal MB, Strong R, Lakshmi VM, Chung HD, Stephenson AH. Prostaglandin H synthetase–mediated metabolism of dopamine: implication for Parkinson's disease. J Neurochem. 1995;64:1645–54.
68. Xu Y, Stokes AH, Freeman WM, Kumer SC, Vogt BA, Vrana KE. Tyrosinase mRNA is expressed in human substantia nigra. Brain Res Mol Brain Res. 1997;45:159–62.
69. Foley JM, Baxter D. On the nature of pigment granules in the cells of the locus coeruleus and substantia nigra. J Neuropathol Exp Neurol. 1958;17:586–98.
70. Zecca L, Wilms H, Geick S, Claasen JH, Brandenburg LO, Holzknecht C, Panizza ML, Zucca FA, Deuschl G, Sievers J, Lucius R. Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: implications for Parkinson's disease. Acta. Neuropathol. 2008;116:47–55.
71. Zhang W, Phillips K, Wielgus AR, Liu J, Albertini A, Zucca FA, Faust R, Qian SY, Miller DS, Chignell CF, Wilson B, Jackson–Lewis V, Przedborski S, Joset D, Loike J, Hong JS, Sulzer D, Zecca L. Neuromelanin activates microglia and induces degeneration of dopaminergic neurons: implications for progression of Parkinson's disease. Neurotox Res. 2011;19:63–72.
72. Banati RB, Daniel SE, Blunt SB. Glial pathology but absence of apoptotic nigral neurons in long–standing Parkinson's disease. Mov Disord. 1998;13:221–7.
73. Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol. 1999;46:598–605.
74. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA–DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology. 1988;38:1285–91.
75. Double KL, Ben–Shachar D, Youdim MB, Zecca L, Riederer P, Gerlach M. Influence of neuromelanin on oxidative pathways within the human substantia nigra. Neurotoxicol Teratol. 2002;24:621–8.
76. Zareba M, Bober A, Korytowski W, Zecca L, Sarna T. The effect of a synthetic neuromelanin on yield of free hydroxyl radicals generated in model systems. Biochim Biophys Acta. 1995;1271:343–8.
77. Zecca L, Casella L, Albertini A, Bellei C, Zucca FA, Engelen M, Zadlo A, Szewczyk G, Zareba M, Sarna T. Neuromelanin can protect against iron–mediated oxidative damage in system modeling iron overload of brain aging and Parkinson's disease. J Neurochem. 2008;106:1866–75.
78. Girotto S, Sturlese M, Bellanda M, Tessari I, Cappellini R, Bisaglia M, Bubacco L, Mammi S. Dopamine-derived quinones affect the structure of the redox sensor DJ-1 through modifications at Cys-106 and Cys-53. J Biol Chem. 2012;287:18738–49.
79. Norris EH, Giasson BI, Hodara R, Xu S, Trojanowski JQ, Ischiropoulos H, Lee VM. Reversible inhibition of alpha-synuclein fibrillization by dopaminochrome—mediated conformational alterations. J Biol Chem. 2005;280:21212–9.
80. Baez S, Linderson Y, Segura-Aguilar J. Superoxide dismutase and catalase enhance autoxidation during one-electron reduction of aminochrome by NADPH-cytochrome P-450 reductase. Biochem Mol Med. 1995;54:12–8.
81. Díaz-Véliz G, Paris I, Mora S, Raisman-Vozari R, Segura-Aguilar J. Copper neurotoxicity in rat substantia nigra and striatum is dependent on DT-diaphorase inhibition. Chem Res Toxicol. 2008;21:1180–5.
82. Segura-Aguilar J, Diaz-Veliz G, Mora S, Herrera-Marschitz M. Inhibition of DT-diaphorase is a requirement for Mn(III) to produce a 6-OH-dopamine like rotational behaviour. Neurotox Res. 2002;4:127–31.
83. Schultzberg M, Segura-Aguilar J, Lind C. Distribution of DT diaphorase in the rat brain: biochemical and immunohistochemical studies. Neuroscience. 1988;27:763–76.

84. Díaz-Véliz G, Mora S, Dossi MT, Gómez P, Arriagada C, Montiel J, Aboitiz F, Segura-Aguilar J. Behavioral effects of aminochrome and dopachrome injected in the rat substantia nigra. Pharmacol Biochem Behav. 2002;73:843–50.
85. Díaz-Véliz G, Mora S, Gómez P, Dossi MT, Montiel J, Arriagada C, Aboitiz F, Segura-Aguilar J. Behavioral effects of manganese injected in the rat substantia nigra are potentiated by dicumarol, a DT-diapase inhibitor. Pharmacol Biochem Behav. 2004;77:245–51.
86. Díaz-Véliz G, Mora S, Lungenstrass H, Segura-Aguilar J. Inhibition of DT-diaphorase potentiates the in vivo neurotoxic effect of intranigral injection of salsolinol in rats. Neurotox Res. 2004;5:629–33.
87. Nicolis S, Zucchelli M, Monzani E, Casella L. Myoglobin modification by enzyme-generated dopamine reactive species. Chem Eur J. 2008;14:8661–73.
88. Shen XM, Xia B, Wrona MZ, Dryhurst G. Synthesis, redox properties, in vivo formation, and neurobehavioral effects of N-acetylcysteinyl conjugates of dopamine: possible metabolites of relevance to Parkinson's disease. Chem Res Toxicol. 1996;9:1117–26.
89. Zhou ZD, Lim TM. Glutathione conjugates with dopamine-derived quinones to form reactive or non-reactive glutathione-conjugates. Neurochem Res. 2010;35:1805–18.
90. Zhang F, Dryhurst G. Effects of L-cysteine on the oxidation chemistry of dopamine: new reaction pathways of potential relevance to idiopathic Parkinson's disease. J Med Chem. 1994;37:1084–98.
91. Wolters ECh, Braak H. Parkinson's disease: premotor clinico-pathological correlations. J Neural Transm Suppl. 2006;70:309–19.
92. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997;276:2045–7.
93. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;392:605–8.
94. Abbas N, Lücking CB, Ricard S, Dürr A, Bonifati V, De Michele G, Bouley S, Vaughan JR, Gasser T, Marconi R, Broussolle E, Brefel-Courbon C, Harhangi BS, Oostra BA, Fabrizio E, Böhme GA, Pradier L, Wood NW, Filla A, Meco G, Denefle P, Agid Y, Brice A. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet. 1999;8:567–74.
95. Hattori N, Matsumine H, Asakawa S, Kitada T, Yoshino H, Elibol B, Brookes AJ, Yamamura Y, Kobayashi T, Wang M, Yoritaka A, Minoshima S, Shimizu N, Mizuno Y. Point mutations (Thr240Arg and Gln311Stop) [correction of Thr240Arg and Ala311Stop] in the parkin gene. Biochem Biophys Res Commun. 1998;249:754–8.
96. Ramirez A, Heimbach A, Gründemann J, Stiller B, Hampshire D, Cid LP, Goebel I, Mubaidin AF, Wriekat AL, Roeper J, Al-Din A, Hillmer AM, Karsak M, Liss B, Woods CG, Behrens MI, Kubisch C. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat Genet. 2006;38:1184–91.
97. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, González-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;304:1158–60.
98. Kachergus J, Mata IF, Hulihan M, Taylor JP, Lincoln S, Aasly J, Gibson JM, Ross OA, Lynch T, Wiley J, Payami H, Nutt J, Maraganore DM, Czyzewski K, Styczynska M, Wszolek ZK, Farrer MJ, Toft M. Identification of a novel LRRK2 mutation linked to autosomal dominant parkinsonism: evidence of a common founder across European populations. Am J Hum Genet. 2005;76:672–80.

99. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. 2003;299:256–9.
100. Kalia LV, Kalia SK, McLean PJ, Lozano AM, Lang AE. A-Synuclein oligomers and clinical implications for Parkinson disease. Ann Neurol. 2013;73:155–69.
101. Bohlega SA, Al-Foghom NB. Drug-induced Parkinson's disease. A clinical review. Neurosciences (Riyadh). 2013;18:215–21.
102. Allen MT, Levy LS. Parkinson's disease and pesticide exposure–a new assessment. Crit Rev Toxicol. 2013;43:515–34.
103. Mullin S, Schapira A. A-Synuclein and mitochondrial dysfunction in Parkinson's disease. Mol Neurobiol. 2013;47:587–97.
104. Exner N, Lutz AK, Haass C, Winklhofer KF. Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. EMBO J. 2012;31:3038–62.
105. Ebrahimi-Fakhari D, Wahlster L, McLean PJ. Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol. 2012;124:153–72.
106. Taylor JM, Main BS, Crack PJ. Neuroinflammation and oxidative stress: co-conspirators in the pathology of Parkinson's disease. Neurochem Int. 2013;62:803–19.
107. Martinez-Vicente M, Vila M. Alpha-synuclein and protein degradation pathways in Parkinson's disease: a pathological feed-back loop. Exp Neurol. 2013;247:308–13.
108. Zafar KS, Siegel D, Ross D. A potential role for cyclized quinones derived from dopamine, DOPA, and 3,4-dihydroxyphenylacetic acid in proteasomal inhibition. Mol Pharmacol. 2006;70:1079–86.
109. Zhou ZD, Lim TM. Dopamine (DA) induced irreversible proteasome inhibition via DA derived quinones. Free Radic Res. 2009;43:417–30.
110. Muñoz P, Huenchuguala S, Paris I, Segura-Aguilar J. Dopamine oxidation and autophagy. Parkinson's Dis. 2012;920953:1–13.
111. Kupsch A, Schmidt W, Gizatullina Z, Debska-Vielhaber G, Voges J, Striggow F, Panther P, Schwegler H, Heinze HJ, Vielhaber S, Gellerich FN. 6-Hydroxydopamine impairs mitochondrial function in the rat model of Parkinson's disease: respirometric, histological, and behavioral analyses. J Neural Transm. 2014. doi:10.1007/s00702-014-1185-3
112. Khaindrava VG, Kozina EA, Kudrin VS, Kucheryanu VG, Klodt PD, Narkevich VB, Bocharov EV, Nanaev AK, Kryzhanovsky GN, Raevskii KS, Ugrumov MV. Experimental modeling of preclinical and clinical stages of Parkinson's disease. Bull Exp Biol Med. 2011;150:566–9.
113. Somayajulu-Niţu M, Sandhu JK, Cohen J, Sikorska M, Sridhar TS, Matei A, Borowy-Borowski H, Pandey S. Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of coenzyme Q10. BMC Neurosci. 2009. doi:10.1186/1471-2202-10-88.
114. Mulcahy P, Walsh S, Paucard A, Rea K, Dowd E. Characterisation of a novel model of Parkinson's disease by intra-striatal infusion of the pesticide rotenone. Neuroscience. 2011;181:234–42.
115. Moretto A, Colosio C. Biochemical and toxicological evidence of neurological effects of pesticides: the example of Parkinson's disease. Neurotoxicology. 2011;32:383–91.
116. Vermeer LM, Florang VR, Doorn JA. Catechol and aldehyde moieties of 3,4-dihydroxyphenylacetaldehyde contribute to tyrosine hydroxylase inhibition and neurotoxicity. Brain Res. 2012;1474:100–9.

Chapter 12
Exploring the Role of Autophagy in the Pathogenesis of Rotenone-induced Toxicity

Gessica Sala, Giovanni Stefanoni, Daniele Marinig and Carlo Ferrarese

Abstract This chapter is intended to provide an organic revision of evidence for the involvement of autophagy in the pathogenesis of rotenone-induced toxicity. The mechanisms underlying rotenone neurotoxicity are elucidated through a description of findings obtained in both *in vivo* and *in vitro* experimental models. Furthermore, this chapter describes the etiological role of rotenone and other pesticide exposure in the pathogenesis of Parkinson's disease, as demonstrated by epidemiological studies performed in the last decades starting from results obtained in animal and cellular experimental models. The specific focus of the present chapter is to explore the effect of rotenone on autophagic pathway, whose dysfunctions are already recognized to play an important pathogenetic role in Parkinson's disease. Specifically, a comprehensive revision of the current available literature on the rotenone-induced dysfunctions of the two main types of autophagy, macroautophagy and chaperone-mediated autophagy, is provided in order to clarify the protective rather than detrimental contribute exerted by autophagy alterations in the cell death induced by rotenone.

Keywords Rotenone · Parkinson's disease · Neurotoxicity · Macroautophagy · Chaperone-mediated autophagy · In vitro models · Animal models

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Abbreviations

3-MA	3-methyladenine
Atg	Autophagy-related genes
ATP13A2	ATPase type 13 A2
cdk5	Cyclin-dependent kinase 5
CMA	Chaperone-mediated autophagy
GBA	Glucocerebrosidase
hsc70	Heat shock cognate protein 70
lamp2A	Lysosomal-associated membrane protein 2 A
LRRK2	Leucine-rich repeat kinase 2
MEF2D	Myocyte enhancer factor 2D
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	Mammalian target of rapamycin
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
ROS	Reactive oxygen species
SOD2	Mitochondrial superoxide dismutase
UCHL1	Ubiquitin carboxyl-terminal esterase L1
UPS	Ubiquitin-proteasome system

Rotenone

Rotenone (molecular formula $C_{23}H_{22}O_6$) is an odorless, colorless and lipophilic compound that occurs naturally in the seeds and stems of several plants (Fig. 12.1). Rotenone was firstly isolated in 1895 from *Robinia nicou* by a French botanist which called it *nicouline*. In 1902, a Japanese chemist isolated the same compound from *Derris elliptica* and, based on the Japanese name of the plant, called it rotenone.

Rotenone is an insecticide, pesticide, and non-selective piscicide, widely used in both agricultural and home settings until 2007, when it was withdrawn from use in the European Union [1].

Toxicity of Rotenone

Based on the fact that rotenone has a limited half-life in the environment (about 6 days) and in natural water (less than 4 days) and that it decomposes when exposed to sunlight, the hazard of rotenone for humans is moderate.

As rotenone is a highly lipophilic compound, it can cross the blood–brain barrier and diffuse in all cell types without the need for specific carriers.

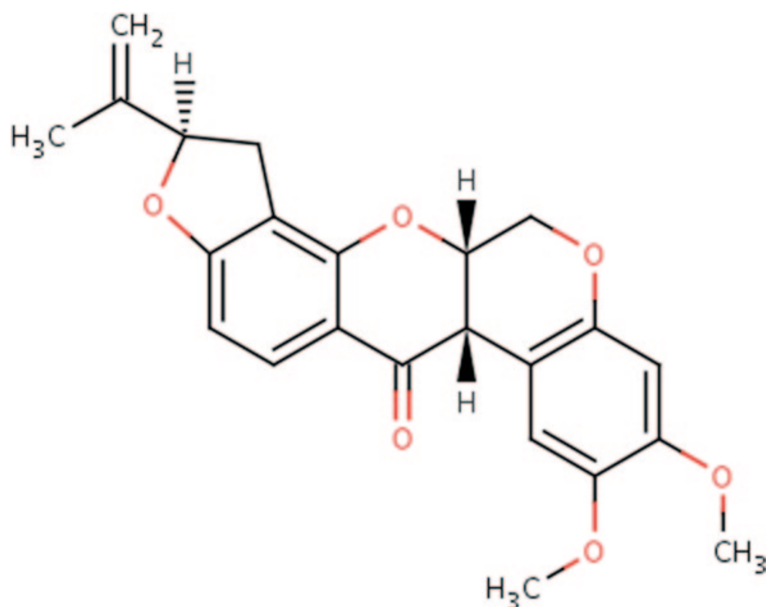

Fig. 12.1 Structure of rotenone (from the database and ontology of Chemical Entities of Biological Interest, ChEBI)

Rotenone is classified as an inhibitor of the mitochondrial electron transport, specifically inhibiting the transfer of electrons from iron-sulfur centers in complex I to ubiquinone. Exposure of cells to complex I inhibitor rotenone results in cascade of detrimental intracellular events that include impaired basal and maximal oxygen consumption, reduced mitochondrial transmembrane potential, depleted mitochondrial-ATP levels, thus creating an environment of oxidative stress in the cell.

Evidence for Rotenone Toxicity in Animal Models

A first important study demonstrated that chronic systemic exposure—through jugular vein cannulation—to rotenone reproduced Parkinson's disease (PD) pathology in rats, as indicated by the degeneration of nigrostriatal dopaminergic neurons and the formation in nigral neurons of α-synuclein-positive cytoplasmic inclusions [2]. Three years later, another study reported that the same PD-related features were reproduced in a less labor-intensive *in vivo* model obtained by a chronic systemic subcutaneous exposure to rotenone [3]. Interestingly, considering the knowledge that rotenone, differently from another PD-related complex I inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is able to enter dopaminergic neurons without a specific carrier, the demonstration that a chronic exposure to rotenone causes a selective death of dopaminergic neurons represents the most convincing evidence supporting the view that these neuronal cell types are particularly vulnerable to a loss of mitochondrial respiration function.

A reevaluation of long-term daily oral rotenone administration in C57BL/6 mice allowed to establish that the protocol at 30 mg/kg rotenone for 56 days more faithfully reproduces the nigrostriatal dopamine neurodegeneration [4]. Furthermore, chronic exposure of rats to rotenone was demonstrated to induce behavioral impairments, to decrease the expression of the cytoprotective proteins parkin and heat shock proteins and to increase caspases [5].

Evidence for Rotenone Toxicity in Cellular Models

Rotenone-induced degeneration of cultured dopaminergic neurons was assessed in primary neuron-enriched and neuron/glia cultures from the rat mesencephalon. While exposure up to 20 nM rotenone for 8 days caused a mild cytotoxicity in neuron-enriched cultures, a significant and selective dopaminergic degeneration was observed in neuron/glia cultures after 20 mM rotenone for 2 days or 1 nM rotenone for 8 days, demonstrating that microglia play a pivotal role in rotenone-induced dopaminergic neuronal death [6]. Further studies from the same Authors evidenced a critical role for superoxide derived from microglial NADPH oxidase in mediating microglia-enhanced rotenone neurotoxicity [7].

Moreover, treating cortical neurons with 500 nM rotenone resulted in an early release of cytochrome c and emergence of mitochondrial permeability transition responsible for a caspase-mediated apoptotic neuronal degeneration [8].

Interestingly, a more recent study demonstrated that mitochondrial complex I inhibition is not required for mediating the neurotoxic effects of PD-related toxins rotenone, 1-methyl-4-phenylpyridinium (MPP+) and paraquat, suggesting that the neurotoxicity of these compounds involves an increase of oxidative stress *in vitro* [9].

Etiologic Role of Pesticide Exposure in PD

Many epidemiological studies have suggested that rotenone, as well as other pesticides, exposure is connected to the development of sporadic PD [10, 11], although till now the precise relationship remains to be clarified.

PD is a neurodegenerative disorder whose pathological hallmark is progressive loss of dopaminergic neurons of the substantia nigra, associated with formation of cytoplasmic inclusions, known as Lewy bodies, within the same neurons. The protein α-synuclein represents the major component of Lewy bodies.

PD represents the second most common neurodegenerative disorder, affecting over 1 % of people older than 60. Patients are usually diagnosed after the appearance of typical motor manifestations, notoriously representing expression of advanced stages of the degenerative process. A late diagnosis partially explains the fact that the disease cannot actually benefit from any neuroprotective treatment.

PD is considered a multifactorial pathology, with genetic and environmental factors synergistically contributing to neurodegenerative changes [12–14]. The lack or a relevant genetic substrate in most of PD cases has provided great support to epidemiologic studies aimed at the identification of environmental etiologies. Among exogenous risk factors, pesticides have been extensively studied, because of the selective dopaminergic neurototoxicity demonstrated by some of them in animal studies. Paraquat has been the pesticide most frequently implicated as a selective dopaminergic neurotoxicant [15, 16]. Besides paraquat, studies on animal models highlighted that pesticides with similar properties, such as maneb and rotenone, can induce α-synuclein accumulation and dopaminergic cell degeneration [2, 15, 17, 18]. Multiple mechanisms have been demonstrated to underlie the role of pesticides in PD. These include mitochondrial dysfunction, oxidative stress, inflammation, triggering of α-synuclein aggregation, alteration of dopamine transporters [19, 20].

The metabolism of exogenous substances is dependent on age and decreases in elderly, thus favoring toxic effects of xenobiotics among older people [21, 22]. Genetic polymorphism in enzymes involved in metabolism of toxics, such as glutathione transferase, may modify the risk of PD by modulating susceptibility to deleterious effects of neurotoxins, such as pesticides [23, 24].

Aberrant DNA methylation and histone acetylation have been described in PD [25]. Since in utero exposure to environmental factors can deregulate gene expression through early epigenetic changes, some authors have proposed that PD may originate from the disruption of epigenetic mechanisms by neurotoxic agents during early development [26–28].

Despite decades of laboratory study, only recently pesticides has been definitely associated with PD in humans. Great interest was aroused by reports of chronic parkinsonism and dopaminergic neurodegeneration in subjects exposed to MPTP, a substance structurally analogue to the commonly used herbicide paraquat [29]. Anyway, definite support of the role of pesticides in etiology of PD comes from a great number of epidemiologic studies, systematic reviews of literature and meta-analysis. Some studies demonstrated a role of life conditions potentially related to pesticide exposure, such as farming, rural living and well-water drinking, as putative risk factors for PD [30–34]. Other studies directly reported a correlation between exposure to single pesticides and an increased risk of PD [19, 20, 35]. Most of the studies are case-control studies, although even ecological and prospective designs have been used. A higher prevalence of PD in rural than in urban areas was observed in different countries [36–39]. In California regions reporting high pesticide use an increased mortality due to PD was reported [40]. A number of case-control studies highlighted a direct association between the duration of pesticide exposure and PD risk [41–45]. Data from a cohort of over 22,000 pesticide applicators suggested an increased risk of PD after exposure to pesticides [46]. A meta-analysis of 19 epidemiologic studies on PD and pesticide exposure reported a combined odds ratio of 1.94 [32].

Recently, an etiologic interaction between exposure to pesticides and a polymorphism in the α-synuclein gene has been demonstrated in early-onset PD [47]. Furthermore, six case–control studies showed a stronger association between pesticide

exposure and the risk of PD among genetically susceptible individuals [24, 48–52]. These data highlight the relevance of gene-environment interactions in PD etiology.

Over the past few years, a great number of studies have been designed to identify specific pesticides or classes of pesticides with a causative role in the development of PD. A recent review of the results of 19 studies [53] showed that insecticides may have a stronger association with PD development than herbicides and fungicides [54–57]. Among herbicides, a role for paraquat has been demonstrated [54, 58], while there is little evidence for an association of PD with fungicides, with the exception of maneb. The insecticide rotenone is instead recognized as a plausible cause of PD because of its mechanism of action. Like MPTP, a toxicant known to cause PD, rotenone directly inhibits mitochondrial complex I [29, 59], thus leading to selective injury of dopaminergic neurons in the substantia nigra, a key pathological feature of PD [60, 61]. Because rotenone is believed to have a relatively short environmental half-life and limited bioavailability, a relationship to human disease has been questioned [34, 62]. Anyway, two epidemiologic studies found it to be associated with significant 10-fold and 2.5-fold increases in PD risk [54, 63]. In particular, the Farming and Movement Evaluation (FAME) study by Tanner et al., a case–control study nested in the Agricultural Health Study (AHS), a prospective study including 84,740 pesticide applicators and their spouses, recruited in 1993–1997 in the USA [64], provided strong evidence for an association between rotenone and PD in humans. An association was observed even when exposure was stopped up to 15 years before diagnosis of PD. Although most participants were exposed to multiple pesticides, the associations observed also remained after adjustment for overall pesticide use.

Whereas parkinsonism due to high-dose exposure to carbon monoxide or manganese has distinctive clinical features, such as symmetric distribution of symptoms, lack of tremor, early postural instability and poor response to dopaminergic therapy [65], PD cases related to rotenone or other pesticides were generally similar to idiopathic PD cases, supporting a role for pesticide exposure in the etiology of typical PD.

Autophagy Dysfunctions in the Pathogenesis of PD

Several mechanisms are implicated in the degeneration of dopaminergic neurons in PD [66]. Among them, a significant role is assigned to the intraneuronal accumulation and aggregation of misfolded α-synuclein, the main component of Lewy bodies, in the substantia nigra of PD patients [67]. Rare hereditary variants of PD, linked to mutations or amplification of α-synuclein gene [68, 69], have provided crucial evidence of the pathogenetic role of accumulation and aggregation of this protein. Experiments with transgenic cellular and animal models reinforced the concept that α-synuclein accumulation can be central in the physiopathologic cascade leading to dopaminenergic degeneration [70–72]. In idiopathic PD the tendency of α-synuclein to aggregate may depend on post-translational modifications related to oxidative stress [73, 74].

Since the accumulation of a potentially neurotoxic protein seems to be a key pathogenetic mechanism in PD, the efficiency of cell machinery responsible for protein catabolism represents an essential component of neuroprotection against cytotoxicity and degeneration. As a consequence, neuronal systems implied in protein degradation have become target of intensive research in the field of PD and other neurodegenerative diseases. Among these systems, autophagy is a finely regulated intracellular process that mediates lysosomal degradation of aberrant proteins and organelles. This catabolic process exerts a cytoprotective role dependent on the clearance of toxic intracellular products and the catabolism of substrates in order to obtain energy during starvation [75]. Therefore, autophagy impairment might be a *primum movens* of the neurodegenerative processes and, at the same time, a limiting factor that is necessary for preventing early onset of neuronal loss.

In mammalian cells autophagy encompasses three main processes: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). Microautophagy is a constitutive, non-selective process consisting on endocytosis of small amounts of cytoplasm into lysosomes through invagination of lysosomal membrane. Macroautophagy and CMA are inducible processes. The first one allows lysosomal degradation of organelles and proteins after their sequestration within a double-membrane-limited vacuole called autophagosome [76]. Macroautophagy proceeds through four main steps, each requiring the presence of specific autophagy related genes (Atg) [77]: nucleation of the autophagosome; sequestration of a region of cytoplasm containing substrates to be degraded; maturation of the autophagosome; fusion with lysosome and degradation of substrates. CMA is a selective device for degradation of aberrant proteins, containing the consensus peptide sequence KFERQ, which are directly transported into the lysosomal lumen by a translocation system constituted by specific carrier proteins. CMA process requires the presence of two main proteins: cytosolic and lysosomal heat shock cognate protein 70 (hsc70) and lysosomal-associated membrane protein 2 A (lamp2A). Cytosolic hsc70 binds the KFERQ sequence of substrate proteins and carries them to the lysosomal membrane, where lamp2A, after interaction with cytosolic hsc70, multimerizes and forms a translocation complex with lysosomal hsc70, thus mediating the transport of the substrate protein into the lysosomal lumen. The binding of the substrate protein to lamp2A represents the limiting step of CMA. Oxidative stress, accumulation of substrates and the lack of nutrients and growth factors are all conditions determining a compensatory and cytoprotective activation of CMA through an increase of lamp2A levels on lysosomal membrane.

Recently, a growing body of neurobiological data has provided evidence of an impairment of autophagy pathways contributing to neuropathologic changes in PD and other neurodegenerative diseases [78–81]. A decrease in the activity of both autophagic-lysosomal pathway and the ubiquitin-proteasome system has been reported during normal aging in every tissue, included neurons [82]. Accumulation of aberrant proteins and mitochondria within senescent neurons contributes to neurodegeneration [73, 83, 84]. The early loss of function of degradative pathways might produce a deleterious effect through induction of the pathogenetic processes directly responsible for death of dopaminergic neurons, such as the accumulation

and oligomerization of α-synuclein, the persistence of damaged mitochondria and the consequent production of reactive oxygen species. In turn, these key mediators of dopaminergic neurodegeneration [85, 86] can further worsen the impairment of clearance machinery and, in particular, of autophagic pathways.

In PD brain, autoptic studies have demonstrated an accumulation of autophagosomes within degenerating dopaminergic neurons [87]. Different alterations in autophagosome dynamics are responsible for this abnormality. In fact, both induction of autophagosome formation [88, 89], and reduction of lysosomal pool [90], can determine autophagosome accumulation.

Several experimental data obtained in animal models clearly demonstrate that the loss of autophagy machinery is a sufficient cause of neuronal death: the deletion of genes encoding for key components of macroautophagy, such as Atg5 or Atg7, leads to diffuse neurodegeneration in mice [91, 92]. The molecular mechanisms linking autophagy impairment to neuronal death are still unclear. Intracellular protein aggregates accumulate following macroautophagy inhibition but they do not seem to be directly toxic for neurons [93]. A key factor may be the reduced turnover of damaged organelles and monomeric or oligomeric misfolded proteins, which are likely to exert a direct neurotoxic effect.

The concept that a down-regulation of CMA may be involved in PD pathogenesis emerged when this pathway was identified as the major degradative system for α-synuclein [89], which contains the aminoacidic sequence KFERQ, necessary for CMA-mediated degradation. The blockage of CMA activity by siRNA-mediated inhibition of lamp2A expression leads to α-synuclein accumulation [94].

Post mortem studies on brain from PD patients provided evidence for a defect in CMA in sporadic PD. In fact, the levels of the two main components of CMA machinery, lamp2A and hsc70, were found to be reduced in the brain of patients with respect to controls [94].

Studies on transgenic cell and animal models of PD have demonstrated that the products of several genes linked to hereditary PD have functional correlations with autophagic-lysosomal pathways. In particular, blockage of CMA is a pathogenetic mechanism involved in hereditary variants of PD [89, 95, 96].

A53T and A30P mutant α-synuclein variants, responsible for autosomic dominant hereditary PD, bind hsc70 and lamp2A with a higher affinity than the wild type protein, thus preventing CMA mediators from the chance to bind other substrates. As a consequence, mutated α-synuclein blocks CMA [89] causing the accumulation of α-synuclein and other potentially neurotoxic proteins. Even the over-expression of wild type α-synuclein suppresses lysosomal degradation of proteins through antagonism for the binding to CMA components [96]. Therefore, -synuclein intraneuronal status is critical for the modulation of the autophagic-lysosomal pathway. These data are consistent with the existence of a strong link between CMA efficiency, α-synuclein toxicity and dopaminergic neurodegeneration.

I93M mutation of PARK5, the gene encoding for the ubiquitin carboxyl-terminal esterase L1 (UCHL1), causes autosomic dominant PD [97]. An interaction of UCHL1 with lamp2A and hsc70 has been demonstrated [95]. The I93M mutation enhances the affinity between UCHL1 and the CMA components, leading to

the suppression of CMA activity and the consequent intracellular accumulation of α-synuclein. This may be a key mechanism underlying the dopaminergic neurodegeneration related to I93M UCHL1 mutation.

Based on the negative modulation that mutations responsible for hereditary PD exert on CMA, the dysfunction of this system is likely to be a central mechanism in the degeneration of dopaminergic neurons [98]. The accumulation of α-synuclein is probably the key mediator of this toxic outcome [99]. Anyway, α-synuclein is not the only CMA substrate that may contribute to dopaminergic neurodegeneration. Myocyte enhancer factor 2D (MEF2D) is a neuronal transcriptional factor with a cytoprotective function [100]. This protein is phosphorylated and inactivated by the apoptotic factor cyclin-dependent kinase 5 (cdk5) [101, 102], which has been found to favor dopaminergic neurodegeneration in MPTP-induced models of PD [103, 104]. The cdk5-mediated inactivation of MEF2D is essential for cdk5-induced cell death [105]. Recently, MEF2D has been identified as a substrate of CMA. CMA efficiency modulates not only the intracellular levels of MEF2D, but also its transcriptional activity. The inhibition of lysosomal degradative function leads to accumulation of cytoplasmatic fraction of MEF2D and reduction of its nuclear levels and DNA-binding capacity. Mutated or over-expressed α-synuclein impairs the CMA-mediated degradation of MEF2D, thus suppressing its function [106]. Sporadic PD patients and α-synuclein transgenic mice show higher cytosolic [106] and decreased nuclear [107] levels of MEF2D in their brain, both alterations consistent with CMA impairment. Based on these data, MEF2D appears as a putative key link between the CMA impairment and dopaminergic neurodegeneration.

The hypothesis that macroautophagy dysfunction might be involved in PD pathogenesis has raised from the identification of the key pathologic role of misfolded proteins [83] and aberrant mitochondria [73], both substrates of macroautophagy. Autoptic studies reported accumulation of autophagic vacuoles in the substantia nigra of PD patients compared to controls [87]. This feature may be due to a selective impairment in autophagosome maturation and fusion with lysosomes [78, 90]. Preclinical studies on PD models have demonstrated neuroprotective effects of drugs activating macroautophagy. The first tested molecule, rapamycin, is known to activate macroautophagy through mTOR (mammalian target of rapamycin) inhibition; this drug has been shown to rescue dopaminergic neurons in various transgenic and sporadic models of disease, both *in vitro* and *in vivo* [108–110]. Trehalose has shown a protective effect in animal models of PD trough mTOR-independent macroautophagy enhancement [111].

The existence of a correlation between genes linked to hereditary PD and macroautophagy has provided elements useful to clarify the role of this system in PD.

Mutated α-synuclein causes inhibition of macroautophagy in transgenic cell and animal models through inhibition of Ras-related protein Rab1A, a cytoprotective factor involved in the regulation of intracellular vesicular trafficking [112]. There is evidence that upregulation of macroautophagy can diminish cytotoxicity of α-synuclein [109, 113, 114]. The degradation of α-synuclein probably becomes more dependent on macroautophagy when its aggregation tendency increases. In fact, macroautophagy shows ability to specifically remove mutated rather than wild

type α-synuclein [114]. Since post-translational modifications produced by oxidative stress favor the tendency of α-synuclein to aggregate [115, 116], the role of macroautophagy in the clearance of α-synuclein may be particularly relevant in oxidative conditions found in the substantia nigra of sporadic PD patients [86].

Leucine-rich repeat kinase 2 (LRRK2) is a mitochondrial kinase linked to autosomal dominant inherited PD [117]. Recent studies highlighted a functional link between LRRK2 and macroautophagy. Indeed, LRRK2 can interfere with the maturation of autophagosomes, thus acting as a negative regulator of macroautophagy. The pathogenetic R1441C mutation of LRRK2 seems to result in an enhancement of this effect [118].

A major feature linking the loss of autophagy function to neurodegeneration may be the accumulation of aberrant mitochondria. Mitochondrial dysfunction is considered a relevant pathogenetic mechanism in PD [119]. Defective mitochondria cause cell damage through oxidative stress and release of pro-apoptotic factors, so that their rapid turnover is necessary for cell viability [120]. The catabolism of mitochondria is mediated by a subtype of macroautophagy named mitophagy. Impairment of mitophagy determines the accumulation of aberrant mitochondria within neurons and may favour neurodegeneration [121]. The hypothesis that mitophagy defect may be a pathogenetic mechanism of PD is supported by the demonstration of a role within mitophagy machinery of PTEN-induced putative kinase 1 (PINK1) and parkin, two proteins linked to recessive hereditary PD [122, 123]. PINK1 is a kinase set in the outer mitochondrial membrane [124], whereas parkin is an E3-ligase involved in protein degradation through the proteasome [125]. In intact mitochondria, PINK1 is rapidly cleaved; mitochondrial damage causes suppression of PINK1 degradation and its accumulation on mitochondrial membrane [126], where it recruits parkin from the cytosol. Parkin determines the ubiquitination of proteins set in outer mitochondrial membrane; this allows targeting of damaged mitochondria to the phagophore [127]. Pathogenetic mutations of PINK1 and parkin cause the loss of the capacity to induce mitophagy after mitochondrial damage. Based on these data, the dysregulation of mitochondrial turnover is likely to be a key mechanism responsible for the dopaminergic neurodegeneration linked to mutations of PINK1 and parkin. Furthermore, PINK1 has been demonstrated to induce macroautophagy through direct interaction with beclin1; pathogenetic mutations of PINK1 impair this function [128].

DJ-1 is an antioxidant protein [129] linked to autosomic recessive PD [130]. Recent data highlight the ability of DJ-1 to induce macroautophagy and mitophagy [131], so that impairment of these processes may be a relevant mechanism even in PD variants caused by DJ-1 mutations.

Since both CMA and macroautophagy are likely to be defective in PD [81] and an accumulation of autophagosomes has been found in PD brains [87], lysosomal dysfunction may be the major mechanism leading to the failure of autophagic pathways in PD. Toxins that inhibit lysosomal proteolysis, such as ammonium chloride and tunicamycin, can reproduce a PD-like neuropathology in animals, thus demonstrating the important role of lysosomal function in dopaminergic neurons [132]. A reduction in lysosomal pool has been found in post-mortem samples of substantia

nigra from PD patients [90]. The induction of lysosomal biogenesis has been demonstrated to restore the intracellular lysosomal pool and to rescue from neuronal death in PD cell and animal models [90].

Once again, further data supporting the role of lysosomal function in PD comes from genetics. Indeed, mutations of ATPase type 13 A2 (ATP13A2) and glucocerebrosidase (GBA), two proteins involved in lysosomal degradative activity, have been demonstrated to modify the risk of developing PD. Mutations inactivating ATP13A2 are responsible for autosomic recessive parkinsonism [133]; experimental data provide evidence that ATP13A2 counteracts α-synuclein accumulation by favoring its lysosomal degradation [134]. GBA is a lysosomal enzyme whose homozygous mutations are responsible for Gaucher syndrome, a multisystem disease characterized by neurodegeneration; carriers of these mutations have higher risk of developing PD [135–137], linked to a reduction in α-synuclein clearance [138].

Effects of Rotenone on Autophagy

Dysfunctions of Macroautophagy Induced by Rotenone

The first evidence for an involvement of autophagy in rotenone-induced toxicity has been reported in 2007 by Chen et al. This study demonstrated that rotenone may induce the formation of autophagic vacuoles (autophagosomes and autolysosomes) and LC3-labeled vacuoles in transformed HEK 293 cells and U87 cancer cells and that these effects are reverted by the autophagy inhibitor 3-methyladenine (3-MA). Moreover rotenone was reported to be able to increase the levels of LC3-II and beclin-1, two commonly used markers of autophagy. Interestingly, transfection of beclin-1 and Atg5 siRNAs in rotenone-treated cells reduced the formation of autophagic vacuoles and LC3-labeled vacuoles as well as cell death, suggesting that rotenone toxicity is also mediated by autophagy induction [139]. This effect seems to be mediated by an increased production of reactive oxygen species (ROS) due to the rotenone-induced mitochondrial electron chain inhibition. This mechanism represents an upstream event of rotenone-mediated autophagy induction as evidenced by the observations that treatment with 3-MA or siRNAs against beclin-1 or Atg5 does not lower ROS production while exposure to ROS scavengers, such as mitochondrial superoxide dismutase (SOD2), is able to reduce both rotenone-induced ROS generation and autophagy induction.

However, another study performed in human neuroblastoma SH-SY5Y cells suggested that rapamycin, a well-known autophagy inducer, exerts a protective function against toxicity induced by a 24 h treatment with 10 μM rotenone [140]. Supporting this evidence, autophagy activation by rapamycin was demonstrated to counteract cell death and ROS formation induced by 500 nM rotenone in overexpressing wild-type or mutant α-synuclein SH-SY5Y cells, while autophagy inhibition obtained using 3-MA and bafilomycin was reported to accelerate cell death [141]. These results seem to be in contrast with the abovementioned findings observed in

transformed and cancer cells, where the autophagy induction triggered by rotenone appears to be rather a cell death mechanism. Collectively, experimental data indicate that rotenone-mediated autophagy induction acts as a toxicity mediator in non-neuronal cell lines and as a protective mechanism in neuronal cells.

Microarray analyses in murine cortical neurons treated with low concentration of rotenone (10 nM) revealed a dysregulated expression of many mitochondrial genes as well as apoptotic signalling, calcium signalling, ubiquin-proteasome system (UPS) and autophagy-lysosomal pathways genes [142]. In more detail, lysosomal proteins were up-regulated, while autophagy machinery components were down-regulated after 15 h treatment, suggesting that rotenone-induced autophagy dysregulation may be a later phenomenon.

Another study showed that a higher rotenone concentration (10 μM) causes an accumulation of autophagic vacuoles (reported as LC3-II increase) after 6 and 24 h treatment in differentiated SH-SY5Y cells. It is likely that the effect on autophagy already evidenced in this study at 6 h can be due to the higher rotenone concentration used. Moreover, monitoring the autophagic flux revealed that the increase of autophagic vacuoles is due to a block of the lysosomal degradation rather than an autophagy induction [143]. This finding is in line with the knowledge that rotenone exposure causes an accumulation of PD-related proteins, such as α-synuclein.

The exact molecular mechanisms underlying rotenone-induced dysregulation of autophagy are still not fully elucidated. Sublethal rotenone concentrations were demonstrated to elicit increased LC3 levels and to reduce p62 levels in rat cortical neurons (25–250 nM for 2 h) and in SH-SY5Y neuroblastoma cells (1 μM for 4 h) [144]. In the same study, rotenone was shown to increase the colocalization of LC3 with mitochondria and this phenomenon was reversed by siRNA knockdown of Atg7 or LC3, suggesting an upregulation of mitophagy. The externalization of cardiolipin, an inner mitochondrial membrane phospholipid, to the outer mitochondrial membrane allows the interaction with LC3 and may represent the signal by which cells recognize mitochondria damaged by rotenone.

Effect of Rotenone on CMA

While the consequences of rotenone exposure on macroautophagy have been at least partially clarified and resumed in the previous section, till now there is only limited information on how this environmental pesticide may affect CMA.

Considering the importance of this selective type of autophagy in the degradation of α-synuclein as well as other potential neurotoxic proteins and considering that CMA assumes a major role during long-term/prolonged stressful conditions, such as chronic exposure to environmental toxins, it is clean-cut the importance of elucidating the role of possible pesticide-induced alterations of this pathway in PD pathogenesis.

A single study performed in SH-SY5Y cells indicated that 48 h treatment with rotenone does not significantly affect lamp2A and hsc70 protein or mRNA levels

[145]. In the same cells, another recent study from our group has explored the effect of rotenone on CMA substrates, α-synuclein and MEF2D, and effectors, lamp2A and hsc70 [146]. This study has evidenced that 24 h exposure to 100 and 200 nM rotenone upregulates α-synuclein and MEF2D mRNA and protein levels, and increases lamp2A mRNA with no change in its protein levels. No effect was evidenced on hsc70 mRNA and protein levels at these rotenone doses and time exposure. Extending the exposure time till 48 h, preliminary and unpublished results from our lab indicate that rotenone causes a reduction of hsc70 mRNA and protein levels, and similar results have been obtained using a higher rotenone concentration (400 nM) for 24 h.

In conclusion, the current deficiency of studies exploring the effect of rotenone, as well as other environmental pesticides associated to an increased risk for PD, on CMA does not allow to have conclusive results on the possible role of specific CMA dysfunctions in mediating rotenone toxicity. Further studies are needed to explore the possible contribution of CMA dysfunctions in rotenone toxicity.

Concluding Remarks

Collectively, literature data indicate that rotenone alters neuronal homeostasis through its ability to increase oxidative stress and damage mitochondria; furthermore, low doses of rotenone increase the number of autophagic vacuoles and cause a mild accumulation of α-synuclein-positive protein aggregates, while high rotenone concentrations impair basal autophagy and compromise lysosomal integrity.

In summary, despite it is now widely accepted that the toxicity of rotenone is at least partially mediated by an autophagy dysregulation, to date the exact molecular mechanisms involved in this phenomenon remain to be clarified. In particular, while a substantial number of studies explored the effect of rotenone exposure on macroautophagy, only two studies have till now assessed the impact of rotenone on CMA, a more selective—with respect to macroautophagy—catabolic pathway whose dysfunction is recognized to play a pathogenic role in PD.

Furthermore, the available literature indicates that rotenone elicits a different modulation of autophagic pathways depending on the specific cell type (neuronal vs. non-neuronal) or toxin concentration/time exposure. This plurality of results obtained after rotenone exposure on autophagy parameters leads to a different and sometimes contrasting interpretation of the role of autophagy in the pathogenesis of rotenone-induced toxicity. As a matter of fact, till now it is not yet well established whether autophagy represents an upstream or downstream effect of rotenone-induced toxicity. Addressing this issue is crucial in order to develop novel PD diagnostics and therapies. Till now several studies have already been performed with the aim of exploring the potential efficacy of different compounds in counteracting rotenone-induced toxicity. In neuronal cells the neuroprotective mechanism of compounds such as iron chelators, flavonoids and terpenoids seems to be mediated by an autophagy induction [147–151]. Supporting this hypothesis, autophagy enhancers,

such as rapamycin and lithium chloride, and potential autophagy enhancer, such as valproic acid and carbamazepine, have been demonstrated to significantly reduce rotenone-induced toxicity *in vitro* [152].

References

1. Schapira AH. Complex I: inhibitors, inhibition and neurodegeneration. Exp Neurol. 2010;224(2):331–5.
2. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 2000;3(12):1301–6.
3. Sherer TB, Kim JH, Betarbet R, Greenamyre JT. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol. 2003;179(1):9–16.
4. Inden M, Kitamura Y, Abe M, Tamaki A, Takata K, Taniguchi T. Parkinsonian rotenone mouse model: reevaluation of long-term administration of rotenone in C57BL/6 mice. Biol Pharm Bull. 2011;34(1):92–6.
5. Sonia Angeline M, Chaterjee P, Anand K, Ambasta RK, Kumar P. Rotenone-induced parkinsonism elicits behavioral impairments and differential expression of parkin, heat shock proteins and caspases in the rat. Neuroscience. 2012;220:291–301.
6. Gao HM, Hong JS, Zhang W, Liu B. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. J Neurosci. 2002;22(3):782–90.
7. Gao HM, Liu B, Hong JS. Critical role for microglial NADPH oxidase in rotenone-induced degeneration of dopaminergic neurons. J Neurosci. 2003;23(15):6181–7.
8. Pei W, Liou AK, Chen J. Two caspase-mediated apoptotic pathways induced by rotenone toxicity in cortical neuronal cells. FASEB J. 2003;17(3):520–2.
9. Choi WS, Kruse SE, Palmiter RD, Xia Z. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. Proc Natl Acad Sci USA. 2008;105(39):15136–41.
10. Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol. 2011;26(Suppl 1):S1–58.
11. Kamel F. Epidemiology. Paths from pesticides to Parkinson's. Science. 2013;341(6147): 722–3.
12. Benmoyal-Segal L, Soreq H. Gene-environment interactions in sporadic Parkinson's disease. J Neurochem. 2006;97(6):1740–55.
13. Ross CA, Smith WW. Gene-environment interactions in Parkinson's disease. Parkinsonism Relat Disord. 2007;13(Suppl 3):S309–15.
14. Bronstein J, Carvey P, Chen H, Cory-Slechta D, DiMonte D, Duda J, et al. Meeting report: consensus statement-Parkinson's disease and the environment: collaborative on health and the environment and Parkinson's Action Network (CHE PAN) conference 26–28 June 2007. Environ Health Perspect. 2009;117(1):117–21.
15. Costa LG, Giordano G, Guizzetti M, Vitalone A. Neurotoxicity of pesticides: a brief review. Front Biosci. 2008;13:1240–9.
16. Franco R, Li S, Rodriguez-Rocha H, Burns M, Panayiotidis MI. Molecular mechanisms of pesticide-induced neurotoxicity: relevance to Parkinson's disease. Chem Biol Interact. 2010;188(2):289–300.
17. Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW, et al. Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiol Dis. 2005;20(2):360–71.

18. Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci. 2000;20(24):9207–14.
19. Brown TP, Rumsby PC, Capleton AC, Rushton L, Levy LS. Pesticides and Parkinson's disease—is there a link? Environ Health Perspect. 2006;114(2):156–64.
20. Dick FD. Parkinson's disease and pesticide exposures. Br Med Bull. 2006;79–80:219–31.
21. Le Couteur DG, Muller M, Yang MC, Mellick GD, McLean AJ. Age-environment and gene-environment interactions in the pathogenesis of Parkinson's disease. Rev Environ Health. 2002;17(1):51–64.
22. Yang MC, McLean AJ, Le Couteur DG. Age-related alteration in hepatic disposition of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and pesticides. Pharmacol Toxicol. 2002;90(4):203–7.
23. López O, Hernández AF, Rodrigo L, Gil F, Pena G, Serrano JL, et al. Changes in antioxidant enzymes in humans with long-term exposure to pesticides. Toxicol Lett. 2007;171(3):146–53.
24. Manthripragada AD, Costello S, Cockburn MG, Bronstein JM, Ritz B. Paraoxonase 1, agricultural organophosphate exposure, and Parkinson disease. Epidemiology. 2010;21(1):87–94.
25. Urdinguio RG, Sanchez-Mut JV, Esteller M. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. Lancet Neurol. 2009;8(11):1056–72.
26. Edwards TM, Myers JP. Environmental exposures and gene regulation in disease etiology. Environ Health Perspect. 2007;115(9):1264–70.
27. Migliore L, Coppedè F. Genetics, environmental factors and the emerging role of epigenetics in neurodegenerative diseases. Mutat Res. 2009;667(1–2):82–97.
28. Perera F, Herbstman J. Prenatal environmental exposures, epigenetics, and disease. Reprod Toxicol. 2011;31(3):363–73.
29. Langston JW, Ballard PA. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983;309(5):310.
30. Kontakos N, Stokes J. Monograph series on aging-related diseases: XII. Parkinson's disease—recent developments and new directions. Chronic Dis Can. 1999;20(2):58–76.
31. Lang AE, Lozano AM. Parkinson's disease. Second of two parts. N Engl J Med. 1998;339(16):1130–43.
32. Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology. 2000;21(4):435–40.
33. Priyadarshi A, Khuder SA, Schaub EA, Priyadarshi SS. Environmental risk factors and Parkinson's disease: a metaanalysis. Environ Res. 2001;86(2):122–7.
34. Li AA, Mink PJ, McIntosh LJ, Teta MJ, Finley B. Evaluation of epidemiologic and animal data associating pesticides with Parkinson's disease. J Occup Environ Med. 2005;47(10):1059–87.
35. Le Couteur DG, McLean AJ, Taylor MC, Woodham BL, Board PG. Pesticides and Parkinson's disease. Biomed Pharmacother. 1999;53(3):122–30.
36. Barbeau A, Roy M, Bernier G, Campanella G, Paris S. Ecogenetics of Parkinson's disease: prevalence and environmental aspects in rural areas. Can J Neurol Sci. 1987;14(1):36–41.
37. Wang SJ, Fuh JL, Liu CY, Lin KP, Chang R, Yih JS, et al. Parkinson's disease in Kin-Hu, Kinmen: a community survey by neurologists. Neuroepidemiology. 1994;13(1–2):69–74.
38. Tandberg E, Larsen JP, Nessler EG, Riise T, Aarli JA. The epidemiology of Parkinson's disease in the county of Rogaland, Norway. Mov Disord. 1995;10(5):541–9.
39. Kuopio AM, Marttila RJ, Helenius H, Rinne UK. Environmental risk factors in Parkinson's disease. Mov Disord. 1999;14(6):928–39.
40. Ritz B, Yu F. Parkinson's disease mortality and pesticide exposure in California 1984–1994. Int J Epidemiol. 2000;29(2):323–9.
41. Hubble JP, Cao T, Hassanein RE, Neuberger JS, Koller WC. Risk factors for Parkinson's disease. Neurology. 1993;43(9):1693–7.

42. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, et al. Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology. 1997;48(6):1583–8.
43. Menegon A, Board PG, Blackburn AC, Mellick GD, Le Couteur DG. Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet. 1998;352(9137):1344–6.
44. Seidler A, Hellenbrand W, Robra BP, Vieregge P, Nischan P, Joerg J, et al. Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. Neurology. 1996;46(5):1275–84.
45. Semchuk KM, Love EJ, Lee RG. Parkinson's disease and exposure to agricultural work and pesticide chemicals. Neurology. 1992;42(7):1328–35.
46. Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, et al. Pesticide exposure and self-reported Parkinson's disease in the agricultural health study. Am J Epidemiol. 2007;165(4):364–74.
47. Gatto NM, Rhodes SL, Manthripragada AD, Bronstein J, Cockburn M, Farrer M, et al. a-Synuclein gene may interact with environmental factors in increasing risk of Parkinson's disease. Neuroepidemiology. 2010;35(3):191–5.
48. Deng Y, Newman B, Dunne MP, Silburn PA, Mellick GD. Further evidence that interactions between CYP2D6 and pesticide exposure increase risk for Parkinson's disease. Ann Neurol. 2004;55(6):897.
49. Dutheil F, Beaune P, Tzourio C, Loriot MA, Elbaz A. Interaction between ABCB1 and professional exposure to organochlorine insecticides in Parkinson disease. Arch Neurol. 2010;67(6):739–45.
50. Elbaz A, Levecque C, Clavel J, Vidal JS, Richard F, Amouyel P, et al. CYP2D6 polymorphism, pesticide exposure, and Parkinson's disease. Ann Neurol. 2004;55(3):430–4.
51. Fong CS, Wu RM, Shieh JC, Chao YT, Fu YP, Kuao CL, et al. Pesticide exposure on southwestern Taiwanese with MnSOD and NQO1 polymorphisms is associated with increased risk of Parkinson's disease. Clin Chim Acta. 2007;378(1–2):136–41.
52. Ritz BR, Manthripragada AD, Costello S, Lincoln SJ, Farrer MJ, Cockburn M. Dopamine transporter genetic variants and pesticides in Parkinson's disease. Environ Health Perspect. 2009;117(6):964–9.
53. Freire C, Koifman S. Pesticide exposure and Parkinson's disease: epidemiological evidence of association. Neurotoxicology. 2012;33(5):947–71.
54. Dhillon AS, Tarbutton GL, Levin JL, Plotkin GM, Lowry LK, Nalbone JT, et al. Pesticide/environmental exposures and Parkinson's disease in East Texas. J Agromedicine. 2008;13(1):37–48.
55. Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B. Well-water consumption and Parkinson's disease in rural California. Environ Health Perspect. 2009;117(12):1912–8.
56. Richardson JR, Roy A, Shalat SL, Buckley B, Winnik B, Gearing M, et al. b-Hexachlorocyclohexane levels in serum and risk of Parkinson's disease. Neurotoxicology. 2011;32(5): 640–5.
57. Weisskopf MG, Knekt P, O'Reilly EJ, Lyytinen J, Reunanen A, Laden F, et al. Persistent organochlorine pesticides in serum and risk of Parkinson disease. Neurology. 2010;74(13): 1055–61.
58. Tanner CM, Ross GW, Jewell SA, Hauser RA, Jankovic J, Factor SA, et al. Occupation and risk of parkinsonism: a multicenter case-control study. Arch Neurol. 2009;66(9):1106–13.
59. Sherer TB, Richardson JR, Testa CM, Seo BB, Panov AV, Yagi T, et al. Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. J Neurochem. 2007;100(6):1469–79.
60. Greenamyre JT, MacKenzie G, Peng TI, Stephans SE. Mitochondrial dysfunction in Parkinson's disease. Biochem Soc Symp. 1999;66:85–97.
61. Langston JW, Forno LS, Rebert CS, Irwin I. Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey. Brain Res. 1984;292(2):390–4.
62. Hatcher JM, Pennell KD, Miller GW. Parkinson's disease and pesticides: a toxicological perspective. Trends Pharmacol Sci. 2008;29(6):322–9.

63. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, et al. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 2011;119(6):866–72.
64. Alavanja MC, Sandler DP, McMaster SB, Zahm SH, McDonnell CJ, Lynch CF, et al. The Agricultural Health Study. Environ Health Perspect. 1996;104(4):362–9.
65. Tanner CM. Occupational and environmental causes of parkinsonism. Occup Med. 1992;7(3):503–13.
66. Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science. 2003;302(5646):819–22.
67. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839–40.
68. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997;276(5321):2045–7.
69. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, et al. alpha-Synuclein locus triplication causes Parkinson's disease. Science. 2003;302(5646):841.
70. Kazantsev AG, Kolchinsky AM. Central role of alpha-synuclein oligomers in neurodegeneration in Parkinson disease. Arch Neurol. 2008;65(12):1577–81.
71. Dawson T, Mandir A, Lee M. Animal models of PD: pieces of the same puzzle? Neuron. 2002;35(2):219–22.
72. Vekrellis K, Rideout HJ, Stefanis L. Neurobiology of alpha-synuclein. Mol Neurobiol. 2004;30(1):1–21.
73. Mancuso M, Coppede F, Migliore L, Siciliano G, Murri L. Mitochondrial dysfunction, oxidative stress and neurodegeneration. J Alzheimers Dis. 2006;10(1):59–73.
74. Zhang Y, Dawson VL, Dawson TM. Oxidative stress and genetics in the pathogenesis of Parkinson's disease. Neurobiol Dis. 2000;7(4):240–50.
75. Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, et al. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005;25(3):1025–40.
76. Brunk UT, Terman A. The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. Eur J Biochem. 2002;269(8):1996–2002.
77. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43:67–93.
78. Nixon RA. Autophagy in neurodegenerative disease: friend, foe or turncoat? Trends Neurosci. 2006;29(9):528–35.
79. Lee JA. Autophagy in neurodegeneration: two sides of the same coin. BMB Rep. 2009;42(6):324–30.
80. Yang Q, Mao Z. Parkinson disease: a role for autophagy? Neuroscientist. 2010;16(4):335–41.
81. Xilouri M, Stefanis L. Autophagic pathways in Parkinson disease and related disorders. Expert Rev Mol Med. 2011;13:e8.
82. Martinez-Vicente M, Sovak G, Cuervo AM. Protein degradation and aging. Exp Gerontol. 2005;40(8–9):622–33.
83. Soto C, Estrada LD. Protein misfolding and neurodegeneration. Arch Neurol. 2008;65(2):184–9.
84. Tatsuta T, Langer T. Quality control of mitochondria: protection against neurodegeneration and ageing. EMBO J. 2008;27(2):306–14.
85. Schapira AH, Gu M, Taanman JW, Tabrizi SJ, Seaton T, Cleeter M, et al. Mitochondria in the etiology and pathogenesis of Parkinson's disease. Ann Neurol. 1998;44(3 Suppl 1):S89–98.
86. Zhang NY, Tang Z, Liu CW. alpha-Synuclein protofibrils inhibit 26 S proteasome-mediated protein degradation: understanding the cytotoxicity of protein protofibrils in neurodegenerative disease pathogenesis. J Biol Chem. 2008;283(29):20288–98.
87. Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, et al. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol Histopathol. 1997;12(1):25–31.

88. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem. 2003;278(27):25009–13.
89. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science. 2004;305(5688):1292–5.
90. Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci. 2010;30(37):12535–44.
91. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441(7095):885–9.
92. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell. 2007;131(6):1149–63.
93. Komatsu M, Wang QJ, Holstein GR, Friedrich VL, Iwata J, Kominami E, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci USA. 2007;104(36):14489–94.
94. Alvarez-Erviti L, Rodriguez-Oroz MC, Cooper JM, Caballero C, Ferrer I, Obeso JA, et al. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch Neurol. 2010;67(12):1464–72.
95. Kabuta T, Furuta A, Aoki S, Furuta K, Wada K. Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. J Biol Chem. 2008;283(35):23731–8.
96. Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L. Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. PLoS One. 2009;4(5):e5515.
97. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, et al. The ubiquitin pathway in Parkinson's disease. Nature. 1998;395(6701):451–2.
98. Bandopadhyay R, de Belleroche J. Pathogenesis of Parkinson's disease: emerging role of molecular chaperones. Trends Mol Med. 2010;16(1):27–36.
99. Pan T, Kondo S, Le W, Jankovic J. The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease. Brain. 2008;131(Pt 8):1969–78.
100. Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci USA. 2003;100(14):8532–7.
101. Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, et al. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron. 2003;38(1):33–46.
102. Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, et al. Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J Neurosci. 2005;25(19):4823–34.
103. Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature. 2000;405(6784):360–4.
104. Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP. Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci USA. 2003;100(23):13650–5.
105. Smith PD, Mount MP, Shree R, Callaghan S, Slack RS, Anisman H, et al. Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. J Neurosci. 2006;26(2):440–7.
106. Yang Q, She H, Gearing M, Colla E, Lee M, Shacka JJ, et al. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. Science. 2009;323(5910):124–7.
107. Chu Y, Mickiewicz AL, Kordower JH. a-Synuclein aggregation reduces nigral myocyte enhancer factor-2D in idiopathic and experimental Parkinson's disease. Neurobiol Dis. 2011;41(1):71–82.
108. Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O, Whitworth AJ. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. Nat Neurosci. 2009;12(9):1129–35.

109. Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, et al. Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. J Neurosci. 2009;29(43):13578–88.
110. Malagelada C, Jin ZH, Jackson-Lewis V, Przedborski S, Greene LA. Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. J Neurosci. 2010;30(3):1166–75.
111. Rodríguez-Navarro JA, Rodríguez L, Casarejos MJ, Solano RM, Gómez A, Perucho J, et al. Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. Neurobiol Dis. 2010;39(3):423–38.
112. Winslow AR, Chen CW, Corrochano S, Acevedo-Arozena A, Gordon DE, Peden AA, et al. a-Synuclein impairs macroautophagy: implications for Parkinson's disease. J Cell Biol. 2010;190(6):1023–37.
113. Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, et al. Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science. 2006;313(5785):324–8.
114. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. 2007;282(8):5641–52.
115. Paxinou E, Chen Q, Weisse M, Giasson BI, Norris EH, Rueter SM, et al. Induction of alpha-synuclein aggregation by intracellular nitrative insult. J Neurosci. 2001;21(20):8053–61.
116. Hodara R, Norris EH, Giasson BI, Mishizen-Eberz AJ, Lynch DR, Lee VM, et al. Functional consequences of alpha-synuclein tyrosine nitration: diminished binding to lipid vesicles and increased fibril formation. J Biol Chem. 2004;279(46):47746–53.
117. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 2004;44(4):601–7.
118. Alegre-Abarrategui J, Christian H, Lufino MM, Mutihac R, Venda LL, Ansorge O, et al. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Hum Mol Genet. 2009;18(21):4022–34.
119. Zhu J, Chu CT. Mitochondrial dysfunction in Parkinson's disease. J Alzheimers Dis. 2010;20(Suppl 2):S325–34.
120. Burbulla LF, Krebiehl G, Krüger R. Balance is the challenge–the impact of mitochondrial dynamics in Parkinson's disease. Eur J Clin Invest. 2010;40(11):1048–60.
121. Tanaka A. Parkin-mediated selective mitochondrial autophagy, mitophagy: parkin purges damaged organelles from the vital mitochondrial network. FEBS Lett. 2010;584(7):1386–92.
122. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998;392(6676):605–8.
123. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 2004;304(5674):1158–60.
124. Zhou C, Huang Y, Shao Y, May J, Prou D, Perier C, et al. The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proc Natl Acad Sci USA. 2008;105(33):12022–7.
125. Tanaka K, Suzuki T, Chiba T, Shimura H, Hattori N, Mizuno Y. Parkin is linked to the ubiquitin pathway. J Mol Med (Berl). 2001;79(9):482–94.
126. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 2010;8(1):e1000298.
127. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol. 2010;12(2):119–31.
128. Michiorri S, Gelmetti V, Giarda E, Lombardi F, Romano F, Marongiu R, et al. The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ. 2010;17(6):962–74.

129. Abou-Sleiman PM, Muqit MM, Wood NW. Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci. 2006;7(3):207–19.
130. Hering R, Strauss KM, Tao X, Bauer A, Woitalla D, Mietz EM, et al. Novel homozygous p.E64D mutation in DJ1 in early onset Parkinson disease (PARK7). Hum Mutat. 2004;24(4):321–9.
131. Krebiehl G, Ruckerbauer S, Burbulla LF, Kieper N, Maurer B, Waak J, et al. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1. PLoS One. 2010;5(2):e9367.
132. Matsui H, Ito H, Taniguchi Y, Takeda S, Takahashi R. Ammonium chloride and tunicamycin are novel toxins for dopaminergic neurons and induce Parkinson's disease-like phenotypes in medaka fish. J Neurochem. 2010;115(5):1150–60.
133. Ramirez A, Heimbach A, Gründemann J, Stiller B, Hampshire D, Cid LP, et al. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat Genet. 2006;38(10):1184–91.
134. Gitler AD, Chesi A, Geddie ML, Strathearn KE, Hamamichi S, Hill KJ, et al. Alpha-synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity. Nat Genet. 2009;41(3):308–15.
135. Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N Engl J Med. 2009;361(17):1651–61.
136. Neumann J, Bras J, Deas E, O'Sullivan SS, Parkkinen L, Lachmann RH, et al. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain. 2009;132(Pt 7):1783–94.
137. Lesage S, Anheim M, Condroyer C, Pollak P, Durif F, Dupuits C, et al. Large-scale screening of the Gaucher's disease-related glucocerebrosidase gene in Europeans with Parkinson's disease. Hum Mol Genet. 2011;20(1):202–10.
138. Cullen V, Sardi SP, Ng J, Xu YH, Sun Y, Tomlinson JJ, et al. Acid b-glucosidase mutants linked to Gaucher disease, Parkinson disease, and Lewy body dementia alter a-synuclein processing. Ann Neurol. 2011;69(6):940–53.
139. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. J Cell Sci. 2007;120(Pt 23):4155–66.
140. Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience. 2009;164(2):541–51.
141. Dadakhujaev S, Noh HS, Jung EJ, Cha JY, Baek SM, Ha JH, et al. Autophagy protects the rotenone-induced cell death in alpha-synuclein overexpressing SH-SY5Y cells. Neurosci Lett. 2010;472(1):47–52.
142. Yap YW, Chen MJ, Peng ZF, Manikandan J, Ng JM, Llanos RM, et al. Gene expression profiling of rotenone-mediated cortical neuronal death: evidence for inhibition of ubiquitin-proteasome system and autophagy-lysosomal pathway, and dysfunction of mitochondrial and calcium signaling. Neurochem Int. 2013;62(5):653–63.
143. Mader BJ, Pivtoraiko VN, Flippo HM, Klocke BJ, Roth KA, Mangieri LR, et al. Rotenone inhibits autophagic flux prior to inducing cell death. ACS Chem Neurosci. 2012;3(12):1063–72.
144. Chu CT, Ji J, Dagda RK, Jiang JF, Tyurina YY, Kapralov AA, et al. Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. Nat Cell Biol. 2013;15(10):1197–205.
145. Alvarez-Erviti L, Seow Y, Schapira AH, Rodriguez-Oroz MC, Obeso JA, Cooper JM. Influence of microRNA deregulation on chaperone-mediated autophagy and a-synuclein pathology in Parkinson's disease. Cell Death Dis. 2013;4:e545.
146. Sala G, Arosio A, Stefanoni G, Melchionda L, Riva C, Marinig D, et al. Rotenone upregulates alpha-synuclein and myocyte enhancer factor 2D independently from lysosomal degradation inhibition. Biomed Res Int. 2013;2013:846725.

147. Wu Y, Li X, Xie W, Jankovic J, Le W, Pan T. Neuroprotection of deferoxamine on rotenone-induced injury via accumulation of HIF-1 alpha and induction of autophagy in SH-SY5Y cells. Neurochem Int. 2010;57(3):198–205.
148. Filomeni G, Graziani I, De Zio D, Dini L, Centonze D, Rotilio G, et al. Neuroprotection of kaempferol by autophagy in models of rotenone-mediated acute toxicity: possible implications for Parkinson's disease. Neurobiol Aging. 2012;33(4):767–85.
149. Wu Y, Li X, Zhu JX, Xie W, Le W, Fan Z, et al. Resveratrol-activated AMPK/SIRT1/autophagy in cellular models of Parkinson's disease. Neurosignals. 2011;19(3):163–74.
150. Deng YN, Shi J, Liu J, Qu QM. Celastrol protects human neuroblastoma SH-SY5Y cells from rotenone-induced injury through induction of autophagy. Neurochem Int. 2013;63(1):1–9.
151. Lin TK, Chen SD, Chuang YC, Lin HY, Huang CR, Chuang JH, et al. Resveratrol partially prevents rotenone-induced neurotoxicity in dopaminergic SH-SY5Y cells through induction of heme oxygenase-1 dependent autophagy. Int J Mol Sci. 2014;15(1):1625–46.
152. Xiong N, Jia M, Chen C, Xiong J, Zhang Z, Huang J, et al. Potential autophagy enhancers attenuate rotenone-induced toxicity in SH-SY5Y. Neuroscience. 2011;199:292–302.

Chapter 13
Autophagic Pathology and Calcium Deregulation in Neurodegeneration

Patricia Gómez-Suaga and Sabine Hilfiker

Abstract Over the last decades, substantial efforts have been made towards understanding the key players underlying neurodegeneration. However, despite extensive research efforts, the exact molecular mechanism(s) remain unclear, and much less is certain about possible common target(s) amongst distinct age-dependent neurodegenerative disorders. Whilst the precise mechanism(s) underlying neurodegeneration amongst the different diseases remain to be determined, a number of cellular processes have been suggested to be involved in all of them, including protein accumulation and aggregation, oxidative stress, mitochondrial deficits, Ca^{2+} dyshomeostasis and impairments in lysosomal degradation pathways including macroautophagy. The various possible pathogenic factors are not mutually exclusive, and the aim of much current research is to elucidate the correlation between them to establish successful strategies in limiting the disease process. Here, we summarize recent data that pinpoint Ca^{2+} dyshomeostasis as a key player underlying neurodegeneration in the context of macroautophagy deregulation. We will provide a brief overview of recent work towards addressing how macroautophagy and Ca^{2+} deregulation may cause cellular dysfunction linked to the pathogenesis of several neurodegenerative disorders, with emphasis on Parkinson's disease (PD).

Keywords Calcium · Autophagy · Endoplasmic reticulum · Mitochondria · Acidic calcium stores · NAADP · Parkinson's disease · LRRK2

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Introduction

Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), which are characterized by progressive nervous system dysfunction, affect millions of people worldwide. There is still no cure for any of them, and treatment is directed towards alleviating some of the symptoms. Slowing the rate of neuronal loss may improve the quality of life for patients; however, this requires considerable knowledge about disease mechanism(s) and approaches able to modify disease course. In this respect, although the majority of neurodegenerative diseases are sporadic, the discovery of mutations that cause the pathological processes has significantly advanced our understanding of the molecular mechanisms involved, as it allows for the generation of cellular and animal models carrying the mutant gene. Hence, even though the brain areas that degenerate and the proteins implicated in each disorder are generally different, the above-mentioned diseases may converge on common cellular processes. The presence of intra-cytoplasmic aggregates within neurons as a hallmark of most neurodegenerative diseases suggests that protein misfolding and aggregation are central features of the pathophysiology [1]. Indeed, most of these pathologies are referred to as protein misfolding diseases [2]. In support of the importance of protein misfolding and aggregation in neurodegeneration, accumulation of tau in AD and tauopathies, α-synuclein in PD, mutant huntingtin in HD and TDP-43 and FUS in ALS seem to mediate neuronal toxicity [1].

Autophagy is a metabolically homeostatic process by which old and/or damaged organelles and protein aggregates are removed from the cell. Thus, the above-mentioned processes which may underlie neurodegeneration, including increased oxidative stress, mitochondrial dysfunction and protein aggregation may all result from impaired autophagic degradation. Indeed, apart from neurodegeneration, autophagic failure has been related to a wide range of additional pathological conditions including cancer and pathogen infection [3]. Alterations in autophagy seem a key event underlying neurodegeneration, and accumulation of autophagic structures is evident in diseased brains as well as in various animal and cellular models [4]. While environmental factors may further impair autophagic clearance, disease-linked mutations reveal possible pathways susceptible for therapeutic interventions. Indeed, studies from animal and cellular models of neurodegenerative diseases indicate a connection between proteins genetically linked to disease and autophagy impairment [5–16]. Therefore, many attempts aimed at treating these diseases are focused on the development of novel therapies towards promoting autophagic clearance. In this respect, pharmacological activation of autophagy has been shown to alleviate the toxicity associated with accumulation of aggregate-prone proteins [17].

Recently, Ca^{2+} handling mechanisms have been recognized to be crucial for proper cellular degradation, suggesting that the role of intracellular Ca^{2+} in regulating autophagy, and alterations in Ca^{2+} signaling in general, have to be considered towards gaining an understanding of the process(es) underlying neurodegeneration. Ca^{2+} homeostasis is essential for neuronal activity and viability. Ca^{2+} is in a

dynamic cellular equilibrium in the cell, and generally kept at low cytosolic concentrations, being either pumped out of the cell, buffered by cytosolic Ca^{2+} buffering proteins, or quickly taken up into a variety of intracellular organelles which act as intracellular Ca^{2+} stores. Highly controlled regulatory mechanisms ensure such normal Ca^{2+} handling, and perturbations lead to a vast array of pathologies. Hence, Ca^{2+} overload, manifested as an increase of cytosolic Ca^{2+}, has pleiotropic effects on a variety of intracellular events and eventually activates both necrotic and apoptotic cell death pathways [18]. There is now compelling evidence for dysfunctional Ca^{2+} regulation in several neurodegenerative disorders including PD [19], AD, HD [21] and ALS [22]. Therefore, Ca^{2+} channels may comprise promising neurodegenerative drug targets, and therapies based on channel blockers are being developed [23–25].

A possible crosstalk between dysfunctional Ca^{2+} handling, autophagy impairment and neurodegeneration may highlight additional therapeutic targets. However, more work is needed to dissect the links between Ca^{2+} and autophagic clearance mechanisms. In this chapter, we will first briefly review the current knowledge of the molecular events of autophagy. Next, we will give an overview of the regulatory role of Ca^{2+} in autophagy, summarizing the mechanisms involved in the dynamic equilibrium of Ca^{2+} uptake and release pathways with a focus on the different Ca^{2+} stores and fluxes as determinants of proper autophagic functioning. Finally, we will focus on the link between autophagic alterations and Ca^{2+} deregulation using PD as an example, and discuss the Ca^{2+}-dependent mechanisms believed to be implicated in the selective degeneration of dopaminergic (DA) neurons [19].

Autophagy

Autophagy likewise targets proteins, protein aggregates or entire organelles for lysosome-mediated degradation. It is important for balancing sources of energy at critical times in development and in response to nutrient stress, and it plays a key role in the homeostatic clearance of defunct or damaged organelles, proteins and protein aggregates [3, 26]. Autophagy normally proceeds at a low basal rate, but basal autophagy is especially high in neurons, and disrupting basal autophagic degradation causes neurodegeneration in mice [27, 28]. Depletion of different autophagic proteins in mice causes an accumulation of cytoplasmic protein inclusions accompanied by progressive deficits in motor function [27, 28].

There are at least three types of autophagy, depending on the delivery route of the material for degradation to the lysosomal lumen: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy [29, 30]. CMA is a selective mechanism responsible for the lysosomal degradation of soluble cytosolic proteins targeted with a consensus motif biochemically related to KFERQ [5]. In CMA, proteins complexed to chaperone proteins are targeted to the lysosomal membrane by binding to the CMA-receptor lysosome-associated membrane protein (LAMP) 2 A (LAMP2A), resulting in their translocation to the lysosomal lumen and subsequent

degradation. Defective CMA has been attributed to underlie different pathologies including lysosomal storage diseases and familial forms of PD [5]. Microautophagy is a mechanism by which a portion of cytoplasm is directly taken up by the lysosome itself through invagination of the lysosomal membrane, but its mechanisms and physiological functions are poorly understood [31]. Finally, a third form of autophagy is macroautophagy (hereafter referred to as autophagy), a process by which cytosolic constituents, including damaged organelles and aggregated proteins, are engulfed within newly formed specialized double-membrane vesicles called autophagosomes. Autophagosomes fuse either with late endosomes (LE) or directly with lysosomes, followed by the hydrolytic degradation of products in lysosomes and reformation of these organelles to maintain cellular degradative capacity. Disrupting any part of this process impairs autophagic flux, accompanied by the accumulation of autophagic substrates.

Autophagy Signaling Pathways

The signaling mechanisms leading to the activation of autophagy under nutrient starvation conditions have been extensively investigated [32]. However, these circumstances are rare under physiological conditions. Nevertheless, autophagy plays an essential role in the maintenance of normal homeostasis at both a cellular and organismal level, and can also be induced by several cellular stresses under normal nutritional conditions. Under normal non-starved conditions, autophagy is regulated by a broad range of extracellular factors including growth factors, cytokines and chemokines, suggesting that there is a mutual exclusive regulation of cell growth and autophagy [33]. These factors are especially important in neurons, because these cells depend almost exclusively on glucose to provide both energy and carbon chains for protein synthesis in an insulin-independent manner. Thus, autophagic responses in neurons may be more related to organelle damage or neuritic remodeling than to the generation of amino acids and energy.

The best known canonical autophagy regulator is the mammalian TOR kinase (mTOR), specifically complex1 (mTORC1, comprised of by mTOR, RAPTOR, GβL/mLst8, PRAS40 and DEPTOR). Given the existence of some mTOR-independent autophagy pathways, autophagy signaling has been divided accordingly into mTOR-dependent and mTOR-independent pathways. mTOR is a serine/threonine protein kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family. mTOR regulates the balance between anabolic (cell growth) and catabolic (autophagic degradation) processes and has recently come to light as a prime modulator of neurodegenerative diseases [34]. The mTORC1 complex is positively regulated by the small GTPase Rheb, which can be inactivated by the GTPase activating protein (GAP) formed by the heterodimer regulator tuberous sclerosis 1 and 2 (TSC1/TSC2). In response to amino acids, mTORC1 has been reported to be recruited to the lysosomal surface by Rag GTPases [35]. Such lysosomal localization seems to be required for activation of the complex by Rheb [36]. The active

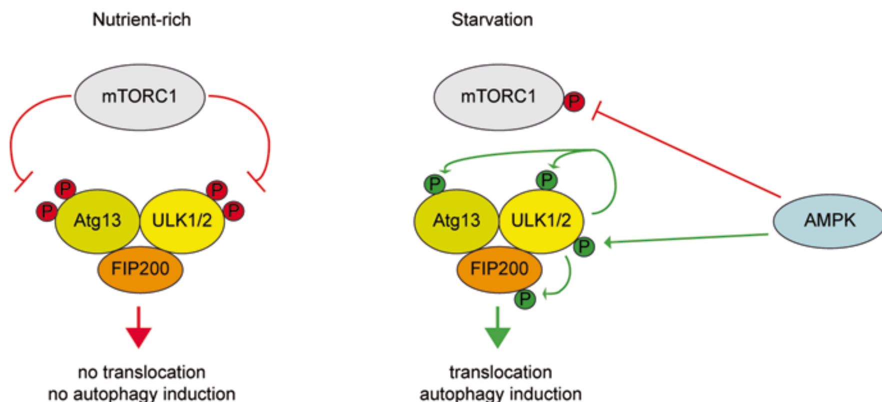

Fig. 13.1 Schematic overview of the regulation of autophagy induction by mTORC1 and AMPK signaling. *Left*: Under nutrient-rich conditions, activated TORC1 inhibits autophagy induction by inhibitory phosphorylation of ULK1/2 and Atg13, thereby inhibiting this complex. *Right*: Under starvation conditions, the mTORC1-dependent phosphorylation sites in ULK1/2 are rapidly dephosphorylated, and ULK1/2 can autophosphorylate itself and phosphorylate Atg13 and FIP200. Alternatively, activation of AMPK under distinct situations can induce autophagy by inhibiting mTORC1 through phosphorylation of a component within this complex. AMPK can phosphorylate and activate ULK1/2, with activation and translocation of the complex to the pre-autophagosomal membrane for autophagy induction. For further details see text

mTORC1 complex promotes cell growth and inhibits autophagy by inhibitory phosphorylation of the mammalian Atg1 orthologues ULK1 and ULK2 (ULK1/2) and Atg13 subunits [37] (Fig. 13.1). mTORC1 activity can be inhibited indirectly by another autophagy-related kinase, adenosine monophosphate-activated protein kinase (AMPK). As mentioned before, autophagy can also be controlled by several additional mTORC1-independent pathways. Direct induction of autophagy can be mediated, downstream of mTORC1, by the activation of AMPK in response to energy loss (an increase in AMP levels), involving various upstream regulators including LKB1 or an increase in cytosolic Ca^{2+} levels and concomitant activation of Ca^{2+}-calmodulin-dependent kinase kinase β (CaMKKβ) [38, 39]. Activated AMPK phosphorylates the ULK1/2 complex and directly induces autophagosome formation [40, 41]. Hence, both mTORC1-dependent and mTORC1-independent signaling pathways converge onto ULK1/2; mTORC1 and AMPK oppositely regulate ULK1/2 kinase activity by direct phosphorylation of the ULK1/2 complex on distinct sites [37, 42, 43] (Fig. 13.1). In both cases, ULK1/2 activation leads to autophosphorylation and stimulatory phosphorylation of both FIP200 and Atg13, which causes translocation of the entire complex to the pre-autophagosomal membrane and autophagy induction (Fig. 13.1).

Autophagic Steps

The autophagic pathway is comprised of distinct steps, including nucleation to generate a phagophore, elongation and formation of the autophagosome, autophagosome fusion with late endosomes or lysosomes, and finally degradation and recycling of macromolecules [44]. The source of the autophagosomal membranes is still controversial, although it is generally accepted that the ER membrane provides the major source of autophagosome membrane [45]. More recently, mitochondria [46, 47], plasma membrane [48], recycling endosomes [49] and the trans-Golgi network (TGN) [50, 51] have all been shown to be cellular sources for autophagophore formation, even though it remains unclear how much of the autophagosome membrane comes from such distinct sources, and under which conditions.

A crucial element for the initiation of the isolation membrane is the transient association of a large core complex including Vps34-Beclin1 [29, 52]. The protein AMBRA1 has been proposed as the link between ULK1/2 and the Vps34-Beclin1 complex [53]. ULK-dependent phosphorylation of AMBRA1 might lead to the release of the Vps34-Beclin1 complex from the dynein motor complex tethered to the cytoskeleton to allow translocation of the core complex to the autophagosome formation-related region [53]. Alternatively, ULK may directly phosphorylate Beclin1 to enhance Vps34 lipid kinase activity [54]. Vps34 is a class III phosphatidylinositol-3-kinase, and the lipid phosphatidylinositol-3-phosphate (PI3P) seems important for the initiation of the autophagosome membrane, with PI3P-enriched membranes recruiting and activating effector proteins containing FYVE or PX PI3P-binding domains [55]. The isolation membrane subsequently elongates, sequestering the cargo for degradation, and finally closes off to form a double-membraned vesicle called the autophagosome. The elongation process is regulated by two ubiquitination-like reactions, the Atg12/Atg5 conjugation and the conjugation of microtubule-associated protein 1 light chain 3 (LC3) to the lipid anchor phosphatidylethanolamine (the autophagosome-associated LC3-II form) [44]. The action of those two ubiquitin-like conjugation systems is modulated by Atg7. The Atg5/12 complex is further associated with Atg16L and LC3, but dissociates from the vesicle once it is fully formed [56]. In contrast, LC3, which is diffuse in the cytoplasm under normal conditions (LC3I), is bound to autophagic membranes (LC3II) upon the above-mentioned conjugation reaction and is classically used as a marker for autophagy (Fig. 13.2). Autophagosomes are then transported on microtubules from the cell periphery to the perinuclear region towards the microtubule-organizing center in a dynein/dynactin-dependent manner [57]. During maturation, the outer membrane of the autophagosome fuses with late endosomes, generating a hybrid organelle called the amphisome, or directly fuses with lysosomes to form an autolysosome (Fig. 13.2). Fusion events during autophagic maturation are similar to endosome-lysosome fusion events, where kiss-and-run, complete fusions or fusion mediated through tubules have been described [58]. Several protein complexes and signaling pathways are involved in the maturation of autophagosomes and their delivery to, and fusion with, lysosomes. Finally, lysosomal hydrolases degrade the

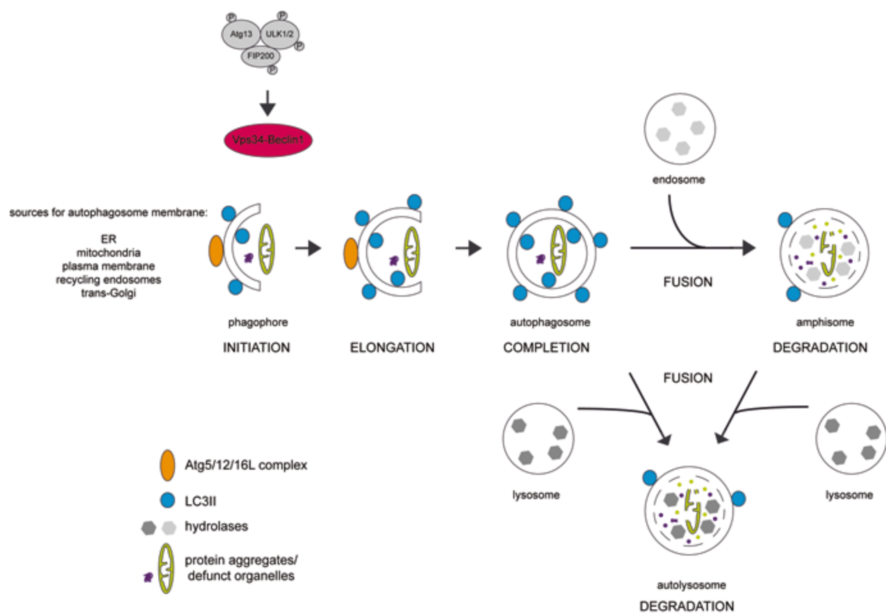

Fig. 13.2 Autophagic steps. Activation of the ULK1/2 complex leads to activation of Vps34-Beclin1 complex, followed by transient association with the isolation membrane and phagophore formation. The Atg5/12/16L complex and LC3II promote elongation and enwrap cytosolic cargoes including defunct organelles such as mitochondria and protein aggregates, finally resulting in the formation of the autophagosome. Autophagosomes then either fuse with endosomes to form amphisomes, or with lysosomes to form autolysosomes, and hydrolases in the interior begin to degrade internal membrane and luminal cargoes. For further information see text

content and the inner membrane of the autophagosome/amphisome. After this process, lysosomal function is restored again by formation of proto-lysosomes budding off from tubules emanating from the autolysosomes. These proto-lysosomes have been reported to be non-acidic and LAMP1 positive [59].

Ca^{2+} as an Autophagy Regulator

The spatiotemporal pattern of Ca^{2+} signaling is crucial for the specificity of cellular responses such as autophagy. Ca^{2+} has an important regulatory function in key steps of the autophagic pathway including during homotypic and heterotypic fusions of late endosomes and lysosomes and during lysosomal reformation [60], analogous to the well-described role of Ca^{2+} in regulated secretion. Moreover, autophagosome formation and maturation may be regulated by Ca^{2+} from different membrane sources [61, 62]. However, Ca^{2+} has been better recognized as an important player for autophagic signaling (see above). Intracellular Ca^{2+} has been proposed to be a regulator of autophagy, even though it is unclear whether there is positive [63, 64]

or negative [65–68] regulation. Dual effects have been reported and may depend on several circumstances such as the spatial and temporal parameters of Ca^{2+} signaling, the source of the Ca^{2+} and the overall cellular state [69].

Changes in intracellular Ca^{2+} caused by the action of Ca^{2+}-mobilizing agents have been reported to lead to autophagic alterations. The first evidence for a positive Ca^{2+}-mediated regulation of autophagy was reported by Jäättelä's group, as Ca^{2+}-mobilizing agents were found to activate the CaMKKβ/AMPK pathway, causing an induction of autophagosome formation [63]. These data suggested that Ca^{2+} may act as a trigger for autophagy, further supported by findings that the ionophore ionomycin, which raises cytosolic Ca^{2+} levels, induced autophagy, whilst a membrane-permeable Ca^{2+} chelator blocked the effects on autophagy. However, Ca^{2+} may also negatively regulate autophagy through acting as a bioenergetic sensor and inhibiting the catabolic mechanism of autophagy under normal cellular growth conditions. Therefore, a coherent understanding of the dual role of Ca^{2+} in regulating autophagy requires an understanding of the precise intraorganellar location of Ca^{2+}, the role which Ca^{2+} plays at these organelles, and the precise cellular status [69].

Neuronal Ca^{2+} entry from the extracellular space can be either mediated by voltage-dependent Ca^{2+} channels or ligand-activated Ca^{2+} influx [20]. Pumping Ca^{2+} out of the cytosol is an energetically costly process, as the removal is carried out by Ca^{2+}-ATPases localized on the plasma membrane (PMCAs) or on intracellular Ca^{2+} stores, such as the endoplasmic reticulum Ca^{2+} pumps named SERCA (sarco/endoplasmic-reticulum Ca^{2+}-ATPase) [70]. Several cellular compartments that have functional Ca^{2+}-release channels and pump mechanisms are essentially capable of acting as Ca^{2+} stores. The ER is the main and best-characterized Ca^{2+} store within the cell, whilst mitochondria can act as additional stores to buffer intracellular Ca^{2+}. At the same time, Ca^{2+} is also important in the normal ER and mitochondrial function, affecting protein folding and mitochondrial metabolism, therefore modulating autophagy. In addition to the ER and mitochondria, a range of acidic organelles, which include endosomes, lysosomes and lysosome-related organelles such as secretory granules also serve as significant Ca^{2+} stores in mammals [71]. Intraluminal Ca^{2+} in acidic stores is crucial for the functioning of the degradative pathway, as chelation of luminal Ca^{2+} blocks membrane fusion [72–74]. Therefore, the overall intraluminal Ca^{2+} concentrations and dynamics between the different Ca^{2+} stores are crucial for autophagy modulation.

ER and Mitochondrial Ca^{2+} and Regulation of Autophagy

The ER produces, delivers and folds newly synthesized proteins in a continuous intracellular network, but also it acts as the major intracellular Ca^{2+} store, setting up cytosolic Ca^{2+} signals. Whilst SERCA transports Ca^{2+} from the cytosol to the ER lumen in an energy-dependent manner [75], the main Ca^{2+}-release channels in the ER are the inositol 1,4,5-trisphosphate (IP_3)-receptor (IP_3R) [76] and the ryanodine-receptor (RyR). Ca^{2+}-binding proteins in the lumen of the ER, such as

Ca^{2+}-dependent chaperones like calnexin and 78-kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78/BiP) further contribute to Ca^{2+} handling in the ER [77]. IP_3R are expressed in all cell types, while RyRs are mainly expressed in neurons and muscle. IP_3 and cyclic adenosine diphosphate-ribose (cADPR) mobilize Ca^{2+} from the ER through activation of these receptors [78, 79], generating local Ca^{2+} signals. The subcellular localization of these receptors, together with their regulation by cytosolic Ca^{2+} and regulatory proteins further contribute to the establishment of highly specific and localized Ca^{2+} signals.

A role for Ca^{2+} as a negative regulator of basal autophagy has been proposed by several reports and implicates the IP_3R. Rubinsztein's group reported that inhibitors of inositol monophosphatases, leading to depletion of IP_3 levels, act as novel chemical inducers of autophagy [65]. These data would suggest that IP_3R-mediated release of Ca^{2+} from the ER, presumably coupled to an increase in cytosolic Ca^{2+} levels, negatively regulates autophagy. In accordance with this concept, they further found that L-type Ca^{2+} channel antagonists induce autophagy through a complex mTORC1-independent signaling pathway impacting upon calpain [66] (Fig. 13.3). Calpains are cytosolic Ca^{2+}-dependent cysteine proteases that have been shown to participate in apoptotic cell death mechanisms [80], but also in the degradation of Ca^{2+} extrusion channels, thereby contributing to increased Ca^{2+} overload [81] and providing a positive feedback loop for autophagy inhibition.

The apparently contradictory data regarding cytosolic Ca^{2+} in stimulating or inhibiting autophagy may be reconciled if considering the combination of alterations in cytosolic Ca^{2+} levels, alterations in intraluminal Ca^{2+} stores and the bioenergetic status of cells. For example, intraluminal Ca^{2+} is crucial for ER function, as ER Ca^{2+} depletion induces ER stress and ER Ca^{2+} overload compromises proteostasis as well. Conversely, normal ER function is necessary for proper ER Ca^{2+} handling, as ER stress disrupts luminal Ca^{2+} homeostasis, causes the accumulation of misfolded proteins and initiates the unfolded protein response (UPR) [82]. The UPR response is activated to restore normal cellular conditions through activation of signaling pathways that reduce protein synthesis and increase protein folding capacity. Autophagy is activated in parallel to the UPS as another pro-survival mechanism, as autophagy has been shown to inhibit tunicamycin- or thapsigargin-mediated cell death [83]. However, the crosstalk between ER stress-induced autophagy and ER stress-induced Ca^{2+} release remains largely unanswered. It has been proposed that ER-mediated Ca^{2+} release induced by ER stress may cause CaMKKβ-dependent activation of AMPK to promote Ca^{2+}-mediated autophagy [63]. Other Ca^{2+}-dependent kinases may be involved in the positive regulation of autophagy as well, including the Ca^{2+}-regulated death-associated protein kinase (DAPK), whose role in ER-stress has been widely reported [84], or PKCθ [85].

The interplay between mitochondria and ER in zones of close contact termed MAMs (mitochondria-associated ER membranes) is crucial for cellular Ca^{2+} handling as well as for mitochondrial dynamics (86). Mitochondria are important Ca^{2+} stores and contain several Ca^{2+} channels, such as the voltage-dependent anion channel (VDAC) and Ca^{2+} uniporters which drive Ca^{2+} entry at high cytosolic Ca^{2+} concentrations reached in these areas. Ca^{2+} signaling regulates mitochondrial traf-

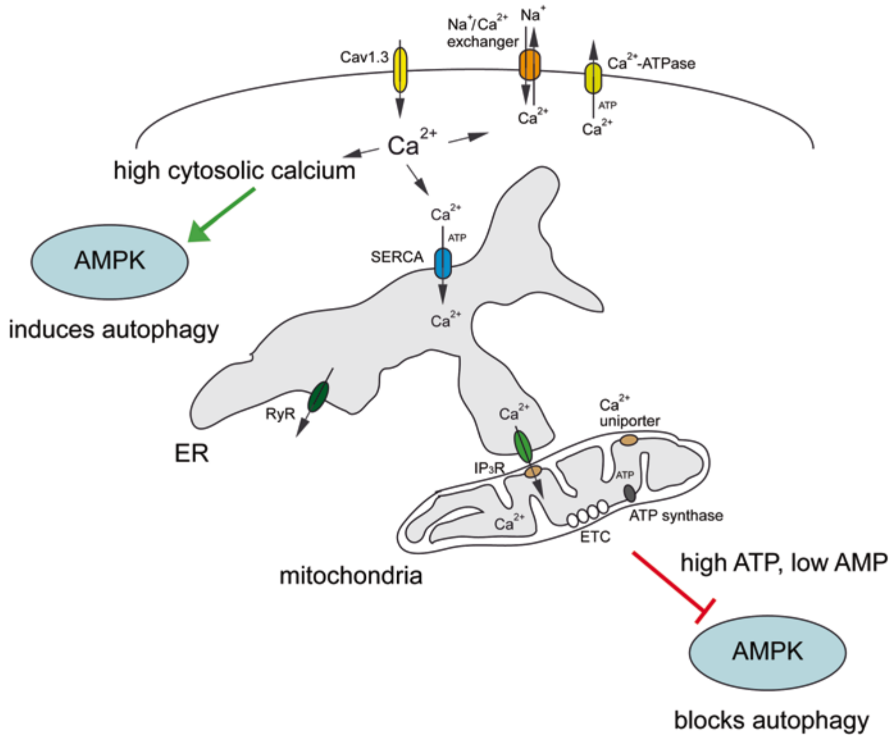

Fig. 13.3 Possible roles for Ca^{2+} in regulating autophagy. Ca^{2+} entry into cells is quickly controlled, either by extrusion or by uptake into the ER. IP_3R mediate a constitutive transfer of Ca^{2+} from the ER to mitochondria, which promotes ATP production, inhibiting AMPK and keeping autophagy to a minimum. Blocking such Ca^{2+} transfer, with the resultant increase in AMP, will stimulate autophagy. On the other hand, increasing cytosolic Ca^{2+} levels, e.g. by Ca^{2+}-mobilizing agents which may target a variety of intracellular Ca^{2+} stores, increases the cytosolic Ca^{2+} concentration. This causes autophagy induction through various pathways, including CaMKKβ and AMPK. For further details see text

ficking and dynamics through the regulation of mitochondrial fusion and fission reactions [87]. Furthermore, release of Ca^{2+} from ER IP_3R is important for mitochondrial activity and bioenergetics, as several mitochondrial dehydrogenases of the Krebs cycle are regulated by Ca^{2+}. Hence, under normal nutritional conditions, constitutive IP_3R Ca^{2+} transfer from ER to mitochondria inhibits autophagy for maintenance of cellular metabolic homeostasis (Fig. 13.3). In the absence of such Ca^{2+} transfer, AMPK is activated due to a decrease in mitochondrial ATP production and concomitant increase in AMP levels, causing AMPK-dependent but mTORC1-independent autophagy induction (67). Conversely, abnormal mitochondrial Ca^{2+} overload leads to the depolarization of the mitochondrial membrane potential with a concomitant decrease in ATP synthesis, autophagy induction and eventually the opening of the permeability transition pore and cell death. Therefore, a tight control and crosstalk of Ca^{2+} dynamics in distinct intracellular organelles, combined

with cellular energy status, may explain the opposing effects of Ca^{2+} on autophagy. Under normal conditions, Ca^{2+} may play a role as a negative regulator of autophagy, but under conditions of ER or mitchondrial stress conditions, Ca^{2+} may activate autophagy to help the cell to cope with a variety of potentially damaging events related to those intracellular organelles. Future experiments aimed at detecting Ca^{2+} changes in the cytosol, the ER and mitochondria will be necessary to dissect the precise events underlying the dual regulation of autophagy by Ca^{2+} signaling.

Acidic Ca^{2+} Stores and Regulation of Autophagy

Endosomes pinching off from the plasma membrane are thought to contain high levels of Ca^{2+} due to the elevated concentrations of this ion in the extracellular fluid, whilst only having a slightly acidic pH (pH 5.9–6.3) [88]. It has been proposed that the uptake of H^+ into endosomes mediated by the proton-pumping ATPase must be balanced to maintain electroneutrality, and that this may happen by Ca^{2+} efflux via endosomal Ca^{2+} channels [89]. Even though direct measurements of luminal lysosomal Ca^{2+} concentrations are technically difficult, the average free Ca^{2+} concentration in lysosomes has been reported to be in a similar range as that of the ER [90], with an average free Ca^{2+} concentration in the range of 500 μM [73]. Whilst less acidic (pH ~ 6.6), the Golgi apparatus also deserves attention as significant intracellular Ca^{2+} store. It contains substantial levels of Ca^{2+}, Ca^{2+} release channels such as the IP_3R as well as Ca^{2+}-binding proteins [91].

Several Ca^{2+}-permeable channels are present on acidic organelles. IP_3R and RyR have been found to be located on acidic Ca^{2+} stores [92]. However, nicotinic acid adenine dinucleotide phosphate (NAADP) has been postulated to be the most powerful endogenous Ca^{2+}-mobilizing messenger known to date and the major regulator of Ca^{2+} release from endolysosomes [93–95]. Two different families of channels have been proposed to be intracellular NAADP targets, including members of the transient receptor potential (TRP) superfamily, particularly the mucolipins (TRPMLs), which are found on acidic stores such as lysosomes and endosomes [96]. Mutations in the TRPMLs channels are responsible for several lysosomal-storage disorders [96]. There are three TRPMLs, displaying differential albeit partially overlapping localization within the endo-lysosomal compartment [71]. TRPML1 has been proposed as the Ca^{2+} channel responsible for the local release of Ca^{2+} crucial for vesicular fusion reactions within the endo-lysosomal system [97]. There is evidence that TPRML1 may be an NAADP target, whilst other studies indicate that $PI(3,5)P_2$, rather than NAADP, may regulate the channel [98, 99]. Interestingly, TRPML1 has been reported to co-immunoprecipitate with the other family of Ca^{2+} channels, the two pore channels (TPCs) [100].

Two TPC isoforms are present in humans. TPC1 localizes to both endosomes and lysosomes, whereas TPC2 is predominantly lysosomal [101]. There have been several independent studies indicating that the TPCs comprise primary NAADP targets [71]. NAADP may not directly bind to TPCs, but rather indirectly through binding

to currently unidentified low-molecular weight proteins which interact with TPCs. Inhibition of the NAADP-mediated Ca^{2+} response occurs upon downregulation of the TPCs or when overexpressing TPCs with a mutation in the pore-forming region [102]. Even though controversy has recently been raised as to the ionic specificity of the TPCs, which have been suggested to behave as a sodium-selective channels activated by PI(3,5)P_2, rather than by NAADP [103, 104], the vast majority of studies indicate that the TPCs can conduct Ca^{2+}.

In analogy to ER-mitochondrial contact sites, membrane contact sites between ER and the endo-lysosomal system have been suggested to correspond to NAADP trigger zones [101]. This interaction has been proposed to be similar or maybe shared with that which mediates cholesterol exchange between the ER and late endosomes [105], and is mediated by the interaction of Rab7 and RILP on the late endosome and the VAMP-associated ER protein (VAP) through ORP1L. In this manner, release of Ca^{2+} from the endo-lysosomal compartment, triggered by NAADP, may evoke local cytosolic Ca^{2+} signals which can by amplified into global Ca^{2+} waves through Ca^{2+}-induced Ca^{2+} release (CICR) from the ER [106].

Several cellular processes are regulated by Ca^{2+} release from acidic stores. NAADP-mediated Ca^{2+} release has been related to cell differentiation and induction of neurite outgrowth [107], and NAADP has also been shown to increase the levels of autophagic markers in cultured astrocytes [104], HEK293 and PC12 cells [13]. Indeed, the rise in free cytosolic Ca^{2+}, likely caused by amplification through CICR from the ER has been shown to induce autophagy by activation of CaMKKβ-dependent activation of AMPK in a mTORC1-independent manner [13], and therefore seems to positively regulate autophagy induction (Fig. 13.3). However, NAADP has also been reported to transiently alkalinize lysosomal pH [13, 94], thereby affecting the recruitment of Rab7 to autophagosomes and disrupting autophagic flux [108]. These data indicate complex effects of NAADP on autophagy regulation, subject to further studies.

Autophagic Pathology and Calcium Deregulation in PD

Whilst the role of Ca^{2+} homeostasis for proper autophagic degradation is increasingly recognized, the precise alterations in Ca^{2+} handling and their effects on autophagic degradation in the context of various neurodegenerative disorders remain to be deciphered. Possibly the links are best understood in PD, where both autophagic failure and Ca^{2+} dysregulation have been extensively shown to play crucial role(s) in the pathomechanism(s) underlying the disease.

PD is characterized by the progressive degeneration of dopaminergic (DA) neurons in the pars compacta of the substantia nigra (SNpc), together with the presence of intraneuronal inclusions called Lewy bodies and abnormal dystrophic neuronal processes called Lewy neurites in surviving neurons [109]. While the exact pathological mechanisms underlying PD remain largely unclear, with most cases being

sporadic or idiopathic, having no apparent familial heritage nor relation to a defined environmental cause, several familial mutations in a variety of genes, with either autosomal-recessive (parkin, PINK1, DJ-1) or autosomal-dominant (α-synuclein, LRRK2) inheritance have been reported for approximately 10 % of PD cases.

Autophagic Failure in PD

Compelling evidence suggests a deficit in autophagic clearance in PD. Increased number of autophagic structures have been found in PD patients, animal and cellular models of PD [110]. Another piece of evidence linking autophagy to PD comes from the reported effects of α-synuclein overexpression in blocking autophagy [12]. In addition, α-synuclein and UCHL-1 mutants have been reported to block CMA [5]. Conversely, pharmacological activation of autophagy has been shown to alleviate the toxicity associated with mutant α-synuclein *in vitro* and *in vivo* [34]. Other PD-related proteins have been related to altered autophagic flux as well. For example, the absence of DJ-1, PINK1 or Parkin has been shown to impair basal autophagic flux and cause accumulation of autophagic markers, mitochondrial fragmentation and depolarization in human DA cells [111, 112]. LRRK2, related to both familiar and sporadic PD has been closely related with autophagy as well.

Neuronal Ca^{2+} Signaling and PD

Currently one of the best working models to explain the selective degeneration of DA neurons in PD relates to perturbations in Ca^{2+} homeostasis [19, 113]. Adult SNpc DA neurons are autonomously active, generating action potentials in the absence of synaptic input, as do many others neurons. However, whilst most neurons use exclusively monovalent cation channels to drive this pacemaking activity, SNpc DA neurons also employ L-type Ca^{2+} channels that allow Ca^{2+} to enter the cytoplasm [114]. Since the spatiotemporal pattern of Ca^{2+} signaling is crucial for the specificity of cellular responses, Ca^{2+} must be under a tight homeostatic control that requires energy. Therefore, Ca^{2+} must be extruded by ATP-dependent processes, either pumped out or rapidly sequestered by intracellular organelles. As previously explained, both processes are mediated by ATP-dependent pumps and exchangers [115, 116]. This additional energy requirement and resultant energy production via mitochondria can generate oxidative stress and production of reactive oxygen species. In addition, the increase in Ca^{2+} uptake by mitochondria from the ER through IP_3R may lead to an increase in the basal generation of oxidant stress in the mitochondria of SNc DA neurons, increasing SNc DA neuron vulnerability [114, 117]. Interestingly, Ca^{2+} channel blockers which have demonstrated good blood-brain barrier permeability and a long record of safe use in humans have been reported to have positive results in rodent PD models [114, 118].

LRRK2: A Possible Link between Altered Ca^{2+} Homeostasis and Autophagy

Autosomal-dominant mutations in LRRK2 are the most common genetic cause of late-onset PD [119]. Approximately 5–8 % of European individuals with a first-degree relative with PD carry mutations in LRRK2. Importantly, variations in LRRK2 increase PD risk, indicating that LRRK2 is an important player for both genetic and sporadic forms of the disease [120].

There are indications that LRRK2 plays an important role in autophagy [110]. PD-linked mutations in LRRK2 seem to cause an impairment in autophagic degradation with a concomitant accumulation of undegraded material, lysosomal-like structures and lipid droplets in cell as well as animal model systems [8, 13, 15, 121–124], even though positive regulatory effects on autophagy have been described as well [125] which warrant further investigations. Deficiencies in Ca^{2+} handling in the presence of autosomal-dominant mutations in LRRK2 have also been reported, leading to mitochondrial depolarization and altered mitophagy [126, 127].

Our recent studies indicate that mutant LRRK2 causes release of Ca^{2+} from acidic stores which activates the CaMKKβ/AMPK pathway and causes autophagy induction, whilst simultaneously partially alkalinizing endolysosomal stores, provoking a possible defect in endolysosomal homeostasis and a defect in autophagic clearance [13]. These events seemed to be mediated by NAADP, as they can be mimicked by lysosomal Ca^{2+} mobilization through NAADP and reverted by an NAADP antagonist or by overexpression of dominant-negative TPC2 receptor constructs. NAADP-mediated release of Ca^{2+} from the endolysosomal compartment can cause a partial alkalinization of acidic stores and induce lipid accumulation, which may occur secondarily to Ca^{2+} release [94]. The deregulation of intraluminal Ca^{2+} levels may then be responsible for the observed changes in lysosomal morphology, distribution and degradative capacity, as described for mutant LRRK2-expressing cells [13, 15, 121, 124]. Indeed, deregulation of endolysosomal Ca^{2+} homeostasis has been proposed as a primary cause of the lysosomal-storage disorder Niemann Pick C1, where the reduced lysosomal Ca^{2+} results in a block in endocytic trafficking to lysosomes [73]. Our recent work further indicates that pathogenic LRRK2 regulates the activity of Rab7, a master regulator of late endocytic membrane trafficking involved in endolysosomal and autophagosome-endolysosomal fusion events [128]. Given the recently reported links between Rab7 and TPC2 [108, 129], further epistasis-type studies are warranted to determine whether LRRK2 regulates Rab7 to modulate NAADP-mediated Ca^{2+} release from endolysosomal stores, or whether LRRK2 regulates NAADP-mediated Ca^{2+} release which then may modulate Rab7 activity for downstream endolysosomal fusion events (Fig. 13.4).

In either case, there is convincing evidence to support the hypothesis that deficiencies in Ca^{2+} homeostasis and protein degradation are crucial elements in the neurodegenerative process underlying several age-dependent neurodegenerative diseases. Whilst Ca^{2+} has been consistently showed to regulate autophagy, the positive or negative link between these two mechanisms in the context of

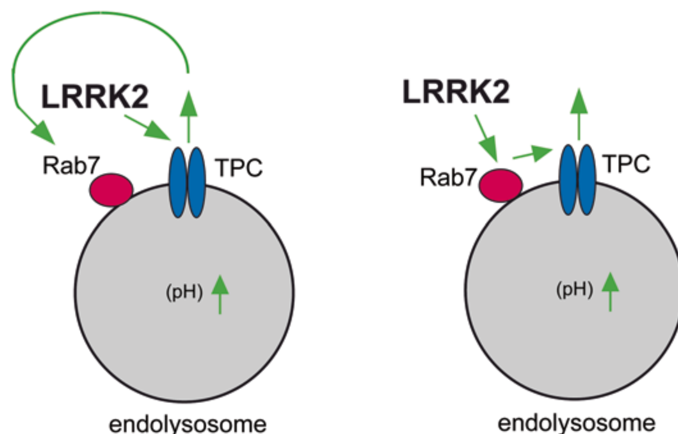

Fig. 13.4 Possible models for the link between pathogenic LRRK2, TPC channels and Rab7. *Left*: LRRK2 may regulate TPC channels, and the resultant alterations in endolysosomal Ca^{2+} exit may regulate Rab7. *Right*: LRRK2 may regulate Rab7 activity, which in turn may modulate TPC channels to alter Ca^{2+} release from endolysosomal stores. For further details see text

neurodegeneration and cellular status remains to be further elucidated, which may help to define novel therapeutic targets.

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References

1. Takalo M, et al. Protein aggregation and degradation mechanisms in neurodegenerative diseases. Am J Neurodegener Dis. 2013;2(1):1–14.
2. Cuervo AM, Wong ES, Martinez-Vicente M. Protein degradation, aggregation, and misfolding. Mov Disord. 2010;25(Suppl 1):S49–54.
3. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132(1):27–42.
4. Lee JA. Autophagy in neurodegeneration: two sides of the same coin. BMB Rep. 2009;42(6):324–30.
5. Cuervo AM, et al. Impaired degradation of mutant a-synuclein by chaperone-mediated autophagy. Science;2004;305:1292–5.
6. Clark IE, et al. *Drosophila pink1* is required for mitochondrial function and interacts genetically with parkin. Nature. 2006;441:1162–6.
7. Shibata M, et al. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J Biol Chem. 2006;281:14474–85.
8. Alegre-Abarrategui J, et al. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Hum Mol Genet. 2009;18(21):4022–34.
9. Boland B, et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. J Neurosci. 2008;28:6926–37.

10. Martinez-Vicente M, et al. Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. Nat Neurosci. 2010;13:567–76.
11. Lee JY, et al. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. J Cell Biol. 2010;189:671–9.
12. Winslow AR, et al. -Synuclein impairs macroautophagy: implications for Parkinson's disease. J Cell Biol. 2010;190:1023–37.
13. Gómez-Suaga P, et al. Leucine-rich repeat kinase 2 regulates autophagy through a calcium-dependent pathway involving NAADP. Hum Mol Genet. 2012;21(3):511–25.
14. Choubey V, et al. Mutant A53T a-synuclein induces neuronal death by increasing mitochondrial autophagy. J Biol Chem. 2011;286:10814–24.
15. Orenstein SJ, et al. Interplay of LRRK2 with chaperone-mediated autophagy. Nat. Neurosci. 2013;16:394–406.
16. Zavodszky E, et al. Mutation in VPS35 associated with Parkinson's disease impairs WASH complex association and inhibits autophagy. Nat Commun. 2014;5:3828.
17. Hochfeld WE, Lee S, Rubinsztein DC. Therapeutic induction of autophagy to modulate neurodegenerative disease progression. Acta Pharmacol Sin. 2013;34(5):600–4.
18. Verkhratsky A. Calcium and cell death. Subcell Biochem. 2007;45:465–80.
19. Surmeier DJ, et al. The role of calcium and mitochondrial oxidant stress in the loss of substantia nigra pars compacta dopaminergic neurons in Parkinson's disease. Neuroscience. 2011;198:221–31.
20. Berridge MJ. Calcium hypothesis of Alzheimer's disease. Pflugers Arch. 2010;459:441–9.
21. Giacomello M, et al. Neuronal Ca(2+) dyshomeostasis in Huntington disease. Prion. 2013;7(1):76–84.
22. Grosskreutz J, Van Den Bosch L, Keller BU. Calcium dysregulation in amyotrophic lateral sclerosis. Cell Calcium. 2010;47(2):165–74.
23. Anekonda TS, Quinn JF. Calcium channel blocking as a therapeutic strategy for Alzheimer's disease: the case for isradipine. Biochim Biophys Acta. 2011;1812(12):1584–90.
24. Schapira AH, et al. Slowing of neurodegeneration in Parkinson's disease and Huntington's disease: future therapeutic perspectives. Lancet. 2014;384(9942):545–55.
25. Bogaert E, d'Ydewalle C, Van Den Bosch L. Amyotrophic lateral sclerosis and excitotoxicity: from pathological mechanism to therapeutic target. CNS Neurol Disord Drug Targets. 2010;9(3):297–304.
26. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol. 2010;12(9):814–22.
27. Hara T, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006;441(7095):885–9.
28. Komatsu M, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 2006;441(7095):880–4.
29. Singh R, Cuervo AM. Autophagy in the cellular energetic balance. Cell Metab. 2011;13(5):495–504.
30. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ. 2005;12(Suppl 2):1542–52.
31. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. Cell Mol Life Sci. 2012;69(7):1125–36.
32. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. Nat Cell Biol. 2013;15(7):713–20.
33. Lipinski MM, et al. A genome-wide siRNA screen reveals multiple mTORC1 independent signaling pathways regulating autophagy under normal nutritional conditions. Dev Cell. 2010;18(6):1041–52.
34. Ravikumar B, et al. Regulation of mammalian autophagy in physiology and pathophysiology. Physiol Rev. 2010;90(4):1383–435.
35. Jewell JL, Russell RC, Guan KL. Amino acid signaling upstream of mTOR. Nat Rev Mol Cell Biol. 2013;14(3):133–9.

36. Groenewoud MJ, Zwartkruis FJ. Rheb and Rags come together at the lysosome to activate mTORC1.Biochem Soc Trans. 2013;41(4):951–5.
37. Jung CH, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009;20(7):1992–2003.
38. Woods A, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol. 2003;13(22):2004–8.
39. Hawley SA, et al. 5'-AMP activates the AMP-activated protein kinase cascade, and Ca2+/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. J Biol Chem. 1995;270(45):27186–91.
40. Loffler AS, et al. Ulk1-mediated phosphorylation of AMPK constitutes a negative regulatory feedback loop. Autophagy. 2011;7(7):696–706.
41. Egan D, et al. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy. 2011;7(6):643–4.
42. Mack HI, et al. AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization. Autophagy. 2012;8(8):1197–214.
43. Chan EY. mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. Sci Signal. 2009;2(84):e51.
44. Mizushima N. Autophagy: process and function. Genes Dev. 2007;21(22):2861–73.
45. Hayashi-Nishino M, et al. A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol. 2009;11(12):1433–7.
46. Hailey DW, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;141(4):656–67.
47. Hamasaki M, et al. Autophagosomes form at ER-mitochondria contact sites. Nature. 2013;495(7441):389–93.
48. Ravikumar B, et al. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol. 2010;12(8):747–57.
49. Puri C, et al. Diverse autophagosome membrane sources coalesce in recycling endosomes. Cell. 2013;154(6):1285–99.
50. Guo Y, et al. AP1 is essential for generation of autophagosomes from the trans-Golgi network. J Cell Sci. 2012;125(Pt 7):1706–15.
51. Ohashi Y, Munro S. Membrane delivery to the yeast autophagosome from the Golgi-endosomal system. Mol Biol Cell. 2010;21(22):3998–4008.
52. Backer JM. The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J. 2008;410(1):1–17.
53. Di Bartolomeo S, et al. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. J Cell Biol. 2010;191(1):155–68.
54. Russell RC, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat Cell Biol. 2013;15:741–50.
55. Gaullier JM, et al. FYVE fingers bind PtdIns(3)P. Nature. 1998;394(6692):432–3.
56. Mizushima N, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol. 2001;152(4):657–68.
57. Eskelinen EL. New insights into the mechanisms of macroautophagy in mammalian cells. Int Rev Cell Mol Biol. 2008;266:207–47.
58. Jahreiss L, Menzies FM, Rubinsztein DC. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. Traffic. 2008;9(4):574–87.
59. Yu L, et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature. 2010;465(7300):942–6.
60. Pryor PR, et al. The role of intraorganellar Ca(2+) in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles. J Cell Biol. 2000;149(5):1053–62.
61. Engedal N, et al. Modulation of intracellular calcium homeostasis blocks autophagosome formation. Autophagy. 2013;9(10):1475–90.

62. Choi S, Kim HJ. The Ca2+ channel TRPML3 specifically interacts with the mammalian ATG8 homologue GATE16 to regulate autophagy. Biochem Biophys Res Commun. 2014;443(1):56–61.
63. Hoyer-Hansen M, et al. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell. 2007;25(2):193–205.
64. Ghislat G, et al. Withdrawal of essential amino acids increases autophagy by a pathway involving Ca2+/calmodulin-dependent kinase kinase-beta (CaMKK-beta). J Biol Chem. 2012;287(46):38625–36.
65. Sarkar S, et al. Lithium induces autophagy by inhibiting inositol monophosphatase. J Cell Biol. 2005;170(7):1101–11.
66. Williams A, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol. 2008;4(5):295–305.
67. Cardenas C, et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca^{2+} transfer to mitochondria. Cell. 2010;142(2):270–83.
68. Ganley IG, et al. Distinct autophagosomal-lysosomal fusion mechanism revealed by thapsigargin-induced autophagy arrest. Mol Cell. 2011;42(6):731–43.
69. Decuypere JP, Bultynck G, Parys JB, A dual role for Ca^{2+} in autophagy regulation. Cell Calcium. 2011;50:242–50.
70. Takenaka H, et al. Calcium fluxes across the membrane of sarcoplasmic reticulum vesicles. J Biol Chem. 1982;257:12649–56.
71. Patel S, Docampo R. Acidic calcium stores open for business: expanding the potential for intracellular Ca^{2+} signaling. Trends Cell Biol. 2010;20(5):277–86.
72. Luzio JP, Gray SR, Bright NA. Endosome-lysosome fusion. Biochem Soc Trans. 2010;38(6):1413–6.
73. Lloyd-Evans E, et al. Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. Nat Med. 2008;14(11):1247–55.
74. Hay JC. Calcium: a fundamental regulator of intracellular membrane fusion? EMBO Rep. 2007;8(3):236–40.
75. Brini M, et al. Calcium pumps: why so many? Compr Physiol. 2012;2(2):1045–60.
76. Missiaen L, et al. Ca^{2+} release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca^{2+} in permeabilized cells. Nature. 1992;357:599–602.
77. Mekahli D, et al. Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harb Perspect Biol. 2011;3(6):a004317.
78. Kiviluoto S, et al. Regulation of inositol 1,4,5-trisphosphate receptors during endoplasmic reticulum stress. Biochim Biophys Acta. 2013;1833(7):1612–24.
79. Lanner JT, et al. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Perspect Biol. 2010;2(11):a003996.
80. Moldoveanu T. A Ca^{2+} Switch aligns the active site of calpain. Cell. 2002;108(5):649–60.
81. Bano D, et al. Cleavage of the plasma membrane Na^{+}/Ca^{2+} exchanger in excitotoxicity. Cell. 2005;120(2):275–85.
82. Høyer-Hansen M, Jäättelä M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell Death Differ. 2007;14(9):1576–82.
83. Ogata M, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006;26:9220–31.
84. Bialik S, Kimchi A. Lethal weapons: DAP-kinase, autophagy and cell death: DAP-kinase regulates autophagy. Curr Opin Cell Biol. 2010;22(2):199–205.
85. Sakaki K, Wu J, Kaufman RJ. Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. J Biol Chem. 2008;283(22):15370–80. doi:10.1074/jbc.M710209200.
86. Marchi S, Patergnani S, Pinton P. The endoplasmic reticulum-mitochondria connection: one touch, multiple functions. Biochim Biophys Acta. 2014;1837(4):461–9.
87. Hoppins S. The regulation of mitochondrial dynamics. Curr Opin Cell Biol. 2014;29:46–52.
88. Yamashiro DJ, Maxfield FR. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. J Cell Biol. 1987;105(6 Pt 1):2723–33.

89. Gerasimenko JV, et al. Calcium uptake via endocytosis with rapid release from acidifying endosomes. Curr Biol. 1998;8(24):1335–8.
90. Miyawaki A, et al. Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. Nature. 1997;388(6645):882–7.
91. Pinton P, Pozzan T, Rizzuto R. The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J. 1998;17(18):5298–308.
92. Gerasimenko JV, et al. Pancreatic protease activation by alcohol metabolite depends on Ca2+ release via acid store IP3 receptors. Proc Natl Acad Sci USA. 2009;106(26):10758–63.
93. Patel S, Churchill GC, Galione A. Coordination of Ca2+ signaling by NAADP. Trends Biochem Sci. 2001;26(8):482–9.
94. Morgan AJ, Galione A. NAADP induces pH changes in the lumen of acidic Ca2+ stores. Biochem J. 2007;402(2):301–10.
95. Calcraft PJ, et al. NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature. 2009;459(7246):596–600.
96. Cheng X, et al. Mucolipins: intracellular TRPML1-3 channels. FEBS Lett. 2010;584(10):2013–21.
97. Zhang F, et al. TRP-ML1 functions as a lysosomal NAADP-sensitive Ca2+ release channel in coronary arterial myocytes. J Cell Mol Med. 2009;13(9B):3174–85.
98. Morgan AJ, et al. Molecular mechanisms of endolysosomal Ca2+ signaling in health and disease. Biochem J. 2011;439(3):349–74.
99. Feng X, et al. Drosophila TRPML Forms PI(3,5)P2-activated cation channels in both endolysosomes and plasma membrane. J Biol Chem. 2014;289(7):4262–72.
100. Yamaguchi S, et al. Transient receptor potential mucolipin 1 (TRPML1) and two-pore channels are functionally independent organellar ion channels. J Biol Chem. 2011;286(26):22934–42.
101. Patel S, Brailoiu E. Triggering of Ca^{2+} signals by NAADP-gated two-pore channels: a role for membrane contact sites? Biochem Soc Trans. 2012;40(1):153–7.
102. Brailoiu E, et al. An NAADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying Ca^{2+} signals. J Biol Chem. 2010;285(49):38511–6.
103. Wang X, et al. TPC proteins are phosphoinositide-activated sodium-selective ion channels in endosomes and lysosomes. Cell. 2012;151(2):372–83.
104. Jha A, et al. Convergent regulation of the lysosomal two-pore channel-2 by Mg^{2+}, NAADP, PI(3,5)P2 and multiple protein kinases. EMBO J. 2014;33(5):501–11.
105. Rocha N, et al. Cholesterol sensor ORP1L contacts the ER protein VAP to control Raby-RILP-p150 Glued and late endosome positioning. J Cell Biol. 2009;185:1209–25.
106. Zhu MX, et al. Calcium signaling via two-pore channels: local or global, that is the question. Am J Physiol Cell Physiol. 2010;298(3):C430–41.
107. Pereira, GJ, et al. Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. J Biol Chem. 2011;286(32):27875–81.
108. Lu Y, et al. Two pore channel 2 (TPC2) inhibits autophagosomal-lysosomal fusion by alkalinizing lysosomal pH. J Biol Chem. 2013;288(33):24247–63.
109. Goedert M, et al. Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy as alpha-synucleinopathies. Methods Mol Med. 2001;62:33–59.
110. Gómez-Suaga P, et al. A link between autophagy and the pathophysiology of LRRK2 in Parkinson's disease. Parkinsons Dis. 2012;2012:324521.
111. Krebiehl G, et al. Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1. PLoS One. 2010;5(2):e9367.
112. Thomas KJ, et al. DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy. Hum Mol Genet. 2011;20(1):40–50.
113. Greenamyre JT, Hastings TG. Biomedicine. Parkinson's—divergent causes, convergent mechanisms. Science. 2004;304(5674):1120–2.
114. Chan CS, Gertler TS, Surmeier DJ. Calcium homeostasis, selective vulnerability and Parkinson's disease. Trends Neurosci. 2009;32(5):249–56.

115. Grace AA, Bunney BS. Intracellular and extracellular electrophysiology of nigral dopaminergic neurons—2. Action potential generating mechanisms and morphological correlates. Neuroscience. 1983;10(2):317–31.
116. Chan CS, et al., 'Rejuvenation' protects neurons in mouse models of Parkinson's disease. Nature. 2007;447(7148):1081–6.
117. Zhang J, et al. Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. Am J Pathol. 1999;154(5):1423–9.
118. Ilijic E, Guzman JN, Surmeier, DJ. The L-type channel antagonist isradipine is neuroprotective in a mouse model of Parkinson's disease. Neurobiol Dis. 2011;43(2):364–71.
119. Paisan-Ruiz C, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron. 2004;44(4):595–600.
120. Hardy J. Genetic analysis of pathways to Parkinson disease. Neuron. 2010;68(2):201–6.
121. MacLeod D, et al. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. Neuron. 2006;52(4):587–93.
122. Tong Y, et al. Loss of leucine-rich repeat kinase 2 causes age-dependent bi-phasic alterations of the autophagy pathway. Mol Neurodegener. 2012;7:2.
123. Ramonet D, et al. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. PLoS One. 2011;6(4):e18568.
124. Tong Y, et al. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. Proc Natl Acad Sci USA. 2010;107(21):9879–84.
125. Bravo-San Pedro, JM, et al. The LRRK2 G2019S mutant exacerbates basal autophagy through activation of the MEK/ERK pathway. Cell Mol Life Sci. 2013;70:121–36.
126. Papkovskaia TD, et al. G2019S leucine-rich repeat kinase 2 causes uncoupling protein-mediated mitochondrial depolarization. Hum Mol Genet. 2012;21(19):4201–13.
127. Cherra SJ III, et al. Mutant LRRK2 elicits calcium imbalance and depletion of dendritic mitochondria in neurons. Am J Pathol. 2013;182(2):474–84.
128. Gómez-Suaga P, et al. LRRK2 delays degradative receptor trafficking by impeding late endosomal budding through decreasing Rab7 activity. Hum Mol Genet. 2014;23(25):6779–96.
129. Lin-Moshier Y, et al. The two-pore channel (TPC) interactome unmasks isoform-specific roles for TPCs in endolysosomal morphology and cell pigmentation. Proc Natl Acad Sci USA. 2014;111:13087–92.

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