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Autophagy, the “Master” Regulator of Cellular Quality Control: What Happens when Autophagy Fails?

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1. Introduction

Autophagy an evolutionarily conserved process, is activated in response to nutrient deprivation, as well as to endogenous and exogenous stresses. It is currently known that the basic mechanism of autophagy has been well conserved during evolution since diverse organisms, including yeast, flies, and mammals, all carry a similar set of autophagy-related genes (ATGs), although there are some significant differences between yeast and higher eukaryotes. As a result, autophagy is a highly regulated process known to contribute to cellular cleaning through the removal of intracellular components in lysosomes showing therefore an important role in cellular quality control. Autophagy ensures that proteins damaged or incorrectly synthesized are removed from the cells by degradation, preventing thus the devastating cellular consequences associated with accumulation of malfunctioning proteins inside cells. Moreover autophagy works as a recycling system where it mediates the breakdown of proteins that are no longer needed into essential components (aminoacids, free fatty acids, sugars), which can then be used in the synthesis of new proteins. This has extreme importance in conditions of nutritional stress or starvation. At optimal physiological conditions in the absence of stressors, basal level of autophagy assures maintenance of cell homeostasis through regular turnover of proteins, lipids, and organelles. Therefore autophagy acts a cautious controller of cellular homeostasis. In addition, it also has an important role in cellular defence as in compromised situations it contributes to the proteolytic breakdown of components of invading pathogens and other types of biological cell aggressors. Autophagy may also modulate synaptic plasticity, which involves structural remodelling of nerve terminals and the trafficking and degradation of receptors and other synaptic proteins [1]. Finally, autophagy is also a key player in cellular adaptation since is able of changing very rapidly the rate of a

particular protein's degradation allowing fast modulation of the proteome in response to stress. The lysosomal system and specifically the autophagic pathway is the principal mechanism for degrading longer-lived proteins and is the only system in cells capable of degrading organelles and protein aggregates/inclusions. Similar to what happens with the proteasome system, protein aggregates and certain organelles have been shown to be tagged with ubiquitination for selective removal by autophagy [2]. An adaptor molecule with an ubiquitin-binding domain engages the ubiquitinated structure and couples it to the pre-autophagosomal isolation membrane for subsequent sequestration. The exact types of ubiquitin motifs recognized by the proteasome and autophagy may differ and the degree to which ubiquitination drives autophagic protein turn-over relative to that by proteasomes is still unclear.

There are three basic forms of autophagy, namely, macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which primarily differ in the way in which cytosolic components are delivered to lysosomes [3]. Moreover, the different types of autophagy share a common endpoint, the lysosome, but differ in the substrates targeted, their regulation, and the conditions in which each of them is preferentially activated.

In macroautophagy and microautophagy there is the sequestration of cytoplasmic components into vesicular compartments. In the case of macroautophagy, vesicles are formed *de novo* from a limiting membrane of non-lysosomal origin that closes and forms a double membrane organelle called autophagosome [4]. Because the autophagosome lacks any enzymes, the trapped contents are not degraded until the autophagosome fuses with a lysosome, which provides all the hydrolases required for degradation of cargo. Autophagosomes are then transported along microtubules to the perinuclear region of the cell (where lysosomes are clustered) to enhance the probability of autophagosome-lysosome fusion to form autophagolysosomes. Lysosomes are single membrane organelles dedicated to degradation of both intracellular and extracellular components. Lysosomal membranes possess vacuolar H⁺ ATPases which are proton pumps that acidify the autophagolysosome contents. This acidification is essential for the activation of lysosomal enzymes (including proteases, lipases, glycosidases, and nucleases), which are responsible for proteolysis of the components in the autophagolysosome and confer this organelle its high degradative capacity [5]. Although autophagy was initially considered to be a nonselective process, cargo-specific autophagy (the selective degradation of certain aggregated proteins and organelles) is now recognized to exist [6]. Numerous different types of cargo-specific autophagic processes have been described, like for instance mitophagy where damaged mitochondria are sequestered and degraded [7]. Macroautophagy occurs constitutively in cells and is markedly induced under stress conditions, such as starvation and has two major purposes: as a source to generate essential macromolecules and energy in conditions of nutritional scarcity, or as a mechanism for the removal of altered intracellular components [8].

In the case of microautophagy the engulfment of cargo occurs through invagination of the lysosomal membrane itself, to form vesicles that invaginate towards the lysosomal lumen [9]. Cytosolic regions are sequestered directly by the lysosome through invaginations or tubulations that "pinch off" from the membrane into the lysosomal lumen where they are rapidly

degraded. Microautophagy participates in the continuous, slow "basal" turnover of cellular components in normal cellular conditions [10]. Furthermore microautophagy has been shown to be responsible for the selective removal of organelles when they are no longer needed [11]. The absence of mammalian homologs for the microautophagy yeast genes has made it difficult in gaining a better understanding of the pathophysiology of this process.

The third type of mammalian autophagy CMA distinguishes from the others because of its distinctive feature of selectivity. Unlike the other forms of autophagy, in which portions of the cytoplasm are typically engulfed, proteins degraded by CMA are identified and transported one-by-one by a cytosolic chaperone that delivers them to the surface of the lysosomes. First there is individual recognition of single proteins in the citosol, then the substrate proteins unfold and cross the lysosomal membrane. Only soluble proteins containing in their aminoacid sequence a pentapeptide motif related to KFERQ, are recognized by heat shock cognate protein of 70 kDa, hsc70, which mediates their delivery to the lysosomes for direct translocation across the lysosomal membrane [3, 12]. These pentapeptide motifs are recognized specifically by the cytosolic protein hsc70, the constitutively expressed member of the hsp70 family of cytosolic chaperones. hsc70 not only targets the CMA substract to the lysosomal membrane where it can interact with the CMA receptor but also facilitates substrate folding [13]. A second chaperone that also localizes on both sides of the lysosomal membrane is the heat shock protein of 90 kDa, hsp90. hsp90 is proposed to participate in substrate protein unfolding [14], and contributes to the stabilization of essential components of the translocation complex as they organize into a multimeric structure [15]. Additionally, the co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) has been shown to mediate lysosomal degradation of proteins such as alpha-synuclein, a known CMA substrate [16], however, it is still undergoing investigation whether CHIP-mediated degradation occurs via CMA or via other forms of autophagy. The lysosome-associated membrane protein type 2A (LAMP-2A) acts as a receptor for substrates of this pathway [17]. This protein is one of the three splice variants encoded by the *lamp2* gene that share identical luminal regions and different transmembrane and cytosolic tails [18]. Once the cytosolic proteins destined for CMA degradation bind to the cytosolic tail of the single-span membrane protein LAMP-2A, they promote its assembly into a high molecular weight complex of about 700 kDa at the lysosomal membrane required for translocation of the CMA substrates into the lysosomal lumen [15]. Multimerization is a required step for substrate translocation. However, the translocation complex is not stable at the lysosomal membrane and, once the substrate reaches the lysosomal lumen, LAMP-2A dissociates into monomers. hsc70 mediates the disassembly generating monomeric forms of LAMP-2A that are required for substrate binding in order to sustain cycles of binding and uptake. Other proteins have been shown to modulate the stability of the CMA translocation complex, such as, glial fibrillary acidic protein (GFAP) and elongation factor 1 a (EF1a). One of the crucial functions of CMA is protein quality control via the selective degradation of damaged or altered proteins. Moreover proteins associated with several neurodegeneratives disorders have KFERQ-like sequences such as, alpha-synuclein, parkin, UCHL1 and Pink1, amyloid precursor protein (APP) and tau and huntingtin. These proteins can be degraded by CMA only when they are in soluble forms; once the insoluble inclusions are formed, they can

only be degraded via other proteolytic pathways [19]. CMA is constitutively active in many cell types and similar to macroautophagy, CMA is maximally activated under stress conditions like nutritional stress or starvation and cellular stresses leading to protein damage [3]. Indeed, upon stressors that cause protein damage, such as oxidative stress, CMA is upregulated [20]. During starvation, macroautophagy is first activated, and if starvation persists, cells activate CMA, which selectively targets non-essential proteins for degradation to obtain the aminoacids required for the synthesis of essential proteins showing that macroautophagy and CMA act in a synchronized manner [21]. There are two lysosomal components that limit CMA activity: LAMP-2A and lys-hsc70. It is known that changes in the number of LAMP-2A molecules, as well as, in the membrane content of LAMP-2A, rapidly upregulate or downregulate CMA activity [17]. Levels of lys-hsc70 increase gradually with the increase in CMA activity, although the mechanisms modulating this increase are still poorly understood [14]. Furthermore, the activity of this autophagic pathway is also directly modulated by changes in other autophagic and proteolytic systems inside the cell. For examples, cells in culture respond to CMA blockage by upregulating macroautophagy [21]. Likewise, blockage of macroautophagy results in constitutive activation of CMA [22].

The identification of ATGs in yeast was a major breakthrough in the study of autophagy [23]. Until now more than 35 ATG genes have been found in yeast and many of them have orthologs in mammals to control the dynamic processes and different stages of autophagy. Indeed, autophagic activities are mediated by a complex molecular machinery including more than 35 ATG-related proteins and 50 lysosomal hydrolases. This dynamic macroautophagic process includes initiation, nucleation, elongation, maturation and degradation. Initiation involves formation of the phagophore, a cup-shaped membrane structure in the cytoplasm. Nucleation involves the recruitment and assembly of several proteins including Beclin-1 and Vps34, among others [24]. Vps34 has a phosphoinositide 3-kinase (PI3K) activity and produces phosphatidylinositol 3-phosphate, which recruits molecular components involved in subsequent vesicle elongation. Subsequently elongation is a critical step in forming the complete autophagosomes and is controlled by two ubiquitin-like conjugation systems. The first involves the Atg7 E1-like enzyme that mediates the covalent linkage of Atg12 to Atg5 followed by formation of a complex of Atg12-Atg5 with Atg16L, which dimerizes and associates with the phagophore membrane. In the second one LC3 has to be cleaved by Atg4 to generate the cytosolic LC3 (that arises from microtubular-associated protein-light chain) that can subsequently be conjugated to a phosphatidylethanolamine tag by Atg7 and the E2-like enzyme Atg3. The resulting lipidated form of LC3, LC3-II, is attached to both sides of the autophagosome membrane. LC3-II is clearly necessary for autophagosome formation and is commonly used as a marker for autophagy. Finally, maturation and degradation steps, involves fusion with endosomes-lysosomes to form autophagic vacuoles (AVs) and requires a number of lysosomal proteins necessary for the degradation of the luminal contents [25]. The transmembrane Atg9 protein also contributes to autophagic vesicle nucleation and elongation, possibly by mediating the transport of lipid molecules. At this stage, LC3 on the outer autophagosome membrane is removed by Atg4 for reuse, while inner membrane LC3-II is digested along with the cargo. The rate-limiting step in the autophagic process is autophagosome formation.

Autophagy can be regulated by mTOR-dependent signaling pathways and mTOR-independent signaling pathways. The serine–threonine kinase mTOR is a master negative regulator of autophagy that acts by blocking the activity of the ULK1/Atg1 complex [26]. mTOR is inhibited when nutrients are scarce, when growth factor signaling is reduced, and under ATP depletion. When mTOR is inhibited and the repressive effect of mTOR on autophagosome formation disappears an increase in autophagosome biogenesis occurs [27]. In pathways that act independently of mTOR, when in nutrient-rich conditions, Beclin-1 is bound to the antiapoptotic protein Bcl-2. Under nutrient starvation, the stress-activated enzyme Jun N-terminal kinase 1 (JNK1) phosphorylates Bcl-2, which induces its dissociation from Beclin-1 [28]. Beclin can then interact with other members of the autophagic machinery and stimulate the induction of autophagy [29-30]. One of the mTOR-independent pathways can be mediated by the IP3 pathway and the Ca²⁺–calpain–G-stimulatory protein alpha pathway [31]. Generation of IP3 induces the release of Ca²⁺ from stores in the endoplasmic reticulum and high levels of cytosolic Ca²⁺ inhibit autophagy by activating calpains. After cleavage by calpains, the Ca²⁺–calpain–G-stimulatory protein alpha pathway becomes activated which, in turn, causes the production of more inhibitory cAMP [31]. On the other hand, decreased levels of IP3 signaling leads to reduced Ca²⁺ release from the endoplasmic reticulum and lower rates of mitochondrial Ca²⁺ uptake, causing a reduction in mitochondrial activity and ATP depletion, which results in 5'-AMP-activated protein kinase (AMPK) activation [32]. Activated AMPK directly phosphorylates ULK1, indirectly inactivating mTOR complex which leads to the induction of autophagy.

2. Autophagic failure at cellular level

Two of the main aggravating factors contributing to failure of autophagic pathways are believed to be oxidative stress and aging. Aging can lead to reductions in autophagosome formation and autophagosome-lysosome fusion [33]. Moreover leads to lysosomal alterations such as increased lysosome volume, decreased lysosomal stability, altered activity of hydrolases and intralysosomal accumulation of indigestible material such as lipofuscin [34]. These alterations are consistent with a decrease in autophagy, more specifically macroautophagy. Consequently, there is a reduction in the turnover of intracellular components as well as a reduction in the ability of cells to adapt to changes in the extracellular environment [35]. Ultimately this contributes to the intracellular accumulation of misfolded proteins in aged organisms [36]. Moreover, also a decline in CMA activity has been described in almost all cell types and tissues. This decrease is primarily due to a reduction in the levels of LAMP-2A at the lysosomal membrane [37]. In addition, the stability of LAMP-2A at the lysosomal membrane is markedly reduced with age, resulting in a decrease in the net content of this protein in lysosomes [38].

On the other hand, oxidative stress overloads the macroautophagic system, and oxidized proteins and damaged organelles engulfed by autophagosomes can become a source of ROS inside either autophagosomes or lysosomes. Subsequently ROS can react and damage lysosomal hydrolases and/or other components required for the lysosome/autophagosome fusion, resulting in the accumulation of undegraded products inside these cellular compart-

ments, such as lipofuscin. Defects in basal autophagy could lead to altered neuronal homeostasis and degeneration through impaired utilization of nutrients or an imbalance of vesicular biogenesis and turnover. Alternatively, neuronal dysfunction might be a more direct result of failed protein degradation with resultant accumulation of ubiquitinated protein aggregates.

2.1. Lipofuscin accumulation

The most abundant pigment in the human brain is lipofuscin, a protein- and lipid-based pigment with broad distribution [39]. Lipofuscin is commonly considered to be a ubiquitous pigment within the brain, being recognized as a hallmark of aging [40]. Lipofuscin is a chemically and morphologically polymorphous and pigmented waste material that is formed exclusively in lysosomes where it can accumulate as well as in other lysosome-related vesicles due to incomplete digestion of engulfed components and subsequent intra-lysosomal oxidation [41]. Ivy and co-workers suggested that lipofuscin accumulation might result from age-related decrease in the activity of lysosomal enzymes, especially cysteine proteases, such as cathepsins B, H, and L [42]. However where some studies showed a decrease in lysosomal cysteine protease activity others did not because lipofuscin starts to accumulate immediately after birth and continues to do so more or less linearly through the life span [41]. Lipofuscin formation appears to depend on the rate of oxidative damage to proteins, the functionality of mitochondrial repair systems, the proteasomal system, and the functionality and effectiveness of the lysosomes. Major factors that contribute and enhance lipofuscin formation are increased autophagic activity, increased oxidative stress or decreased antioxidant defences, and high concentrations within the lysosomes of redox-active iron [43].

The cell recycles many of useful substrates by autophagic degradation to simple molecules, such as aminoacids, fatty acids and simple sugars, which may be reused after relocation to the cytosol. Many of these macromolecules that are autophagocytosed contain iron, which is released from a variety of metalloproteins during their intralysosomal degradation [43]. Moreover ROS (mainly H_2O_2 , which is produced in mitochondria by dismutation of $O_2^{\bullet-}$) easily diffuse into lysosomes and by interacting with iron results in Fenton-type reactions which leads to excessive production of ROS and peroxidation of lysosomal protein content. Moreover the newly generated ROS are then able to interact with the proteins and lipids within the lysosome and form a complex array of Schiff bases and cross-linking of surrounding macromolecules culminating in lipofuscin formation [44]. This is supported by data that demonstrates that oxidative stress, high iron or decreased antioxidant systems stimulate lipofuscin formation whereas antioxidants and iron chelators attenuate lipofuscin formation [39]. Furthermore, oxidatively damaged mitochondria may contain some already peroxidized, undegradable macromolecules. In addition, such ineffective mitochondria are not only ferruginous but also may generate larger amounts of $O_2^{\bullet-}$ than do functional mitochondria. Inside the lysosome, such production of $O_2^{\bullet-}$ may continue for a while, as well as, iron-catalyzed oxidative modification of mitochondrial components. Moreover ATP synthase subunit c, a mitochondrial protein is a predominant component of lipofuscin from aged neurons [45]. Indeed, autophagocytosed mitochondria seem to be a major source for both macromolecular components of lipofuscin and the low mass iron that catalyzes the peroxidative reactions

resulting in its formation. Therefore the primary constituents of lipofuscin are oxidatively modified protein residues, which are bridged into polymer complexes by acids, and lipid residues such as reactive aldehydes originating from the breakdown of triglycerides, free fatty acids, cholesterol and phospholipids, while carbohydrates form only a minor structural component [33]. Proteins within lipofuscin are linked by intramolecular and intermolecular cross-links; many of these cross-links are caused by nonproteineous compounds including oxidation products of other cellular components, such as 4-hydroxy-2-nonenal. Overall, the composition of lipofuscin is variable and dependent upon cell type, but in neurons, lipofuscin appears to be derived primarily from autophagocytosed mitochondria [46].

Hence, lipofuscin accumulation in post-mitotic tissues is both a hallmark of normal senescence and symptomatic of numerous age-related diseases including Alzheimer's disease (AD) and age-related macular degeneration. In addition, lysosomal storage diseases (neuronal ceroid lipofuscinosis, or Batten's disease) are also associated with the accumulation of lysosomal pigment displaying properties similar to that typical of lipofuscin. Lipofuscin complex is undegradable as the result of the excessive oxidation and cross-linking that occurs during its formation leading to the inability of the lysosome to degrade all incorporated materials. Lipofuscin inherently causes toxic effects, in part because of its ability to bind metals, such as iron, copper, zinc, manganese, and calcium, in a concentration up to 2% [47]. Being rich in heavy metals such as iron lipofuscin may jeopardize lysosomal stability under severe oxidative stress, causing enhanced lysosomal rupture and consequent apoptosis/necrosis. The only known mechanism allowing cells to get rid of lipofuscin is mitotic activity, which results in the dilution of the pigment. When lysosomes accumulate lipofuscin, lysosomal enzymes increasingly go to lipofuscin loaded lysosomes in an attempt to degrade the non-degradable material. Since the capacity to produce lysosomal enzymes for autophagy is not unlimited, the lack of lysosomal enzymes for autophagy leads to reduced ability to recycle other cellular organelles, such as mitochondria. Hence lipofuscin acts as a sink for newly produced lysosomal enzymes. If damaged mitochondria are not eliminated this subsequently results in a lower rate of ATP production and increased ROS production. Moreover, the release of the abundant iron from the aged intralysosomal compartment by free-radical-mediated membrane damage will also stimulate free radical production via Fenton chemistry, possibly leading to apoptotic cell death [46]. On the other hand, large numbers of lipofuscin-containing lysosomes, which also contain active hydrolases, may promote cellular damage if lysosomal membranes are destabilized by pathogenic factors (including oxidative stress), resulting in leak of hydrolytic enzymes into the cytosol. Because lipofuscin is separated from the rest of the cytoplasm by the lysosomal membrane, it cannot react directly with extralysosomal constituents. However, within lysosomes loaded lipofuscin iron may promote ROS production, sensitizing cells to oxidative injury through lysosomal destabilization. So, lipofuscin a yellowish-brown, autofluorescent, nondegradable polymeric substance that cells cannot get rid of it [48], reduce the lysosomal degradative capacity [34]. Interestingly, the rate of lipofuscin formation is inversely related to age and lysosomes containing lipofuscin have a reduced ability to fuse with autophagic structures [43].

Moreover, lipofuscin is able to decrease not only lysosomal degradation but also proteasomal degradation, perhaps as a result of binding proteasomal complexes in unsuccessful attempts of degradation [49]. Therefore, lipofuscin directly decreases cellular proteolysis by inhibition of the proteasomal turnover, resulting in reduced proteasomal activity [50]. Another reason for lipofuscin toxicity is the gradual filling of the cytoplasmic space over time, [51] resulting at first in decreased cellular functional capability, later in apoptotic cell death. In addition, also intracellular trafficking as well as cytoskeletal integrity may be compromised by the presence of large intracellular aggregates.

2.2. Defective autophagy

Defects in autophagy have been described to occur at very different levels within the cell and at very different diseases. Therefore understanding how autophagy step or steps are altered in specific disorders is a priority. There are several examples of defects that can arise in autophagy.

For example, initiation of autophagy may be compromised because of altered signaling through the insulin or mTOR pathways, which is tightly bound to activation of autophagy. Since autophagy can be cargo-specific which implicate cargo adaptors molecules that enable autophagosomes to identify specific substrates, alterations in the organelle-specific markers for degradation or in the autophagic machinery can impair autophagic turnover. For instance, if autophagosomes not recognize damaged mitochondria, defects in mitophagy occur which contributes to disease pathology, since removal of damaged mitochondria is impaired leading to high levels of ROS. Cells are then more susceptible to proapoptotic insults. Defects in cargo recognition occurs for instance in Huntington's disease (HD). HD is caused by gain-of-function mutations that confer neurotoxic effects on the ubiquitously expressed protein huntingtin [52]. This gain-of-function impairs the ability of autophagosomes to recognize certain cargoes, while mutant huntingtin is efficiently incorporated into autophagosomes, it may reduce autophagic sequestration of other cargoes [53].

In other conditions, autophagosomes form correctly and sequester relevant cargo but they fail to be cleared from the cytosol. Problems with clearance could result from alterations at very different levels. For example, problems with vesicular trafficking could indirectly interfere with the mobilization of autophagosomes toward the lysosomal compartment [54]. The cytoskeleton is extremely important in the trafficking of organelles and in fact has the role to maintain the spatial organisation for autophagy by conducting the trafficking of organelles involved in different interactions during autophagy. Therefore, autophagy is microtubule-dependent [55]. When microtubules are disrupted by colchicine, autophagosome-lysosomal fusion is also disrupted leading to an increase in the number of autophagic vacuoles (AVs) [56].

Pathogenic proteins can also interfere with the fusion step, which, although still not completely elucidated at the molecular level, is known to depend on different SNARE proteins, the actin cytoskeleton and the histone deacetylase 6 (HDAC6) [57]. Furthermore, if organelle traffic through the axon is "jammed" we will have organelle transport problems [58].

It is also possible that problems in maturation of autophagosomes and their fusion with lysosomes also occur. The maturation of autophagosomes and their fusion with lysosomes is dependent on the motor protein dynein, which mediates autophagosome movement along the cytoskeleton towards the microtubule-organizing center where the lysosomes are clustered. Loss of dynein function also results in the accumulation of autophagosomes and reduction of the clearance of intracytoplasmic aggregation-prone proteins [54].

Furthermore, lysosomal defects also have a negative impact on clearance of autophagosomes. Autophagosomes can form properly and sequester the usual cargo but they are not eliminated through the lysosomal system. The reasons for lysosomal failure could be multiple. For instance in most lysosomal storage disorders (LSDs) the accumulation of undegraded products inside lysosomes limits their degradative capacity [59]. Undigestible material in lysosomes could build up or otherwise inhibit hydrolases, and could also dilute or divert the delivery of lysosomal hydrolases decreasing their efficiency. Interestingly, accumulation of β -amyloid (A β) 1-42, which has a high propensity to aggregate and therefore is less efficiently degraded, causes leakage of lysosomal enzymes into the cytosol [60-61].

Moreover the build up of AVs filled with undigestible material could inhibit secretory pathways interfering with nutrient uptake and response to growth factors or recovery from stress. Additionally, it could also inhibit organelle fusion or block the supply of aminoacids from autophagic protein breakdown, inducing cell starvation [62]. Additionally, conditions that alter lysosomal membrane stability, decreases lysosomal biogenesis or changes lysosomal pH inhibiting lysosomal proteolysis (because of the acidic pH requirements of their degradative enzymes) could also alter autophagosome clearance. Lysosomal hydrolases inhibition by defective acidification of the lysosomal lumen can be due to the inability to target to lysosomes one of the subunits of the proton pump that usually acidifies this compartment, or by direct inhibition of cathepsins that leads to reduced rates of autophagy. Changes in the lysosomal pH could be due to enhanced activity of the V-ATPase. The V-ATPase is a holoenzyme consisting of a membrane bound V_o and cytosolic V_1 components, and both V_1 and V_o are composed of multiple subunits. The V_o subunit a_1 is required for acidification of degradative competent lysosomes [63]. Preservation of low pH is important for cargo release, lysosomal hydrolase and vesicle maturation, autophagy and neurotransmitter loading into synaptic vesicle [64].

Accelerated endocytosis also increases protein and lipid accumulation in endosomes and slows lysosomal degradation of endocytic cargoes [65], leading to lysosomal instability.

The process from protein sorting to endosomal-lysosomal fusion is maintained by the sequential interaction of four complexes termed the endosomal sorting complexes required for transport (ESCRT complexes). The four ESCRT complexes, numbered 0–III, are required for the degradation of aggregate-prone proteins by autophagy [66]. Mutations or depletions affecting ESCRT related genes results in deficient maturation of autophagosomes or in their inability to fuse with lysosomes and endosomes. This leads to autophagosomes accumulation without degradation of their cargo, as well as, ubiquitin-positive aggregates accumulation leading to neurodegeneration in many cases [67]. As proteasome is thought to be turned over

by autophagy [68], blockade of proteasome turnover could disrupt additional degradative pathways.

More specifically alterations of CMA can also occur. Different pathogenic proteins have been shown to directly interfere with CMA activity [69]. For instance, mutant forms of alpha-synuclein fail to translocate to the lysosomal lumen whereas the wild-type protein binds to the lysosomal receptor and rapidly reaches the lumen for degradation. Consequently, mutant forms of alpha-synuclein block access of other cytosolic proteins to lysosomes via CMA by abnormally bind to the lysosomal receptor. Moreover, the accumulation at the surface of lysosomes of oligomeric forms of pathogenic proteins targeted via CMA destabilizes the lysosomal membrane and results in leakage of lysosomal enzymes into the cytosol, which often triggers cellular death.

2.2.1. *Consequences of defective autophagy*

Healthy cells harbour a high autophagic clearance capacity preventing the “traffic jams” of endosomes, autophagosomes, as well as, aggregated proteins and damaged organelles. Only unfolded monomer proteins can undergo degradation through any of these two systems: autophagy, specifically CMA and ubiquitin-proteasome system (UPS). Consequently, once organized in oligomers, protofibrils, and fibrils, proteins can only be removed by in-bulk degradation, such as via microautophagy or macroautophagy. Thus, oligomers and fibrils of particular proteins can block the proteolytic activity of the UPS and of CMA. These alterations in intracellular proteins can be due to exposure to intracellular or extracellular stressors, such as oxidative stress, endoplasmic reticulum stress, ultraviolet radiation and other toxic insults. In addition, genetic mutations can generate proteins that cannot fold properly or are prone to aggregation. This can lead to the generation of misfolded proteins or modified soluble proteins resulting from protein cross-linking and oligomerization. The most toxic forms of altered proteins are complex organized structures, such as fibrils or oligomers, although the mechanism by which they exert their cellular toxicity is still controversial. In the absence of a properly functioning quality control system, and as a last attempt to prevent toxicity, cells favor formation of protein aggregates rather than fibrils and oligomeric complexes. The maturation of misfolded or unfolded protein into protein aggregates can vary across different disorders, but generally protein aggregation results from proteins that fold into an abnormal conformation, leading to the formation of oligomeric intermediates [70]. These smaller aggregates, both structured and unstructured, continue to grow and multimerize into larger aggregates or inclusions. Larger cytoplasmic inclusions can evolve further and coalesce into an aggresome, a pericentriolar, membrane-free cytoplasmic inclusion formed specifically at the microtubule organizing center. It has been proposed that the aggresome is a protective structure, formed to sequester proteins that cannot be degraded by the proteasome and packaged for degradation by autophagy. Moreover they sequester toxic monomeric or oligomeric species diluting its toxicity and facilitating the removal of these toxic species. However, aggregates are not completely harmless, because they interfere with normal cellular trafficking and become a sink for still-functional proteins that usually get trapped in these aggregates [71]. Their prolonged presence in neurons is indicative of some failure in fundamental cellular processes.

Taking this into account protein aggregation occurs and enhances the complexity that each neurodegenerative disorders presents. Interestingly, despite the unique features of each neurodegenerative disease, protein aggregation also shares several common characteristics. Generally, the major component of the inclusions is often ubiquitously expressed, such as huntingtin, alpha-synuclein and tau. Moreover the inclusions are found throughout the brain, and they do not correlate only to the pattern of neurodegeneration that occurs in each neurodegenerative disorder [6].

In addition, accumulation of defective, no longer functional organelles is also deleterious for the cells [72]. Whole organelles including peroxisomes and mitochondria are degraded by macroautophagy. If AVs containing damaged mitochondria or peroxisomes are not degraded, damaged organelles will gradually accumulate [41, 73]. Mitochondrial degradation by autophagy, known as mitophagy plays an important role in the regulation of mitochondrial function and remodeling. Mitophagy may also be important in attenuating apoptosis or necrosis, by clearance of damaged mitochondria. This could then prevent the release of cytochrome c, AIF (apoptosis-inducing factor) and other apoptotic factors that lead to cell death [74]. Clearly, autophagic removal of defective mitochondria is of crucial importance for cell survival. Inhibition of autophagy by 3-methyladenine in growth-arrested human fibroblasts, as a model of cell aging, results in the accumulation of lipofuscin-like material and of mitochondria, with a low-membrane potential [75]. Enlarged and structurally deteriorated mitochondria, showing swelling and disrupted *cristae* often result in the formation of amorphous material [76-77]. These mitochondria are defective in ATP production and produce increased amounts of ROS which are harmful for cells [78]. One would think that damaged mitochondria should be degraded, but their accumulation with age implies that they either acquire replicative advantage over normal mitochondria, or instead they accumulate due to a decrease in autophagic-lysosomal pathway.

Overall, autophagy is directly responsible for the maintenance of a proteome free of alterations [79]. Importantly, autophagy by mediating the removal of damaged organelles after stress restores organelles homeostasis being essential for the maintenance of cellular homeostasis and to guarantee cellular survival during stress. When quality control systems fail to accomplish its function is the basis for protein conformational disorders. In many late-onset neurodegenerative disorders, including Parkinson's disease (PD) and HD, there is accumulation of intracellular protein aggregates in the brain [6]. The elimination of these intracellular protein aggregates is often correlated with amelioration of symptoms of the disease [6]. Indeed, in mice with deficiencies in either Atg7³³ or Atg5³⁴ constitutive autophagy is required for the clearance of cytosolic aggregate-prone proteins from neurons [80].

3. Autophagy in disease

The broad array of physiological functions attributed to autophagy justifies why alterations in this catabolic process lead to cellular malfunctioning and often cell death. Autophagy is closely

involved in the etiology of several human diseases contributing to its pathogenesis, including cancer, neurodegenerative diseases and metabolic disorders [81-82].

3.1. Autophagic dysfunction in neurodegenerative disorders

Alzheimer's disease

As AD progresses, either due to AD related genes or environmental/aging factors, several pathological changes of the lysosomal network occurs, such as deregulation of endocytosis and increased lysosomal biogenesis culminating in a progressive failure of lysosomal clearance mechanisms [83]. Enlargement of Rab5 and Rab7 positive endosomes is one of the earliest specific pathology reported in AD brain tissue which reflects a pathological acceleration of endocytosis. Interestingly, it develops in pyramidal neurons of the neocortex at a stage when plaques and tangles are restricted only to the hippocampus. Furthermore genes involved in endocytosis are up-regulated in AD and their corresponding proteins are abnormally recruited to endosomes promoting fusion and enlargement of early and late endosomes, which is a specific characteristic of AD and is not seen in normal aging brain. Acceleration of endosome pathology is also seen in individuals who inherit the $\epsilon 4$ allele of APOE, the major risk factor for late-onset AD [84-85]. Lipinski and co-workers recently reported that transcription of factors that promote autophagy are up-regulated in the brains of AD patients, while negative regulators of autophagy are down-regulated [86]. Indeed, cellular ultrastructural changes have been described in AD brain biopsies revealing a high level of AVs within dystrophic neurites [87]. AVs and lysosomes constitute more than 95% of the organelles in dystrophic neuritic swellings in AD. This means that autophagy initiation is up-regulated or its progression is either delayed or impaired. However the profuse and selective accumulation of AVs in dystrophic neurites indicates a defect in the clearance of AVs by lysosomes rather than an abnormally augment of autophagy. In the case of familial AD, for instance, presenilin 1 (a ubiquitous transmembrane protein involved in diverse biological roles) mutations hinder lysosome proteolysis and accelerate neuritic dystrophy which also supports a primary role for failure of proteolytic clearance. Presenilin 1 is required for lysosome acidification which is needed to activate cathepsins and other hydrolases that carry out digestion during autophagy. Mutations in Presenilin 1 result in impaired targeting of the $\alpha 1$ subunit of V0-ATPase from the endoplasmic reticulum to the lysosome. As V0-ATPase is required for acidification of the autolysosome contents, mutations in Presenilin 1 are proposed to be involved in the defective proteolysis of autophagic substrates in patients with AD [88]. Furthermore, Zhang and colleagues reported for the first time, a role for presenilins in regulating lysosomal biogenesis [89]. The role of impaired lysosomal degradation in the etiology of AD was underscored in a study of a transgenic mouse model of this disease. In these mice, deletion of cystatin B (an endogenous inhibitor of lysosomal cysteine proteases) stimulated the turnover of proteins by the lysosome, enhanced the clearance of the autophagic substrates (including $A\beta$), and rescued the deficient cognitive phenotype of the animals. Furthermore, in APP transgenic mouse models of AD, undigested autophagic substrates including LC3-II, p62, and ubiquitinated proteins accumulate in neuronal AVs [90]. This general failure to clear autophagy substrates affects clearance of various proteins relevant to AD pathogenesis, including the protein $A\beta$

and tau promoting cell death [91]. These results indicate that mutant APP overexpression alone can lead to autophagic-lysosomal pathology. However the mechanism by which overexpression of mutant APP may lead to impaired autophagy and neuritic dystrophy, is not well understood. One possibility is that APP may affect the endosomal-lysosomal system. Strong overexpression of human A β 42 in *Drosophila* neurons induces age-related accumulation of A β in AVs and neurotoxicity which is further enhanced by autophagy activation and is partially rescued by autophagy inhibition [92]. These authors propose that the structural integrity of post-fusion AVs may be compromised in A β 42 affected neurons, leading to subcellular damage and loss of neuronal integrity in the A β 42 flies. Moreover, expression of an APOE ϵ 4 allele, but not the APOE ϵ 3 allele, in a mouse AD model increases levels of intracellular A β in lysosomes, altering their function and causing neurodegeneration [93]. Interestingly, inhibition of A β aggregation rescues the autophagic deficits in the TgCRND8 mouse model of AD [94]. Autophagy sequesters and digests unneeded or damaged organelles, some of which are APP-rich [95]. Autophagosomes are enriched in APP as well as APP substrates and enzymes that are responsible for processing APP into A β . Under normal circumstances A β is subsequently degraded by lysosomes [96-97]. Therefore autophagosomes are a site of intracellular production of A β , thus upon their cellular accumulation amyloid deposition occurs [98].

Failure of the autophagic system also compromises the elimination of aggregate forms of tau, a protein that also accumulates in AD neurons [99]. In fact, for certain types of tau mutations, pathogenic tau could contribute to the failure of macroautophagy, due to the toxic effect that the still soluble forms of the protein exert in the membrane of lysosomes when they are delivered to this compartment by CMA. Furthermore, particular mutant forms of tau have been shown to abnormally interact with components of the lysosomal CMA translocation machinery [100]. More interestingly, stimulation of autophagy is neuroprotective in a mouse model of human tauopathy [101]. In addition to the defects in late stages of autophagy, evidence suggests that autophagy might be disrupted at the level of autophagosome formation in patients with AD. Compared with healthy individuals, the brains of patients with AD show reduced expression of Beclin-1, which could lead to an impairment in the initiation of autophagy [95]. Transgenic mice that expressed a mutated form of the human APP on a Beclin-1 haploinsufficient background had disrupted autophagy, as well as, increased intracellular A β accumulation and neurodegeneration, compared with mice that expressed the mutated human APP in the context of a normal Beclin-1 background [95]. Caspase 3-mediated cleavage of Beclin-1 occurs in the brains of patients with AD; thus, increased activity of this enzyme might contribute to the loss of Beclin-1 function in individuals with this disease [102].

Parkinson's disease

In PD the most common pathogenic protein, alpha-synuclein, is usually degraded by different autophagic and non-autophagic pathways. Soluble forms of the protein are substrates of both the UPS and of CMA [103]. However, macroautophagy is the only plausible way for the elimination of the pathogenic variants of this protein once they aggregate [104]. When alpha-synuclein aggregates can no longer be degraded by either the UPS or CMA, macroautophagy becomes the only proteolytic pathway able to remove these proteinaceous deposits from the

neuronal cytosol. As in the other disorders, post-translational modifications and pathogenic forms of alpha-synuclein promote the formation of oligomeric species that interfere with the normal functioning of the UPS [105], CMA [103] and even macroautophagy at the level of autophagosome formation [106]. In fact, upregulation of wild-type alpha-synuclein leads to significant inhibition of macroautophagy [107] and mutant forms of alpha-synuclein A30P and A53T have been shown to be poorly degraded by CMA, because they are not translocated into the lysosome although they bind to the lysosomal membrane with high affinity. Furthermore, because of their high-affinity binding to the CMA receptor, they block the uptake and degradation of other CMA substrates, leading to a general CMA blockage [103]. Indeed, dopamine-modified alpha-synuclein, which is modified by non-covalent binding of oxidized dopamine, not only is poorly degraded by CMA but also block degradation of other substrates [108]. Subsequently, normal substrate proteins for CMA can no longer be turned over through this pathway and also end up accumulating inside the affected cells. Interestingly, inhibition of the UPS and CMA promote macroautophagy upregulation, [21] which maintains normal levels of protein degradation and removes the cytosolic toxic and aggregated alpha-synuclein [109]. AVs have also been described in melanized neurons of the substantia nigra in PD [110]. This accumulation is consistent with either an overproduction or impaired turnover of AVs. Additionally, LC3-II levels were increased in patients with diffuse Lewy Body Disease and PD, and LC3 co-localized with alpha-synuclein in most Lewy Bodies and Lewy neurites, suggesting an increase in macroautophagy, as well as, an attempt to clear alpha-synuclein pathology by up-regulating autophagic activation [111-113]. Furthermore, Alvarez-Erviti et al. revealed that CMA activity, as well as, LAMP-2A and hsc70 levels were significantly decreased in the substantia nigra from PD patients when compared to controls [114]. Likewise, cathepsin D immunoreactivity was significantly reduced in substantia nigra neurons of PD patients with an even greater decrease in alpha-synuclein inclusion-bearing cells [115]. This suggests the presence of abundant and dysfunctional autophagosomes and lysosomes in PD and diffuse Lewy Body Disease. In several cell models, such as SK-SH5Y [116], BE-M17 [117], PC12 cells [118], as well as, in primary mesencephalic [117] or cortical neurons [119], 1-methyl-4-phenylpyridinium (MPP⁺) has been shown to perturb the autophagy flux, as mirrored by an evident increase in the number of LC3-positive autophagic vesicles, in association with apoptotic or nonapoptotic cell death. More interestingly, cells harboring PD patient's mitochondria or in mitochondrial DNA-depleted cells show compromise quality control autophagic response and defective clearance of the AVs [120]. The recently identified physiological role for Parkin and Pink1, two PD-causative genes, in mitophagy, suggests that alterations of this selective form of autophagy could also contribute to the pathogenesis of this disease [121]. PINK1 encodes the serine-threonine kinase and is localized to the outer mitochondrial membrane, and PARK2 encodes Parkin, an E3 ubiquitin ligase. Under basal conditions, Parkin is mainly found in the cytosol where its ubiquitin ligase activity is inhibited by an unknown mechanism. Loss of mitochondrial membrane potential drives the recruitment of Parkin to the mitochondria. In fact, under physiologic and pathologic stress, Parkin is selectively recruited from the cytosol to damaged mitochondria in a PINK-1-dependent manner where ubiquitinates membrane proteins such as voltage-dependent anion channel 1 and mitofusins 1 and 2, mediating the elimination of the mitochondria by mitophagy [122-124].

Neurodegeneration in PINK-1 and Parkin-positive familial forms of PD may result from a defect in mitophagy, leading to the accumulation of damaged mitochondria and excessive ROS production. In fact, Parkin-deficient mice accumulate dysfunctional mitochondria and oxidative damage [125] while *Drosophila* flies with mutated Parkin are particularly sensitive to oxygen radical stress [126]. In addition, other PD causative gene, DJ-1 also plays a protective role in autophagy. Loss of DJ-1 has been shown to result in decreased basal autophagy [127]. Moreover, Irrcher and co-workers demonstrated that DJ-1 deficiency leads to altered autophagy in murine and human cells [128]. McCoy and Cookson also showed that loss of DJ-1 leads to mitochondrial phenotypes including reduced membrane potential, increased fragmentation and accumulation of autophagic markers [129]. Moreover, *Lrrk2* gene which is mutated in certain dominant forms of familial PD was shown to modulate autophagy. For instance, G2019S mutation induces autophagy via MEK/ERK pathway and inhibition of this exacerbated autophagy reduces the sensitivity observed in G2019S mutant cells [130]. Furthermore, *Lrrk2* was shown to co-localize with markers of the endosomal-lysosomal pathway in cases of diffuse Lewy Body Disease [131]. Loss of function of *Lrrk2* mutants have been shown to decrease neuritic arbor and to cause the accumulation of autophagic vesicles and swollen lysosomes containing tau inclusion bodies [132]. However, the precise mechanism(s) by which LRRK2 regulates autophagy are still elusive. Interestingly, recent data suggest a mechanism involving late steps in autophagic-lysosomal clearance in a manner dependent on NAADP (nicotinic acid-adenine dinucleotide phosphate)-sensitive lysosomal Ca^{2+} channels [133]. Other connection between autophagy and PD has been established in studies where mutant forms of the ubiquitin carboxyl-terminal esterase L1 (UCHL-1) described in some familial forms of PD were shown to interact abnormally with CMA components, namely hsc70, hsp90, and LAMP-2A. The abnormally binding between UCHL-1 and these three CMA components was proposed to interfere with CMA activity [134].

3.2. Autophagic dysfunction in lysosomal storage disorders

Lysosomes are ubiquitous organelles that have crucial roles in cellular clearance. Individually rare, it is now known that about 1 in 7500 live-born infants in many populations will have a lysosomal disease [135]. The LSDs are a group of over 60 diseases, clinically characterized by a progressive phenotype involving multiple organs and tissues, including severe neurodegeneration [136]. Indeed, LSDs involving primary defects in lysosome function commonly exhibit prominent neurodegenerative phenotypes, including neuritic dystrophies closely resembling the ultrastructural morphologies of dystrophic neurites in AD and, in some of these disorders, neurofibrillary tangles as well as increased amyloidogenic processing of APP and diffuse A β deposits [137]. Walter and colleagues demonstrated in LSDs an accumulation of sphingolipids, as occurs in lysosomal lipid storage disorders and, decreases the lysosome-dependent degradation of APP C-terminal fragments that stimulates γ -secretase activity increasing the generation of both intracellular and secreted A β . Notably, primary fibroblasts from patients with different storage diseases show strong accumulation of potentially amyloidogenic APP C-terminal fragments [138]. LSDs are characterized by progressive accumulation of undigested metabolites such as glycogen, ceramide, heparin sulphate and glucocerebroside, among others, within the cell due to lysosomal dysfunction. Loss-of-

function of lysosomal proteins causes accumulation of autophagic and endosomal substrates, and as a result lysosomal storage. Such proteins include lysosomal enzymes, lysosomal integral membrane proteins, and proteins involved in the post-translational modification and trafficking of lysosomal proteins. Defects in these proteins involved in lysosome regulation or function induce the accumulation of undigested molecules that can subsequently alter many cellular processes. These include lysosomal pH regulation, synaptic release, endocytosis, vesicle maturation, autophagy, exocytosis and Ca^{2+} homeostasis [139-140]. As a consequence, many tissues and organ systems are affected, including brain, viscera, bone and cartilage, with early onset central nervous system dysfunction predominating. Although individuals with LSDs can display early symptoms, the majority are clinically normal at birth which suggests that lysosomal dysfunction per se does not impact significantly the events of early brain development. Furthermore children typically meet early development milestones signifying that lysosomal storage does not affect neuronal function and maturation at early developmental stages. In patients with these disorders, dysfunction of a lysosomal enzyme (usually one of the many lysosomal hydrolases) causes impaired degradation of the enzyme's substrate, which accumulates within lysosomes [136]. An abundance of AVs is a common feature of cells from patients with LSDs. Lysosomal storage impairs autophagic delivery of bulk cytosolic contents to lysosomes. This can be attributed to a defect in autophagosome-lysosome fusion as a result of dysfunction of the lysosomal compartment. This, in turn, leads to a progressive accumulation of poly-ubiquitinated protein aggregates and of dysfunctional mitochondria, suggesting that neurodegeneration in LSDs may share mechanisms with some neurodegenerative disorders in which the accumulation of protein aggregates is a prominent feature [141]. This defect in autophagosome-lysosome fusion can result from an impairment of vesicular trafficking due to a microtubule-based transport deficiency. The motor protein dynein mediates the movement of mature autophagosomes towards lysosomes. Indeed, mutations affecting dynein impair the autophagic degradation of aggregate-prone proteins [54]. Another possible mechanism involves changes in the lipid composition of lysosomal membranes. In fact, lipids have been demonstrated to accumulate in a wide range of LSDs [142]. These lipids are the principal constituents of lipid-rafts. Since lipid-rafts play a critical role in membrane physiology influencing their plasticity, it is possible that the abnormal accumulation of lipids in LSDs leads to an increase in lipid-rafts that affects the dynamics of lysosomal membranes and specifically their ability to fuse with autophagosomes. Indeed, in conditions that are similar to what is found in Niemann Pick type C diseases-1 and-2, cholesterol accumulation in late endosomes perturb the intra-endosomal trafficking affecting most likely autophagosome maturation [143].

Impaired autophagy has been reported in several models of LSDs, including Pompe disease, Niemann-Pick disease, the neuronal ceroid lipofuscinoses, multiple sulphatase deficiency, and GM1-gangliosidosis. Additionally, lysosomal function is intimately linked to exocytosis (removal of cellular cargo by fusion of vesicle with the plasma membrane), and multiple LSDs, such as mucopolipidosis type I, Niemann-Pick disease and sialidosis have been shown to have impaired exocytosis [144].

The first LSD in which an involvement of autophagy was reported was Danon disease (also called “glycogen storage disease due to LAMP2 deficiency” or “lysosomal glycogen storage disease with normal acid maltase activity”) [145]. It was reported an accumulation of AVs in several tissues, particularly the muscle, from a mouse model of the disease [146]. The disease, extremely rare, is inherited as an X-linked trait and the disease phenotype is characterized by severe cardiomyopathy and variable skeletal muscle weakness often associated with mental retardation.

Another LSD is Multiple sulfatase deficiency which is attributed to deficiencies in the activity of sulfatase enzymes. The sulfatases are a family of enzymes that catalyze the hydrolysis of sulfate ester bonds in a wide variety of substrates, ranging from complex molecules, such as glycosaminoglycans, to sulfolipids and steroid sulfates. These enzymes can be divided, at least in mammals, into two main groups based on their subcellular localization: those found in lysosomes (acting at an acidic pH) and those found in the endoplasmic reticulum, the Golgi apparatus and at the cell surface (acting at a neutral pH) [147]. Sulfatases are activated upon a post-translational modification by sulfatase modifying factor 1. The gene encoding sulfatase modifying factor 1 in humans is mutated in this rare autosomal recessive disorder, in which the activity of all sulfatases is profoundly impaired [148]. As for other types of LSDs, the pathogenic mechanisms that lead from enzyme deficiency to cell death in Multiple sulfatase deficiency is not completely understood. An impairment of autophagy is postulated to play a major role in disease pathogenesis. In mouse models of multiple sulfatase deficiency, it was observed an accumulation of autophagosomes resulting from defective autophagosome-lysosome fusion which was demonstrated by the inefficient degradation of exogenous aggregate-prone proteins (expanded huntingtin and mutated alpha-synuclein) and defective organelles [59]. Moreover in chondrocytes from multiple sulfatase deficiency mice show a severe lysosomal storage defect and a defective autophagosome digestion leading to a defect in energy metabolism and to cell death [149]. Interestingly, the mechanisms underlying the impairment of the fusion between lysosomes and autophagosomes in multiple sulfatase deficiency seems to involve abnormalities of membrane lipid composition and SNARE protein distribution [150]. Overall, it seems that a global lysosomal dysfunction leads to the impaired autophagy observed in the pathogenesis of multiple sulfatase deficiency.

Niemann-Pick type C disease is caused by mutations in the NPC1 or NPC2 genes, [151] whose protein products are thought to act cooperatively in the efflux of cholesterol from late endosomes and lysosomes [152]. In the Niemann–Pick type C disease defects in lysosomal trafficking and biogenesis occur. Moreover, a marked accumulation of autophagosomes occurs in the brains of Niemann–Pick disease type C mice and in skin fibroblasts from Niemann–Pick disease type C patients [153-155]. Using human embryonic stem cell-derived neurons engineered to mimic the cholesterol lysosomal storage disease Niemann Pick type C, we have shown that excessive activation and impaired progression of the autophagic pathway lead to abnormal mitochondrial clearance [156]. NPC1 deficiency leads to both an induction of autophagy and an impairment of autophagic flux. The impairment in degradation of autophagic substrates may contribute to several aspects of NPC neuropathology, including the accumulation of ubiquitinated proteins and the generation of ROS.

Gaucher's disease is an autosomal recessive condition, due to deficient activity of lysosomal β -glucosylceramidase. The pathologically enlarged and often multinucleate 'storage' cell is a striking feature of Gaucher's disease. Similarly to Niemann-Pick type C disease, also models of Gaucher disease show both an induction of autophagy and an accumulation of autophagosomes and autophagic substrates [157-158].

In LSDs the primary alteration of the lysosomal compartment can indirectly affect CMA activity. However, there are two LSDs for which alterations in CMA are not merely a consequence of the lysosomal alteration, but rather are due to the possible functional association of the mutant protein with CMA, such as galactosialydisosis and mucopolipidosis type IV. The first one is caused by a mutation or loss of cathepsin A gene which is a protease involved in CMA regulation and contributes to lysosomal activity and stability. Moreover is also involved in the degradation of LAMP-2A [159]. Loss of cathepsin A enzymatic activity leads to slower LAMP-2A degradation resulting to an increase in CMA activity which contributes to an extreme weight loss that characterizes these patients. In the second LSD the mutated protein, the transient receptor potential mucolipin-1 (TRPML1) which is a endolysosomal cation channel, interacts with HSPA8 (HSC70) and DNAJB1 (HSP40), two components of the CMA molecular machinery leading to decreased CMA activity in response to serum removal [160]. Moreover it has been suggested that TRPML1 mediates Ca^{2+} efflux from late endosomes and lysosomes indicating that TRPML1 is a key regulator of membrane trafficking along the endosomal pathway [161]. In fact, in TRPML1-deficient cells the delivery of cargo from the cell surface to the lysosome and fusion of lysosomes with the plasma membrane is impaired [162-163].

In spite of all the above mentioned differences among diseases in most cases there is an impairment of autophagic flux, causing a secondary accumulation of autophagy substrates and on the other hand an increase in factors involved in autophagosome formation, such as Beclin1, as an attempt to compensate for the impaired autophagic flux. Accordingly, LSDs can be seen primarily as "autophagy disorders." [164].

3.3. Autophagic dysfunction in diabetes

In response to a variety of metabolic stressors such as nutrient starvation, growth factor withdrawal, high lipid content challenges or hypoxia macroautophagy and CMA can be induced [165]. Moreover autophagy is also capable of mobilizing energetically efficient molecules, such as lipids, glycogen and nucleic acids which can be used by TCA cycle, gluconeogenesis and glycolysis to produce ATP [165-166]. This capability of autophagy to maintain ATP production and support macromolecular synthesis makes it a pro-survival pathway of particular importance in organs with high energetic requirements, such as the heart or skeletal muscles. Therefore, alterations of this specific autophagic function can constitute the basis of some common metabolic disorders such as diabetes. Type II diabetes is caused, in most cases, by the inability of the body to buffer the free fatty acid concentration. This increases the redox pressure on the mitochondrial respiratory chain, increases ROS production, reduces mitochondrial function, and increases apoptosis of beta-cells [167]. Moreover it is characterized by insulin resistance and failure of beta-cells

producing insulin. The most potent fatty acid causing insulin resistance is palmitate. As palmitate is the precursor of ceramide and sphingolipid biosynthesis, the sphingolipid pathway has been implicated in the etiology of insulin resistance [168]. Interestingly, ceramide has been shown to activate autophagy by upregulating beclin1 [169]. Moreover a decrease in intracellular aminoacid concentrations and a decrease in mTOR-dependent signaling may also contribute to the activation of autophagy by ceramide [170]. If in fact autophagy turns out to be up-regulated in type II diabetes, we could speculate that the onset of insulin resistance in elderly people could be an adaptive mechanism aiming to increase autophagy and helping to improve the ability to remove damaged organelles [171]. Autophagy is inhibited by the insulin -mTOR signaling pathway and can be activated either upon aminoacid depletion or by rapamycin administration. Insulin inhibits autophagy in two ways: first by activating mTOR in synergy with aminoacids, which results in the phosphorylation and inhibition of the protein kinase Atg1 (ULK1 in mammals); and second by protein kinase B-mediated phosphorylation and inhibition of the transcription factor FoxO3, which is responsible for the expression of ATG genes [172]. Because insulin inhibits autophagy in insulin-sensitive cells, such as beta-cells, autophagy might be increased. In addition, as a result of free fatty acid induced oxidative stress insulin resistance develops in response to over-feeding, which is one of the major causes of insulin resistance. Interestingly, ROS are known to be required to trigger autophagy, probably because they oxidize a critical cysteine residue in Atg4 [173]. A report from 2007 showed that oxidative stress induced by diabetes leads to ubiquitination and storage of proteins into cytoplasmatic aggregates that do not co-localize with insulin. Because accumulation of ubiquitinated protein may damage cells, such data suggests that autophagy may contribute to the regulation of beta-cell survival and death acting as a defense to cellular damage during diabetes [174]. To prove that a relationship between autophagy and beta-cell death indeed exists experiments were performed in a mouse model whose beta-cells were deficient in autophagic activity. In beta-cell specific Atg7 knockout mice it was reported by two different studies islet degeneration, decreased glucose tolerance, insulin secretion and accumulation of large ubiquitin-containing protein aggregates indicating that autophagy was impaired. This happened even with overexpression of LC3-binding protein p62, which is required for polyubiquitinated protein aggregates to be delivered to the autolysosome [175-176]. In beta-cells from diabetic animals the levels of autophagosomes was significantly increased in both diabetic *db/db* mice and in non-diabetic control mice fed with a high-fat diet. This can occur either as a result of impaired autophagosome/lysosome fusion, or impaired function of the lysosomal proton pump. Interestingly when Atg7(-/-) mice were fed with a high-fat diet their glucose tolerance decreased indicating once again the important role of autophagy in maintaining proper beta-cell function under stressful conditions. Ebato and colleagues also showed that free fatty acids which can cause peripheral insulin resistance associated with diabetes induced autophagy in beta-cells [176]. These findings suggest that basal autophagy is important for maintenance of normal islet architecture and function and protects beta-cells against cell damage [177]. Interestingly in autophagy-deficient beta-cells ubiquitinated proteins accumulated inside cells. Moreover also p62, a specific autophagy substrate also accumulated in autophagy-deficient beta-cells [178]. Furthermore, exposure of human

neuroblastoma SH-SY5Y cells to sera from type 2 diabetic patients with neuropathy is associated with increased levels of autophagosomes [179]. Jung and co-workers showed that the presence of defective beta-cell mitochondria and endoplasmic reticulum presumably contributed to the reduced ability to produce insulin, indicating that decreased function and mass of beta-cells can result from mitochondrial dysfunction and endoplasmic reticulum stress, since both organelles are autophagic substrates [175]. Moreover, deregulated autophagy may be involved in insulin resistance. In fact, mitochondrial impairment and defective endoplasmic stress response have been implicated in insulin resistance [180-182]. In pancreatic beta-cells, the endoplasmic reticulum is the crucial site for insulin biosynthesis. Consequently, perturbations to endoplasmic reticulum function of the beta-cell, such as those caused by high levels of free fatty acid and insulin resistance, can lead to an imbalance in protein homeostasis and ER stress, which has been recognized as an important mechanism for type 2 diabetes. Macroautophagy is activated as a novel signaling pathway in response to ER stress [183]. In the brain of young patients with poorly controlled type I diabetes mellitus and fatal diabetic ketoacidosis was demonstrated increased levels of macroautophagy-associated proteins as well as increased levels of the ER-associated GRP78. Therefore probably chronic metabolic instability and oxidative stress may cause alterations in the autophagy-lysosomal pathway [184].

Interestingly, insulin resistance, one of the major components of type II diabetes mellitus is a known risk factor for AD. Son and co-workers observed that insulin resistance promotes A β generation in the brain via altered insulin signal transduction, increased BACE1/ β -secretase and γ -secretase activities, and accumulation of autophagosomes. These authors proposed that the insulin resistance that underlies the pathogenesis of type II diabetes mellitus might alter APP processing through autophagy activation, which might be involved in the pathogenesis of AD [185].

4. Targeting autophagy

The evidence indicating that induction of autophagy is cytoprotective in neurodegenerative disease models raises the possibility that this intracellular catabolic pathway may be exploited to clear toxic disease proteins and provide therapeutic benefit for patients. Consequently, novel approaches to manipulating autophagy in human patients are desirable.

Using a yeast screen, one recent study identified several small molecules capable of augmenting autophagy in mammalian cells and further demonstrated therapeutic benefit of these compounds in a *Drosophila* model of neurodegeneration [186].

Knockdown of autophagy blocks the protective effects of delayed aging, suggesting that neuroprotection associated with manipulation of aging pathways is autophagy dependent [97]. These results are consistent with the notion that protein aggregation neuronal dysfunction might occur coincidentally with age-related decline in cellular mechanisms to deal with misfolded protein species. Thus, the age-related onset of pathology in neurodegenerative conditions might be correlated with a decline in autophagic capacity beyond a critical thresh-

old. However, additional studies will be required to define the precise relationship of aging, autophagy and neurodegeneration. In the case of some diseases autophagy is initially induced as a neuroprotective response in stressed or injured neurons, but is subsequently overwhelmed or impaired by disease-related factors. This could partly account for evidence that autophagy seems to be both induced and impaired in several major neurodegenerative diseases. Overexpression of HDAC6, a cytoplasmic deacetylase containing a ubiquitin-binding domain, was found to suppress neurodegeneration in a model of polyglutamine disease and to compensate for defects in the UPS by facilitating autophagic protein degradation [187]. These results suggest that HDAC6 functions at the intersection of the UPS and autophagy and identify HDAC6 as a promising target for pharmacological manipulation in neurodegeneration.

Pharmacological activation of autophagy can be achieved with rapamycin, a lipophilic macrolide antibiotic. Chronic low-level stimulation of autophagy through peripheral administration of rapamycin or other agents [188], or enhancing lysosomal proteolysis selectively [90, 189], can markedly diminish A β levels and amyloid load in APP transgenic mice, underscoring the importance of lysosomal clearance of A β . In both APP and triple transgenic mouse models of AD for instance peripheral administration of rapamycin significantly reduces A β deposition and tau pathology [188, 190-191]. Furthermore, by deleting an endogenous inhibitor of lysosomal cysteine proteases (cystatin B) in the TgCRND8 APP mouse model lysosomal pathology is rescued and abnormal autolysosomal accumulation of autophagy substrates including A β is decreased and learning and memory deficits also ameliorate [90]. In addition, inhibition of mTOR by rapamycin improves cognitive deficits and rescues A β pathology and intraneuronal neurofibrillary tangles by increasing autophagy [192]. In a cell model of HD treatment with rapamycin reduced cellular toxicity by reducing both levels of soluble mutant huntingtin and the formation of intracellular aggregates [193]. Likewise this was also replicated in vivo in both *Drosophila* and mouse models of HD [194]. Furthermore, treatment of animals with rapamycin ameliorated neuronal toxicity in models of both Frontotemporal Dementia, spinocerebellar ataxia type 3, and PD [195-199]. However rapamycin shows autophagy-independent functions, such as regulation of ribosome biogenesis and protein translation. Taking into account that rapamycin may not be a good choice for the treatment in humans, other compounds that are independent of mTOR pathway are being studied. Moreover, long-term massive degradation of intracellular components as a result of the upregulation of macroautophagy can have possible negative consequences. Current efforts are now focused on the identification of other methods that can activate macroautophagy.

The mood stabilizer lithium used in bipolar disorder induces autophagy independently of mTOR through the inhibition of inositol monophosphatase therefore depleting the intracellular signaling molecule IP3 [200-201]. Lithium enhances clearance of aggregate-prone proteins, such as mutant forms of huntingtin and alpha-synuclein [202]. Likewise, sodium valproate and carbamazepine are other mood stabilizing drugs that can also reduce the accumulation and toxic effects of aggregation-prone mutant proteins in cell models and protect against neurodegeneration in vivo. Enhanced clearance of mutant alpha-synuclein and neuroprotective effects through lithium were demonstrated in cell-culture models and mice, underscoring the potential of lithium and related molecules for further evaluation [203-204].

An interesting study has shown that lithium (when given in addition to riluzole), significantly delayed the onset of disability and death in human Amyotrophic lateral sclerosis patients, compared to those administered riluzole alone [205]. In addition, prolonged lithium treatment alleviates memory deficits and reduces A β production in AD mouse models [206]. On 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism mice valproate combined with lithium carbonate rescued dopaminergic neurons and ameliorated the loss of DOPAC, likely via activation of autophagic/lysosomal pathways [207].

The disaccharide trehalose is a stable disaccharide with unique physicochemical properties. This disaccharide directly acts as a chemical chaperone that can stabilize misfolded proteins but it also facilitates the clearance of aggregate-prone proteins. Trehalose was shown to augment autophagy and enhance the clearance of mutant forms of huntingtin and alpha-synuclein from neuronal cells in culture [208]. In APP(swe) mutant mice co-treatment with trehalose increases the expression of autophagic markers as well as the expression of chaperones and reduces the levels of A β peptide aggregates, tau plaques and levels of phospho-tau [209]. Moreover in PC12 cells overexpressing wild-type or A53T mutant alpha-synuclein trehalose promoted the clearance of A53T alpha-synuclein but not wild-type alpha-synuclein in PC12 cells, and increased LC3 and Lysotracker RED positive AVs by using lysotracker and LC3 staining [210].

These findings have opened the possibility of using autophagy modulators as therapeutic approaches for these types of pathology. Therefore the search for chemical autophagy modulators more selective and potency is ongoing. Nevertheless, there are certain limitations in the use of up-regulation of macroautophagy for anti-neurodegenerative purposes. One of the limitations is that they all act on early steps of macroautophagy enhancing autophagosome formation, but will not have a beneficial effect in those pathological conditions in which the autophagic defect is in steps past autophagosome formation. Moreover, it has been reported that not all protein aggregates are recognized by the macroautophagic machinery. Further studies are needed to address whether expression of other cellular components or specific post-translational modifications in the aggregated proteins could enhance their recognition by the autophagic systems. For example, pharmacological induction or inhibition of macroautophagy alters the rate of turnover of polyglutamine-expanded proteins, polyalanine-expanded proteins, as well as, wild-type and mutant forms of alpha-synuclein [193, 211].

A patent by Harvard and Cambridge Universities disclosed novel small molecule enhancers of autophagy, named SMERs (small molecule enhancers of rapamycin) [President and Fellows of Harvard College, Dana Farber Cancer Institute, Cambridge Enterprise Ltd. Regulating autophagy. WO2008122038 (2008)]. In mammalian cells, the three most active compounds: SMER10, SMER18 and SMER28, augmented autophagosome formation in an mTORC1-independent fashion and without affecting the levels of Beclin-1, Atg5, Atg7 and Atg12, or enhancing the conjugation of Atg12 to Atg5. Importantly, SMERs enhanced the clearance of aggregation-prone mutant forms of alpha-synuclein and huntingtin in mammalian cells [186]. In addition, in cell lines and primary neuronal cultures SMER28 reduced the levels of A β and APP C-terminal fragments [188]. In addition, drugs that involve the serine/threonine-protein kinase mammalian target of rapamycin (mTOR) are

being currently examined for the treatment of metabolic disorders, such as diabetes mellitus. However future investigations are necessary [212]. Drugs that take advantage of the potential protective effect of autophagy in ER stress, such as glucagon like peptide-1, will be a promising avenue of investigation [183, 213].

5. Concluding remarks

The progression of research in the field of autophagy has taken the understanding of autophagy from a means of survival during starvation to a possible means of treating pharmacologically diseases where autophagy holds an important role. Progress in understanding autophagy has emphasized the importance of autophagy in cellular homeostasis and quality control; prevention of neurodegeneration in several animal models, and the significance of its deficiency in the development and pathogenesis of several disorders. This may provide further insights into the role that autophagy has in disease and how it may be possibly use it as a therapeutic strategy.

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