Alzheimer's Disease & Treatment

Chapter 4

Autophagy Dysregulation in Alzheimer's Disease: Plant Polyphenols as A Possible Preventive and Therapeutic Treatment

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Abstract

The self-degradative process of autophagy plays a fundamental role in cellular, tissue, organismal homeostasis and in balancing sources of energy at critical times in development and in response to nutrient stress. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing dysfunctional organelles and intracellular pathogens. Autophagy is generally considered as a pro-survival mechanism although its deregulation has been linked to non-apoptotic cell death. Autophagy efficiency declines with age, with consequent accumulation of harmful protein aggregates and damaged mitochondria and increased oxidative stress.

Accordingly, abnormalities in the autophagic flux may contribute to many different pathophysiological conditions, as cancer, cardiomyopathy, diabetes, liver disease, autoimmune diseases, infections and neurodegeneration, particularly to the onset of Alzheimer's disease

(AD); the latter is the most common form of dementia in the elderly with increasing prevalence in developed countries. Indeed, a number of studies have revealed that the maturation of autophagolysosomes and the inhibition of their retrograde transport creates favourable conditions for iperphosphorylation of the tau protein and the accumulation of the Aβ peptide, with their aggregation into intracellular tangles and extracellular plaques, respectively, the main responsible for neuronal damage in AD.

The potential therapeutic benefits of naturally occurring phytochemicals as pharmacological modulators of autophagy have been addressed since several years. Recent data have shown that OleA, a key component of extra virgin olive oil (EVOO), stimulates cell defences against plaque-induced neurodegeneration and triggers autophagy. After ingestion, OleA is metabolized to hydroxytyrosol (HT), the most powerful antioxidant compound in the olive tree. Recent reports indicate that OleA protects against cognitive impairment murine models of plaque deposition and that HT inhibits both enzymatic and spontaneous oxidation of endogenous dopamine and mitigates its oxidation during monoamine oxidase (MAO) action, thus slowing the progression of Parkinson's disease. In this chapter, we summarize the biological functions of autophagy and the positive effects of polyphenols as modulators of autophagic processes from the perspective of understanding—and potentially preventing/reversing— the pathophysiology of aging-associated human diseases.

1. Introduction

Autophagy is a key process involved in homeostasis of cellular proteins (proteostasis), lipids and organelles, particularly mitochondria (mitophagy), and contributes to the clearance from the cell of materials of endogenous or exogenous origin. Autophagy encompasses the different routes used by cells to deliver cytoplasmic substrates to lysosomes for degradation; the latter requires the formation of autophagosomes that subsequently fuse with lysosomes into autophagolysosomes. Autophagy efficiency declines with age, with consequent accumulation of harmful protein aggregates and damaged mitochondria, which leads to increased ROS production. Autophagy abnormalities may contribute to many different pathophysiological conditions and is increasingly considered as a promising target to treat a number of pathologies, particularly those associated with neurodegeneration and ageing. Alterations of the autophagic machinery correlate with the onset of Alzheimer's disease (AD), the most common form of dementia in the elderly whose occurrence in the developed countries is dramatically increasing. Indeed, studies have revealed that the maturation of autophagolysosomes and the inhibition of their retrograde transport provide favourable conditions for deposition of the $A\beta_{40/42}$ peptides in

target CNS areas such as the hippocampus, the entorhinal and pre-frontal cortex. Furthermore, increasing signalling by the mammalian target of rapamycin (mTOR) reduces autophagy with concomitant upregulation of the expression of tau protein and increase of its phosphorylation level. Both Aβ and tau are involved in formation and deposition of amyloid plaques and fibrillary tangles, respectively, in AD patients, where a compromised mitophagy, possibly associated with dysfunctional mitochondria, is also observed. Overall, the existing literature supports the idea that establishing treatments aimed at restoring proper autophagy can be considered as an emerging and valid therapeutic strategy against aging-associated neurodegeneration.

2. The Autophagy Flux

Together with the ubiquitin-proteasome system, autophagy participates to cellular proteostasis as one of the two main pathways of intracellular proteolysis, particularly misfolded aggregates that cannot enter the proteasome due to the reduced size of its pore [1]. Autophagy is a main route to deliver cytoplasmic substrates to lysosomes for enzymatic degradation through the formation of autophagosomes and their subsequent fusion with lysosomes to form autophagolysosomes [2]. Depending on the way of degradation of the cargo material, it is possible to distinguish three different types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [3]. Microautophagy is characterized by cargo engulfment by direct invagination of the lysosomal membrane, but relatively little information is presently available about this process, its regulation and its involvement in human health and disease [4]. One of the few studies on this issue has reported that soluble cytosolic proteins and/or intact organelles can be transported to the lysosome membrane through an endosomal microautophagy-like process [5]. The CMA pathway appears to be quite different; in fact, it does not use membranous structures to degrade cellular materials but exploits chaperones (such as Hsc-70) to identify proteins that contain a particular pentapeptide motif related to KFERQ, a consensus sequence for CMA or microautophagy also found at the C-terminus of APP the protein precursor of the Aβ peptides [6]. CMA is highly specific for substrates, such as glycolytic enzymes, transcription factors, calcium- and lipid-binding proteins [7] that contain this motif that targets them to the lysosomal-associated membrane protein 2A (LAMP-2A), a lysosomal membrane receptor responsible for their internalization for degradation [8].

Microautophagy and CMA are minor contributors to the overall autophagix flux whereas macroautophagy, most often indicated as "autophagy", is the main pathway, used to eradicate damaged cell organelles or aged/damaged proteins [9]. Macroautophagy has been extensively studied and is by far the most known type of autophagy; different steps leading to the formation of mature autophagosome characterize the macroautophagyc pathway. It can divided into three phases: autophagosome formation, substrate recognition and autophagosome trafficking and degradation. The process starts with the formation of precursor vesicles arising from a variety of membranes including endosomal intermediates, the Golgi and specific regions of

the endoplasmic reticulum (initiation or nucleation). These intermediates fuse generating pre-autophagosomal structures (phagophores) lipid-protein assemblies characterized by a double membrane containing receptors that bind cargo materials (precursor formation, the most dynamic step of autophagy). The phagophores grow further and close, eventually generating double-membraned autophagosomes, relatively transient assemblies which enclose the material to be degraded (maturation) [10,11]. Then, the autophagosomes are trafficked through the cytoplasm by dynein motors along microtubules to the perinuclear region where they fuse with lysosomes to form autophagolysosomes, where their contents are degraded by acidic hydrolases [12].

Macroautophagy is executed by highly conserved autophagy-related (Atg) genes, first identified by genetic screens in *Saccharomyces cerevisiae* [13-15]. Macroautophagy is thoroughly regulated and its regulators undergo post-translational modifications, including ubiquitination, phosphorylation and acetylation that facilitate the delivery of cytoplasmic materials to the autophagosomes and provide an additional level of control [15]. Macroautophagy is a very complex and highly regulated process. In brief, in mammals, amino acid sensing and additional signals regulate the activity of two kinases that activate or block the autophagy machinery. The protein kinases complexes mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) provide an opposite regulation of autophagy through the phosphorylation of the quaternary complex ULK1-ATG13-ATG101-FIP200 whose activation controls phagosome formation [16] In particular, activation of ULK1 and ULK2 (two of the five *Atg1 homologues* identified in mammals) [17,18] results in phosphorylation and activation of beclin-1 (mammalian homologue of Atg6) [19]; the latter, in turn, activates the VPS34 complex, with ensuing accumulation of phosphatidylinositol-3-phosphate (PtdIns(3)P) in the phagophore and recruitment of other binding proteins (ATG5-ATG12-ATG16L1) harbouring a PtdIns(3)P binding motif, a complex needed for phagophore elongation (Fig. 1). In fact, beclin-1 serves as a core component of the class III phosphatidylinositol 3-kinase (PI3KC3) complex as shown in **Figure 1**. The active ULK and Beclin-1 (Becn1) complexes form a stable core in the phagophore, where they contribute to the activation of downstream autophagy components [20,21].

The "nucleation" and "precursor formation" steps described above are followed by "phagophore expansion and elongation" steps. They involve the ATG5-ATG12-ATG16L1 complex, particularly two ubiquitin-like conjugation systems: Atg12 and Atg8 (LC3 and GABARAP in mammals). In the expansion step, the phagophore covalently binds to the microtubule-associated protein 1 light chain 3 (LC3), widely used as a marker for the microscopic detection and quantification of autophagosomes. LC3 results in the active form, LC3-II, after conjugation steps; LC3II forms an E3-like complex with Atg16 for phagophore membrane elongation [23]. In detail, after synthesis, LC3 is first cleaved by ATG4B to LC3-I that is conjugated to phosphatidylethanolamine (PE) by a ubiquitination-like enzymatic

reaction including ATG7 and ATG3, to become LC3-II that associates with both membranes of the phagophore to remain in the complete autophagosome after closure [24].

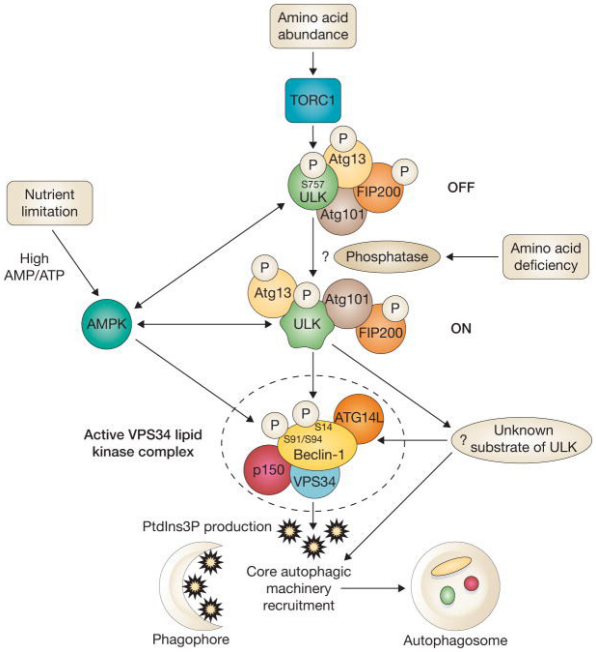

Figure 1: Formation of autophagosomes. Activation of the ULK kinases complex and Beclin-1. Beclin 1 is a core component of the class III phosphatidylinositol 3-kinase (PI3KC3) complex. (Image from Nazarko and Qing Zhong 2013)) [22].

The second step (substrate recognition) depends on the presence of several adaptor proteins (p62, NDP52, optineurin) that recognize specifically the cargo material to be engulfed into the autophagosome. Actually, many evidences indicate that autophagy is a selective process [25-27] and that the substrates to be degraded bind to the inner surface of the growing phagophore through adaptor proteins [28]. One of the key adaptors for cargo degradation is p62, also known as sequestosome 1 (SQSTM1), that binds both LC3 and polyubiquitinated cargo proteins to be degraded. Considering that p62 is also selectively degraded by autophagy [29], it turns out that p62 plays a central role in selective autophagy involving the clearance of misfolded proteins [30]. Since p62 accumulates when autophagy is inhibited or does not proceed correctly, and decreases when autophagy is triggered and proceeds correctly, it may be used as a marker to study the autophagic flux.

The third step (autophagy trafficking and degradation) requires the fusion of a "mature" autophagosome with a lysosome through the actions of multiple proteins, including SNAREs [31] and UVRAG [32]. It requires autophagosome transport to the perinuclear region by dynein through the microtubules before fusion. Following fusion, an autophagosome with a single lipid membrane, named autophagic body can be found inside the lysosome (autophagolysosome, autolysosome). These autophagic bodies are efficiently degraded and their content hydrolyzed to their simplest components such as amino acids that are recycled for synthesis of essential components of the cell upon release through permeases [33]. The degradation of this material depends on the acidic pH found in the lysosomal lumen and on the action of various enzymes, including cathepsins such as proteinase B [34]. The undigested material is usually discharged

from the cells through exocytosis, or it remains inside the vacuoles that will acquire the biochemical features of wear pigments or lipofuscins [35].

The autophagic flux and lysosomal biogenesis are under transcriptional control by several transcription factors including TFEB, a master regulator of lysosome biogenesis [36] and FOXO3, a member of the FOXO family of transcription factors, critical regulators of cellular homeostasis, aging and stem cells [37] which controls the expression of many genes involved in autophagy [38]. Recently, peroxisome proliferator activated receptor alpha (PPARα), a transcription factor that regulates genes involved in fatty acid metabolism and activates hepatic autophagy has also been shown to reglate autophagy in the nervous system and that PPARα-mediated autophagy affects AD [39].

3. Autophagy Dysfunction is a Main Determinant of Alzheimer's Disease

The efficiency of the autophagy flux declines with age, with consequent accumulation of harmful protein aggregates and damaged mitochondria resulting in increased ROS production and oxidative stress. Extensive literature exists supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD together with the existence of a link between mitochondrial dysfunction and autophagy in AD. Disfunctions of autophagy may contribute to many different pathophysiological conditions; there is substantial evidence indicating that senile plaques and neurofibrillary tangles (NFT), the histopathological hallmarks of AD, resulting from accumulation of amyloid β peptides (notably $A\beta_{42}$) and tau hyperphosphorylation, respectively, are related to the alteration of the autophagic pathway [40,41]. Thus, autophagy is increasingly considered as a promising target to prevent ageing and to prevent and, possibly, to treat several pathologies, particularly those associated with neurodegeneration.

`Present knowledge suggests that any impairment of autophagy accelerates neuronal degeneration and that inflammation, autophagy and AD are interconnected processes, as reported in a study by Francois *et al*. (2013), where an example of cross-talk between them was provided. Those authors showed that $A\beta_{42}$ influences the expression and activation of some proteins involved in autophagy including p62, mTOR and Becn1 [42,43]. It has also been reported that mTOR signaling is inhibited in the cortex and the hippocampus of adult AD model mice [43] with reduction of $A\beta_{42}$ levels [44,45] and protection against memory loss in AD model mice [45], and that neuroinflammation might influence autophagy following stress-induced hypertension [46]. Another study reported that adult mice bearing mutations of the *App* and *Psen1* genes displayed higher brain levels of inflammatory mediators (including Il-1β) together with accumulation of autophagic vesicles within dystrophic neurons in the cortex and hippocampus [43].

In AD it was observed also an alteration of the AMPK pathway. AMPK is a phylogenetically conserved serine/threonine protein kinase, a regulator of cellular energy

homeostasis and a central player in glucose and lipid metabolism [47]. As such, AMPK has opposite metabolic and regulatory effects respect to the mTOR complex that, under those conditions is inhibited. AMPK is activated in response to stresses that deplete cellular ATP supplies such as nutrient shortage, low glucose and heat shock resulting in increase of the AMP/ATP ratio. As a cellular sensor responding to low ATP levels, AMPK regulates several intracellular systems including the cellular glucose uptake, and mitochondria [48]. Perturbations of brain energy metabolism are critically involved in the neurodegeneration process occurring in AD; they may also correlate with early cognitive impairment, including increased insulin resistance, mitochondrial dysfunctions and alteration of Ca^{2+} homeostasis [49]. AMPK activity also correlates with autophagy activation through direct phosphorylation of ULK1 [50] and emerging studies indicate that the AMPK pathway can regulate mTOR activity also through ULK1 [51]. Recent reports show that a transient increase in cytosolic Ca^{2+} can induce autophagy by inhibition of mTOR through the CaMKKβ-mediated activation of AMPK [52].

The above reported issues are only a part of the much more complex network of players involved in the balance of proteostasis in cells; therefore, much more elements should be taken into consideration. For example, it has been widely reported that neurodegenerative disorders are linked with the alteration of endosomal trafficking and that active multivesicular bodies are a prerequisite for efficient clearance of misfolded proteins by autophagy. This alteration correlates with autophagosome accumulation eventually culminating with neurodegeneration [53]. The correlation between autophagy disfunction and onset of neurodegenerative diseases is also associated to a reduced activity of lysosomal hydrolases or to an impaired lysosomal acidification [54].

4. Autophagy and Amyloid Aggregation of Tau Protein and $A\beta_{40/42}$ Peptides

The histopathological hallmarks of AD involve the presence of two main types of proteinaceous deposits in the brain that are considered the main culprits of the loss of neuronal function: extracellular plaques, mainly composed of fibrillar deposits of $A\beta_{42}$, and intracellular neurofibrillary tangles (NFTs) formed by polymerization of hyperphosphorylated tau. The Aβ peptides are produced by the cleavage of the amyloid precursor protein (APP) by proteolytic complexes referred to as α−, β- and γ-secretase [55]. The cleavage sequence α, γ-secretase yields harmless non-aggregating peptides, whereas the cleavage sequence β, γ-secretase under normal conditions produces a mixture of Aβ peptides mainly composed of of $A\beta_{40}$ (90%) and $A\beta_{42}$ (10%). The latter peptide is much more amyloidogenic than $A\beta_{40}$ and undergoes easily the aggregation path, being the main responsible of the extracellular plaques found in AD patients. Of course, any factor that alters such a balance with increased presence of $A\beta_{42}$ promotes plaque deposition. Autophagy can hinder the process in several ways: it sequesters Aβ peptides, notably $A\beta_{42}$, in autophagic vacuoles (AVs) thus reducing its concentration, even though sequestration decreases the efficiency of vacuole degradation [56]. The accumulation of

both AVs and incompletely degraded material correlates with the parallel occurrence of aging and metabolic or oxidative stress [44,45]. The less amyloidogenic $A\beta_{40}$ peptide is degraded by intra- or extracellular proteases such as nephrilysin or insulin-degrading enzyme (IDE) [57] and therefore does not interfere remarkably with the efficiency of autophagy [58].

The alteration of the autophagy system in early stages of AD can also result from a deficit of Beclin1 activity [59]. Data obtained in AD transgenic mice have shown that a strong reduction of Beclin1 expression by genetic manipulation resulted in increased deposition of extracellular $A\beta_{42}$ and neurodegeneration. It also caused alterations in the microglia and profound neuronal ultrastructural abnormalities. These alterations were recovered following administration of a lentiviral vector expressing Beclin1 [60]. These data, together with other similar findings, confirmed that the activation of Beclin1 reduces $A\beta_{42}$ accumulation, whereas its loss of function increases both APP and $A\beta_{42}$ levels [61]. Recent studies have reported that $A\beta_{40}$ also acts as a modulator of some intrinsic checkpoint of autophagy, as in the AKT-dependent pathway or by increasing mitochondrial ROS [61,62].

Autophagy is also involved in the degradation of neurofibrillary tangles (NFTs), composed by hyperphosphorylated tau aggregated into bundles of filaments [63,64]. Tau is the major microtubule associated protein (MAP) of a mature neuron and two of its major functions are to promote microtubule assembly and to maintain their structure [65]. Under pathological conditions, tau reduction inhibits microtubule assembly favouring their disruption through the lack of the microtubule-promoting tau-tubulin assembly [65]. In AD, it has been reported that the main causes of dysfunctional tau are its conformational changes [66] and truncation [67] following hyperphosphorylation [68], such that six tau isoforms have been shown to be involved in NFT formation [69]. Distinct autophagic pathways are involved in tau degradation; the full-length molecule is preferentially degraded through macroautophagy, whereas a truncated tau is degraded by the CMA machinery [70]. The clearance of soluble tau species is likely to involve selective autophagy adaptor proteins to target tau to the autophagic machinery. Indeed, Hsc70 recognizes two CMA targeting motifs of tau which are involved in tau delivery to the lysosomal LAMP-2A receptor [71]. In this case, not only autophagosome-lysosome fusion and degradation appear to be important for amyloid clearance, but also the autophagic cascade. Indeed, mTOR signalling activation or inhibition correlate with tau levels and phosphorylation. It has been reported that treatment with rapamycin, an mTOR inhibitor and autophagy inducer through AMPK activation, ameliorated tau pathology and the associated behavioural deficits, indicating a role for autophagy in modulating tau levels [72]. All these results confirm that the autophagy-lysosomal pathway is heavily involved in Aβ and tau clearance and that aggregation and accumulation of these proteins/peptides result in autophagy dysfunction, leading to consider autophagy as a promising therapeutic target for AD and other degenerative diseases.

5. Autophagy and Mitochondria Dysfunction

Autophagy in AD is altered also for what mitochondria homeostasis is concerned. Mitochondria are double-membraned subcellular organelles mainly responsible for the processes of metabolic respiration. They provide most of the cellular adenosine triphosphate (ATP) demand by oxidative phosphorylation, generating the energy that fuels normal cellular function while monitoring, at the same time, cellular health to rapidly decide (if needed) to initiate programmed cell death. Unfortunately, the high demand for energy that drives ATP mediated cellular functions, also generates a significant amount of reactive oxygen species (ROS) as a by-product of ATP biogenesis [73]. Recently, mitochondria have been assigned the status of key players contributing to normal aging and onset of neurodegenerative diseases, notably AD [74]. Indeed, mitochondria are involved in neuronal differentiation and play key roles in developmental and adult neuroplasticity. For example, mitochondria regulate the differentiation and growth of the axon by buffering cytosolic Ca^{2+}, thus promoting polymerization of axonal microtubules [75]. The regulation of Ca^{2+} homeostasis is a very important issue, because perturbed neuronal Ca^{2+} levels result in neuronal death and are implicated in neurodegenerative diseases, including AD [76]. In the latter, the clearance of impaired mitochondria is hindered and is accompanied by increased oxidative stress, resulting in dramatic neuronal loss and cognitive dysfunction [77]. Indeed, mitochondria dysfunction and oxidative stress occur before Aβ plaque formation not only in AD-vulnerable brain regions [78,79] but also peripherally [80], such that it is considered an early event contributing to AD progression [81].

Physiologically, the damaged mitochondria fuse with a lysosome producing an autolysosome in which they are degraded by proteases. In AD brain, an accumulation of AVs, as described above, with reduced lysosome activity and consequently altered mitochondrial transport have been found [82]. Mitophagy, the molecular machinery that mediates targeting of mitochondria to lysosomes has been actively investigated, leading to identify two pathways of mitophagy activation. The first, PINK1/Parkin pathway, involves the protein PTEN-induced kinase 1 (PINK1) that stabilizes the outer mitochondrial membrane when mitochondria are damaged, that is when depolarization of the inner membrane occurs [83]. This activation involves also some ubiquitin-binding proteins such as p62 and NBR1 that recruit the mitochondria to the autophagy pathway and are needed for Parkin-mediated autophagy [83]. The second pathway includes other mitophagy receptors, AMBRA1 and Nix/BNIP3L. AMBRA1 can bind to LC3 inducing mitophagy in either a Parkin-dependent or a Parkin-independent way [84]. Nix/BNIP3L was originally reported as a mitophagy receptor needed to clear mitochondria in erythroid cells [85]; however, recent studies suggest that Nix can also induce mitophagy in other cell types, such as neurons, possibly as a downstream executor of the PINK1/Parkin pathway [86]. These mitophagy receptors bind to proteins associated with nascent autophagosomes via

LC3-interacting region (LIR) motifs in LC3 and GABARAP family proteins that are covalently bound to phosphatidylethanolamine in the phagophore membrane [87]. The formation of the protein bridges between the outer mitochondrial membrane and the membrane of the phagophore results in LC3-mediated elongation and in the closure, by GABARAP proteins, of the phagophore membrane, thus completely engulfing the mitochondrion inside the closed vesicle. The final stage of mitophagy is the fusion of the autophagosome with a lysosome, whose hydrolases degrade the mitochondrion.

Accumulating evidence suggests that mitophagy is important for prevention of age-related disease, including neurodegenerative diseases. The mitochondrial–lysosomal axis theory proposes that, during brain aging, many mitochondria undergo enlargement and structural disorganization with decreased ATP production, release of apoptotic factors and eventually cell death. These effects result not only from continuous oxidative stress, with oxidation of mitochondrial constituents and autophagocytosed material, but also from the inherent inability of cells to completely remove oxidatively damaged materials [88]. Consistent with these observations, microscopic analysis showed that granular immunoreactivity is found in neurons from AD people, consistent with autophagic vacuoles and increased cytoplasmic staining respect to age-matched and young controls [89].

Altogether, these observations support the notion that, in AD, increased mitochondrial degradation products are found in vulnerable neurons; this finding suggests either a greater turnover of mitochondria by autophagy within the cell body and/or a reduction of further proteolytic degradation leading to accumulation of intermediate products of mitochondria degradation. The investigations on these essential organelles in AD suggest that mitochondria dysfunction and oxidative stress are early contributors to AD progression, which makes mitochondria an appealing target for AD therapeutic strategies.

6. Epigenetic Regulation of Autophagy in AD

In addition to the activation of several transcription factors (see above), different epigenetic mechanisms, such as chromatin modulation, histone modification, and microRNAs (miRNAs) can regulate the expression of autophagy genes [90,91]. The most well-known modulator of genomic stability, DNA repair and transcriptional regulation is the NAD^+-dependent deacetylase sirtuin 1 (SIRT1). In mammalian cells seven different sirtuins (SIRT1-7) are present, structurally similar in their catalytic- and NAD-binding-domain but with different in their N- and C-terminal domains [92]. SIRT1 is involved in the regulation of autophagy genes expression through various ways of action. Firstly, SIRT1 deacetylates Lysine 16 on histone H4, (H4K16) its primary deacetylation target [93]. H4K16 deacetylation inhibits the transcription of genes involved in the early and late steps of autophagy. SIRT1 also plays an important role in modulation of the AMPK pathway [94]. Two hypothesis of AMPK activation have been

proposed: SIRT1 could activate AMPK directly [95] or by increasing and activating, trough deacetylation, the expression of PGC-1α-mediated gene, a substrate of SIRT1 [96]. In addition to the AMPK-SIRT1 relation in autophagy modulation, SIRT1 can stimulate autophagy by deacetylation of FOXO1, which subsequently triggers autophagy by increasing the expression of Rab7, a small GTP-binding protein that mediates autophagosome-lysosome fusion [97]. Moreover, SIRT1 deacetylates directly and NAD-dependently Atg5, Atg7 and Atg8 forming a complex that, deacetylates these proteins promoting autophagosome formation [98].

SIRT1 activity depends on the presence of NAD^+ but in AD models a drastic reduction of its levels has been observed, with effects on ATP production and impairment of cell functions. In the central nervous system, the reduction of NAD^+ levels under stress conditions correlates with excessive activation of poly(ADP-ribose)polymerase-1 (PARP-1), that induces alterations of mitochondria function, neuronal death and Aβ toxicity [99]. Furthermore, under pathological condition such as in the brain of AD patients, PARP-1 activation may lead to poly(ADP-ribose) (PAR) build-up particularly in neurons of the frontal and temporal lobes and in skin fibroblasts and lymphoblasts.

In this scenario, PARP1-SIRT1 cross talk has been proposed, considering that SIRT1 can inhibit PARP1, through deacetylation. Thus, it can be suggested that these two proteins might be able to counterbalance each other's activity to contribute to the control of the balance between cell survival and death [100].

Finally, intriguing connections between autophagy and the RNA research have become evident in recent literature. Post-transcriptional regulation of autophagy via microRNA (miRNAs) has been widely documented. miRNAs are small (21–25 nucleotides) single- stranded RNAs that bind to complementary nascent mRNAs making them susceptible to degradation prior to translation, thereby inhibiting the expression of specific target genes. miRNAs are highly conserved, and to date, thousands miRNA have been identified in plants and animals. In particular, miR-34 is linked to autophagy and longevity in several species. Recently, an extended life span has been reported in the *C. elegans* model carrying miR-34 loss-of-function mutations, but that extension was eliminated by RNA interference of autophagy genes such as bec-1/Becn1/VPS30/ATG6, atg-9, and atg-4 [101]. Several additional studies have related miR34 to autophagy regulation in mammalian cells, but its contribution to aging in mammals remains unclear. For example, in human cells, miR34 targets the cell death-regulating protein Bcl2, which directly binds to, and inhibits, the autophagy protein BECN1/VPS30 [102]. miR34 also inhibits the expression of SIRT1 [103]. Thus, age-related upregulation of miR34 could contribute to the aging process by directly modulating autophagy-related proteins expression. During physiological aging and in neurodegenerative diseases such as AD, Huntington, and Parkinson diseases, the expression of several autophagy-regulating miRNAs is altered [104]. However, it is not yet known whether disease pathogenesis is directly affected by such

deregulated miRNAs.

7. Autophagy A New Target for AD Therapy

Considering the different autophagy alterations observed in AD and other neurodegenerative disorders, several autophagy modulator drugs have been proposed for possible AD prevention/therapy (**Figure 2**).

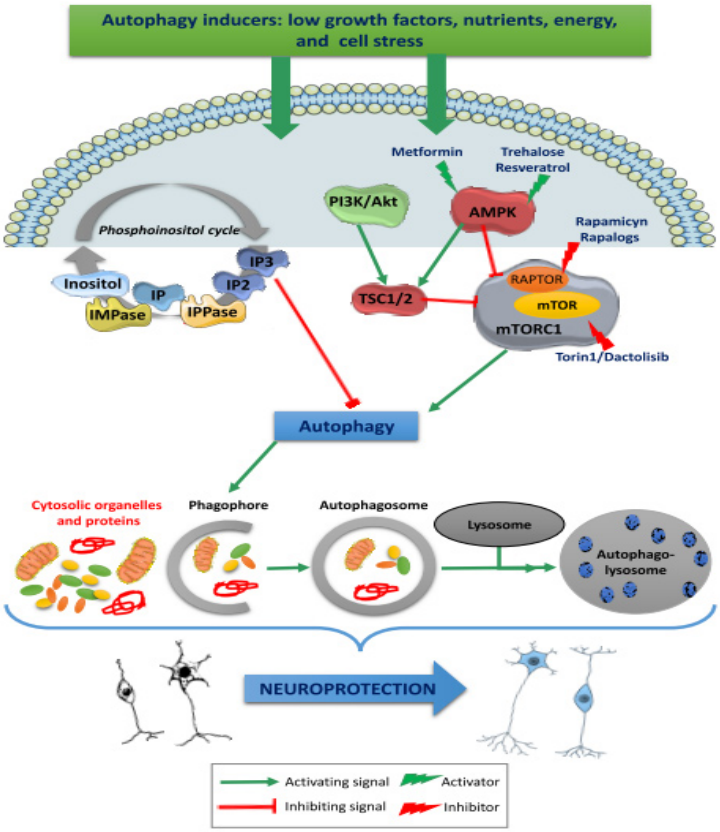

Figure 2: Some drugs activity on autophagy pathway (modified from Ma S et al. 2019) [105].

The most investigated autophagy modulators are rapamycin and its analogues. There is substantial evidence that the activation of the autophagic flux induced by all rapamycin analogues provides neuroprotective effects in experimental models of neurodegenerative diseases, preventing the accumulation of aggregation-prone proteins and increasing neuronal viability [106, 44]. Rapamycin and rapalogues enhance autophagy by stabilizing the raptor-mTOR association thus inhibiting mTOR. The efficacy of rapamycin as an inducer of autophagy is unquestionable, but the benefits of its chronic administration in a clinical setting are significantly hampered by undeniable and common adverse reactions, including infections of the respiratory and urinary tracts, gastrointestinal pain, and thrombocytopenia [107]. Nevertheless, rapalogues are currently under investigation for their ability to reduce the severity of neuronal loss in several proteinopathies, in view of their potential extension to long-term therapeutic treatment of neurodegenerative diseases [108]. Temsirolimus, an esterified rapamycin derivative displaying milder side-effects has recently been approved for treatment of advanced renal cancer [109].

The autophagy flux can also be modulated using mTOR/PI3K inhibitors and pan-mTOR inhibitors such as ATP analogues [110]. Pan-mTOR inhibitors, showing better antitumor properties than rapamycin, have also been studied for their neuroprotection. Among these, Torin1, an interesting novel compound yet with pharmacokinetic limitations, inhibits specifically the kinase domain of both TORC1 and TORC2 complexes without appreciable effects on PI3K; as a dietary supplement, Torin1 has revealed anti-aging properties [111,112].

Metformin, the first line drug against type II diabetes, whose long-lasting practice in human therapy has evidenced excellent tolerability, though with frequent gastrointestinal side-effects, is now attracting significant interest as an anti-aging molecule. Metformin pro-autophagic activity is mediated by the activation of the AMPK, which results, among others, in mTOR inhibition and stimulation of autophagosome formation [113]. Interestingly, metformin and other AMPK activators can also restore mitophagy-modulating PARKIN activity [114].

8. Autophagy Modulation by Plant Polyphenols

The importance of nutrition for disease prevention has been recognized since the days of Hippocrates 460-377 B.C. who said: "Let food be your medicine and medicine be your food". Diet is becoming an important factor for human health and can no longer be considered simply nutrition; rather, in the light of recent advances in research, especially nutrigenomics, it has been shown to be intimately linked via evolution and genetics to cell health status by modulation of autophagy/apoptosis, detoxification, and appropriate gene response.

The positive actions of nutritional supplements, minerals, and plant extracts in disease prevention are now mainstream and commercially important, such that their health claims are subject to strict regulation in most countries. Recently, epidemiological studies suggest that a role in maintaining health and protection against disease is not only played by the deficiency, in the diet, of a particular element but also by the presence of adequate amount of various other compounds from fruits and vegetables [115]. In particular, the Mediterranean (MD) and Asian diets are traditionally high in fruits, vegetables, legumes and cereals, with moderate consumption of oily fish and dairy, low in meat, sugar and saturated fat and with moderate alcohol, taken mainly with meals.

An intriguing novelty in the research of neuroprotective compounds was the description of the anticancer, cardioprotective, antiaging and antioxidant properties of the plant polyphenols, either flavonoids, such as kaempferol, quercetin, resveratrol and curcumin [106-119] and secoiridoids such as oleuropein (see next section) and ligstroside. It was reported that resveratrol induces autophagy through the stimulation of AMPK- and PARKIN-mediated mitophagy [120,121,122], as confirmed in cellular and mouse models of AD, where it has been shown to provide neuroprotection [123]. Kaempferol has been reported to improve the autophagy flux by enhancing the autophagosomal marker LC3-II and activating mitophagy by promoting

mitochondrial fission [120]. Quercetin has been shown to potentiate proteasomal function and the autophagic cascade [118]. Curcumin has been reported to increase the expression of LC3-II and Beclin-1 but also to provide endoplasmic reticulum stress with calcium release and destabilization of the mitochondrial compartment eventually resulting in apoptosis, thus providing a molecular basis of the crosstalk between autophagy and apoptosis [124].

Recently, it was reported how polyphenols could act also on epigenetic regulation of autophagy. Resveratrol was shown to be involved in the activation of the PGC1α/SIRT1/AMPK axis and in the regulation of miR-34a and miR-30a that targets transcripts encoding Beclin1 [119,125]. The same results on the SIRT1 path were reported also for the (2)-epigallocatechin-3-gallate (EGCG). Moreover, EGCG leads to re-expression of autophagy genes due to inhibition of a specific DNA methyltransferase 2 (DNMT2) methylation activity, that resulted significantly expressed in macrophages derived from aging mice in association with hypermethylation of the promoter region of Atg5 and LC3B [125,26].

Most of the lipid content in the MD comes from olive oil, whose pharmacological properties, especially those associated with the olive fruit and leaves of the variety *Olea europaea L.*, have been recognized as important for a healthy diet for their phenolic content [127]. Over 100 different polyphenols have been described in olive samples, including hydroxythyrosol (HT), tyrosol and their secoiridoids: oleuropein (OLE), ligstroside, oleocanthal and their aglycones, particularly of oleuropein (OleA) [128,129]. OleA and its main metabolite, HT are the main polyphenols in the EVOO and extensive research on their positive effects on health has been carried out. OleA is one of the best characterized polyphenols as autophagy inducers [130]; it possesses several pharmacological activities, including antioxidant [131], anti-inflammatory [132], anti-atherogenic [133], anti-cancer [134], antimicrobial [135], and antiviral [136] properties. In addition, it has been shown to be cardioprotective against acute adriamycin cardiotoxicity [137] and has been shown to exhibit anti-ischemic and hypolipidemic activities [138]. For these reasons, it is commercially available as food supplement. It should also be considered that OleA interaction with cell membranes would increase its local concentration, which could be particularly relevant considering the beneficial effects that would be attained above critical concentration of the molecule and its reduced bioavailability.

In terms of neuroprotection, recent studies have shown that OleA reduces cognitive impairment and improves synaptic function in animal models. This is due to the inhibition of the aggregation and toxicity of Tau [138] and Aβ [139], the epigenetic modulation by histone acetylation [140], the reduction of astrocytosis and modulation of astroglia activity, and the induction of autophagy [141]. The activity of OleA as an autophagy inducer was described in wild-type and TgCRND8 mice a model of Aβ deposition, fed with OleA (50 μmg/kg of diet) for 8 weeks [141,142]. In this study, the treated mice exhibited a remarkable increase of autophagic vesicles in the soma and dendrites of neurons from different parts of cerebral

cortex, as shown by the increased levels of Beclin-1 and LC3II/LC3I ratio respect to untreated littermates. OleA also improved the autophagosome-lysosome fusion measured as increased p62 and cathepsin B levels in OleA-fed mice, suggesting that the functional degradation phase of autophagy was at work. Similar results in the same murine model were obtained by diet supplementation with the same amount of HT [143] or with a polyphenol-rich cake obtained by drying waste water of olive mills [144].

The molecular mechanism of autophagy induction by OleA was also investigated. It was shown that it proceeds through the increase of cytosolic Ca^{+2} and the subsequent activation of AMPK by phosphorylation by Ca^{2+}/Calmodulin Protein Kinase Kinase β (CaMKKβ), with ensuing ULK1 activation and mTORC1 inhibition, with induction of autophagic vacuoles [145]. Similar effects were reported in the case of resveratrol, which was shown to activate PKA that, in turn, activated AMPK by phosphorylation [146]. It was also shown that the treatment of TgCRND8 mice with OleA reduced the activation of PARP1 at both RNA and protein levels as well as the subsequent accumulation of PAR polymers [147]. PARP1 activation, as described above, causes a reduction of NAD^+ levels that result in inhibition of SIRT1 [148] deacetylases that act on many transcription factors such as p53, NF-κB, and FOXO. Therefore, the increased NAD^+ levels through OleA-mediated PARP1 reduction induced SIRT1 activation in TgCRND8 mice [149]. These effects were confirmed in N2a cells treated with OleA for 24 h, where PARP1 activation by methylnitronitrosoguanidine (MNNG), a mutagen that activates PARP1 expression, was reversed and SIRT1 and Beclin-1 levels were increased [148].

In summary, the cognitive improvement of plaque deposition in animal models such as TgCRND8 mice indicates that diet supplementation with OleA may also display beneficial effects in slowing cognitive decline in humans. This conclusion is also supported by the studies showing that OleA, either as such or through its derivatives crosses the blood-brain barrier and develops neuroprotection in the brain, where a decrease of deposited plaques and a significant activation of autophagy were observed. Actually, OleA has been found in the brain of rats fed with this polyphenol, even though in minute amounts [150]; in addition HT and some metabolites such as homovanillic acid were found in CRND8 mice 2 h after an acute gavages of administration of OleA (50 mg/Kg) [148]. Finally a recent report shows brain accumulation of HT and some metabolic derivatives after 21 h of diet supplementation to rats of HT, oleuropein or a secoiridoid extract(5 mg/kg rat weight/day) [151]. These results show that, in spite of their reduced bioavailability, these molecules are effectively absorbed , circulate in the blod strean for a contenient time and cross the blood brain barrier in significant amounts. They also suggest a shared molecular mechanism underlying the healthy effects of these substances against ageing and neurodegeneration implying autophagy dysfunction.

Overall these data currently available show that polyphenols can help to counteract aging and aging-associated neurodegeneration, notably preventing or treating cognitive impairment,

even though most of the pleiotropic effects of polyphenols remain largely unexplained. These results have prompted the researchers to translate studies with polyphenols from cell and animal models to humans. Curcumin and resveratrol are currently the most investigated compounds in clinical trials [152].

9. Conclusion

Neuronal deregulation of autophagy impairs the balance of energy and nutrient homeostasis as well as of proteostasis, which likely contribute to AD pathogenesis. Given recent findings, the regulation of autophagy to reduce the deposition of aberrant protein aggregates and to improve normal brain cell homeostasis represents a new and promising pharmacological target for drug development.. Further knowledge regarding the mechanisms of autophagy and the therapeutic relevance of polyphenols will help to identify novel therapeutic strategies with clinical relevance. Accurate studies on the effective daily dose of polyphenols to be administered to humans to get significant protection are still lacking. Data about the bioavailability of these molecules are scarce due to several factors hindering its absorption, tissue distribution and intracellular penetration; yet the presence in the brain of animal treated with doses not far from those available with dietary supplementation of these molecules has been reported. Our knowledge is far from complete and there is still much to learn about autophagy, its role in AD and the beneficial effects of plant polyphenols for AD prevention and therapy in humans. Nonetheless it appears evident that therapeutic strategies that enhance autophagy have the potential to be beneficial in AD.

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