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The autophagy-lysosomal degradation pathway: role in neurodegenerative disease and therapy

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1. ABSTRACT

Alterations in the autophagy-lysosomal degradation pathway have been described in normal brain aging and in age-related neurodegenerative diseases including Alzheimer's (AD) and Parkinson's (PD). An improper clearance of proteins in AD and PD may result either from a compromise in the autophagy-lysosomal degradation pathway or induce alterations in this pathway, and may result in neuron dysfunction and neuron loss. This review provides an overview of AD and PD with a specific focus on macroautophagy, chaperone-mediated autophagy and lysosome function in human and experimental models of AD and PD. Potential therapies for AD and PD are also discussed that may promote survival by regulating the autophagy and lysosomal degradation pathway.

2. INTRODUCTION

Neurodegenerative diseases, including Alzheimer's disease (AD), Dementia with Lewy bodies (DLB), and Parkinson's disease (PD), affect more than 4 million people in the US. These diseases are age-dependent and progressive and exhibit characteristic protein inclusions, synapse loss and brain region-specific neuron death. Apoptosis or type I cell death (1) has been extensively studied in AD and PD in an attempt to provide a mechanistic link to neuron loss and to delineate potential therapies that combat against neuron loss in these diseases. Molecules that promote or mediate apoptosis have been found to be activated in AD and PD, and thus have been studied as potential targets for AD and PD therapy (2). However, the role of apoptosis as the principal death

mediator in neurodegenerative diseases has been questioned (3; 4). For one reason, there are many cell death pathways that are either caspase-independent or activate multiple death pathways that are both caspase-dependent and –independent which have been implicated in the etiology of age-related neurodegenerative disease. Type II cell death, or autophagic cell death (1) results from alterations in the autophagy-lysosomal degradation pathway, a complex and tightly regulated series of signaling events that promotes the efficient delivery of macronutrients and organelles to lysosomes for degradation by acidic hydrolases (5). Interestingly, the autophagy-lysosomal degradation pathway has been shown to be altered as a function of normal human aging and may also play a role in the onset and progression of age-related neurodegenerative disease. Furthermore, multiple cell death pathways have been linked to alterations in the autophagy-lysosomal degradation pathway, suggesting that therapies that target this pathway may prevent neuron loss due to multiple death stimuli. In this review we provide an overview of the autophagy-lysosomal degradation pathway, discuss alterations in this pathway specific to AD and PD, and discuss candidate therapies in AD and PD that may act by regulating the autophagy-lysosomal degradation pathway.

2.1. The autophagy-lysosomal degradation pathway

Autophagy is defined as the homeostatic delivery of macronutrients and organelles to the lysosome for pH-dependent degradation, and has been shown to play an important role in responding to intracellular energy demands and maintaining energy balance. To date, three major types of autophagy have been described (6): macroautophagy, whereby bulk cytoplasm and organelles are enclosed within vesicles and are delivered via a series of vesicular fusion events to the lysosome for degradation by lysosomal hydrolases that function optimally at low pH; chaperone mediated autophagy (CMA), whereby proteins with "KFERQ" motifs are selectively shuttled via molecular chaperones including hsc70 to lysosomes, where lysosomal membrane-bound receptors (Lamp2a) selectively internalize these proteins into the lumen of lysosomes for degradation; and microautophagy, a process of direct nutrient uptake by lysosomal membranes. Recent evidence suggests that the degradation of mitochondria by macroautophagy, termed "mitophagy", may be a more selective form of macroautophagy and has been implicated in neurodegenerative disease (7-9). Both macroautophagy and CMA have been extensively characterized in mammalian systems, and alterations in both have been shown to be altered in models of normal aging and in age-related neurodegenerative disease (10; 11). As such, macroautophagy and CMA will be the principal focus of this review (Figure 1).

During macroautophagy, a double-membraned vesicle is synthesized that encircles cytoplasm including long-lived proteins and/or damaged organelles, forming an autophagosome, which is considered an immature form of autophagic vacuole (AV). The limiting double membrane is thought to arise from the endoplasmic reticulum, although the golgi complex has also been implicated as a source (5; 12). These double-membraned vacuoles subsequently undergo a series of fusion events with different types of vesicles, including endosomes or multivesicular endosomes and ultimately with lysosomes, forming a single-membraned autophagolysosome (Figure 1). The autophagolysosome has a lower pH resulting from fusion with acidic lysosomes, which allows for the degradation of macromolecules by lysosomal hydrolases that act optimally at low pH.

Macroautophagy is regulated by a complex series of interactions between "Atg" proteins, which were first characterized in yeast with many homologs found subsequently in mammalian systems (5; 13). Atg proteins have been implicated in regulating specific facets of macroautophagy (Figure 1). The binding of Atg1 with Atg13 is thought to be important for the induction of macroautophagy. In addition, Atg6 (known also as Beclin-1), by binding to Vps34, a class III phoshpatidylinositol 3-kinase, has been implicated in the "vesicle nucleation" step of macroautophagy which induces formation of the limiting double membrane around cytoplasmic contents. Two distinct groups of Atg proteins, Atg3/4/7/8 and Atg5/7/10/12, mediate a series of ubiquitin-like conjugation events which are important for the expansion and completion of the double membraned autophagosome (Figure 1). LC3 is the mammalian homolog of yeast Atg8 and the cleavage of its C-terminal arginine by Atg4 exposes a glycine residue that is utilized for subsequent conjugations with Atg 7 and Atg 3, resulting ultimately with its attachment to a phosphatidylethanolamine group (14). The cleaved and lipidated form of LC3 is called LC3-II, and its lipidation allows for its insertion into the membrane of autophagosomes (14). LC3-II is considered a selective marker for autophagosomes and its immunodetection is used widely as a marker of autophagosome accumulation. The importance of macroautophagy for cell survival has been highlighted in studies of Atg gene manipulation. Atg5 and Atg7 gene-deficient mice develop severe neurodegeneration (15; 16) whereas over-expression of Atg1 leads to apoptotic cell death (17).

The principal function of macroautophagy is the maintenance of intracellular energy requirements, and the induction of macroautophagy appears to be negatively regulated by activation of the protein mammalian target of rapamycin (mTOR) (Figure 1). During nutrient starvation, macroautophagy is induced by a decrease in trophic factor signaling and a resultant decrease in class I PI3-K/Akt signaling (Figure 1). Akt phosphorylates and inactivates tuberous sclerosis complex (TSC)1/2 proteins, which when active negatively regulate activation of mTOR (18). In its active state, mTOR inhibits macroautophagy by stimulating the phosphorylation of Atg13, which leads to a decreased affinity of Atg13 for Atg1 and a subsequent inhibition in the induction of macroautophagy. Rapamycin is a potent inducer of macroautophagy due to its inhibition of mTOR and has been proposed in therapies where a stimulation of macroautophagy may be cytoprotective (19). Homeostatic levels of amino acids are also known to negatively regulate macroautophagy, likely through mechanisms that maintain activation of mTOR. PTEN catalyzes the hydrolysis of

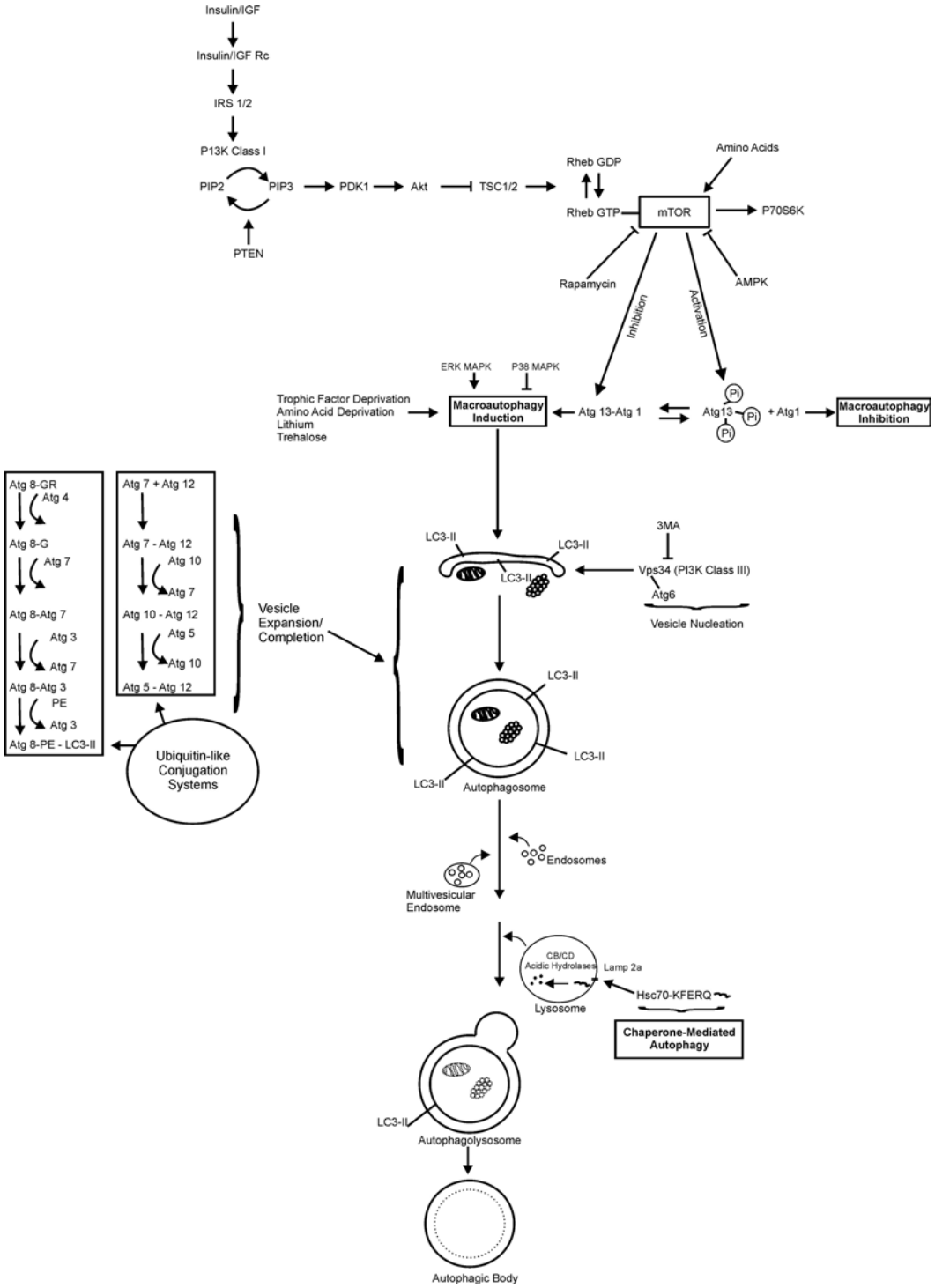

Figure 1. The autophagy-lysosomal degradation pathway. Macroautophagy is exquisitely regulated by a cascade of signaling events that lead to either mTOR inhibition (macroautophagy stimulation) or mTOR activation (macroautophagy inhibition). Upon macroautophagy stimulation, a complex series of events is triggered, including "vesicle nucleation" mediated by the Vps34/Atg6 complex, and "vesicle expansion and completion" mediated by the ubiquitin-like conjugation of Atg proteins, leading to the formation of a double-membraned autophagosome around cytoplasmic contents. Through a series of fusion and maturation events the autophagosome ultimately fuses with the lysosome, forming the autophagolysosome that has lysosomal hydrolases that act at low pH to effectively degrade macromolecules. In chaperone-mediated autophagy, specific proteins with "KFERQ" sequences are shuttled to the lysosome via cytoplasmic molecular chaperones such as hsc70, where proteins are internalized into the lumen of the lysosome following their binding to membrane-bound Lamp-2a receptors.

phosphatidylinositol trisphosphate (PIP3) to phosphatidylinositol bisphosphate (PIP2), and as such negatively regulates class I PI3-K (18) and serves as a potential inducer of macroautophagy. An induction in macroautophagy can account for as much as 75% of the overall protein degradation in nutrient starved hepatocytes (20-23). Interestingly, the stimulation of ERK MAPK has been shown recently to induce macroautophagy in an Atg6/PI3-K-independent manner (9; 24), suggesting that there are multiple pathways leading to the induction of macroautophagy (Figure 1). Inhibition of p38 MAPK has also been implicated in the inhibition of macroautophagy (24); (Figure 1).

The lysosome is an acidic membrane-bound organelle containing several different lysosomal hydrolases that are important in protein degradation. Lysosomal enzymes are synthesized in the endoplasmic reticulum, sorted to the trans-Golgi network by mannose 6-phosphate receptors, transported through the endosomes to arrive at their lysosomal destination, where they are activated upon exposure to the acidic environment. Lysosomal function declines with age in the human brain (25), and may contribute to a decrease in macroautophagy and CMA observed with aging and in age-related neurodegenerative disease (10). Agents that disrupt lysosome function (26-28) inhibit the completion of macroautophagy and result in a robust accumulation in AVs that is typical of many age-related neurodegenerative diseases (29). Enhancing macroautophagy itself, such as through treatment with rapamycin, may help clear aggregated proteins (30; 31) and may be a response of neurons in age-related neurodegenerative disease to an increased demand for protein degradation. However, because both macroautophagy and CMA are dependent on intact lysosomes for proper function, the progressive impairment of lysosome function in aging and in age-related neurodegenerative disease may override any long-term benefits derived from the over-stimulation of macroautophagy. In fact, excessive stimulation of macroautophagy resulting from nutrient starvation was shown *in vitro* to be initially protective, but ultimately resulted in "autophagic" cell death (32).

An interrelationship exists between macroautophagy and CMA, but the relative roles of each pathway in regulating intracellular degradation is still unclear. For instance, inhibition of CMA was shown to cause an up-regulation of macroautophagy (33). However, a deficiency in the lysosomal membrane receptor Lamp-2, which is important for CMA, was shown to have little effect on the overall rate of protein degradation (34). While macroautophagy is known to be induced by nutrient starvation, hypoxia, neurotrophic factor deprivation, excitotoxins and accumulation of protein aggregates through PI3K and ERK-mediated pathways (9; 35), the regulation of CMA is unclear. Conceivably, levels of hsc70, Lamp-2a, CMA substrates or lysosomal proteases may all influence the rate and effectiveness of CMA. An important focus for future studies will be to delineate the interplay between macroautophagy and CMA in the hopes of developing effective therapies that maximize the clearance of potentially toxic protein aggregates.

3. AUTOPHAGY AND LYSOSOME FUNCTION IN AD

3.1. Overview of AD

AD is the most prevalent human neurodegenerative disease and the most common form of dementia. AD is characterized by several neuropathological hallmarks including widespread neuron loss and decreased synaptic density, concomitant with the accumulation of extracellular amyloid-containing senile plaques and neurofibrillary tangle-bearing neurons. Atrophy of the cortex, hippocampus and amygdala are commonly observed in AD in addition to neuron loss in the nucleus basalis of Meynert of the basal forebrain (36; 37). Cholinergic neurons are prominently affected in AD (37), and are known to play a major role in memory formation and consolidation (38). The vast majority (95%) of AD cases are sporadic, without a direct genetic link. However, the apolipoprotein E ε4 allelle (Apo E ε4) has been identified as a risk factor in the development of sporadic AD. The remaining 5% of AD cases are familial in nature and exhibit a much earlier age of onset. Familial AD has been clearly linked to specific mutations in several genes including beta amyloid precursor protein (APP), presenilin 1 (PS1) and PS2. A major focus in AD research has been to delineate the consequences of these mutations and as such we now have a better understanding of the pathophysiology of familial AD, which has also provided clues to the pathogenetic mechanisms contributing to sporadic AD. Despite major advances in our understanding of AD pathophysiology, therapeutics that are currently available for clinical management have at best only slightly delayed the progression of AD by months and not years. Taken together it is clear that major gaps still exist in our understanding of this devastating disease.

There is a wide divergence among the AD scientific community regarding which neuropathological hallmarks of AD are causal and/or contributory to its clinical onset and progression, and as such which should be the target of therapeutics development. For instance, many investigators believe that neuronal dysfunction and death in AD are critically mediated by disturbances in the cytoskeletal protein tau, while others purport the importance of altered processing of APP and an aberrant increase in specific Aβ cleavage products. In addition, the contribution of neuron loss vs. synaptic dysfunction to the onset vs. progression of AD is unknown, and whether therapies which promote neuron survival in AD also restore neuronal function is unclear. Neuron loss in AD has been hypothesized to be caused by a specific increase in apoptosis, but there is a general discordance in the literature regarding the detection of apoptotic markers and morphology in AD brain, studies which are complicated by the asynchronous nature of apoptotic cell death in age-related neurodegenerative diseases including AD. Over the last twenty years a growing body of literature has implicated aberrant macroautophagy and lysosome function

as reliable markers of AD pathophysiology, which may be contributing factors to multiple types of neuron death and dysfunction in AD. Aberrant macroautophagy has also been linked to AD mutations and as such may provide a mechanistic link between familial and sporadic onset AD. In the following sections we will review the evidence for aberrant macroautophagy and lysosome dysfunction in AD and will discuss potential AD therapeutics that may act by regulating macroautophagy and lysosome function.

3.2. Disruption of the endosomal-lysosomal system in AD

Alterations in the endosomal-lysosomal system are one the earliest findings in AD brain, and precede the formation of plaques and tangle-associated neuropathology in susceptible neuron populations (39). Endosome enlargement occurs early in sporadic AD, and is associated with increased endocytosis and endosome recycling (40). Evidence suggests that endosome alterations may be specific to sporadic AD, since endosome populations are normal in appearance in AD brains of patients with PS1 or PS2 mutations (39), suggesting that endosomal alterations are not critical for Aβ formation and deposition. Nevertheless, Aβ immunoreactivity is evident in these populations of enlarged endosomes prior to Aβ deposition (41), indicating their potential importance for Aβ formation in early AD brain. Interestingly, the inheritance of the ApoE ε4 allele accelerates endocytic pathway alterations in sporadic AD, suggesting a mechanistic link for this risk factor in the disruption of endosomal-lysosomal signaling in AD. Abnormal endosomes in AD brain have been shown to exhibit an increase in β-secretase, which may lead to increased formation of Aβ within lysosomes. These endosome populations also exhibit increased levels of cathepsin D (CD) (42; 43), which has been shown to possess inherent β and γ secretase activity (44-47),although CD deficiency was shown previously to have no effect on APP processing in an animal model (48). The increased accumulation of CD in endosomes of sporadic AD patients may be related to the increase in mannose 6-phosphate receptors in AD brain, which are important for delivering CD to early endosomes (49).

Lysosome proliferation has also been documented in AD concomitant with enhanced activity and levels of the lysosomal hydrolases CD and CB (40; 42; 50-54). In addition, both CB and CD have been localized to amyloid-containing plaques (50; 51). At present it is not entirely clear if an increase in lysosomal proliferation and alterations in cathepsins are pro-survival or pro-death in AD brain. An up-regulation in the autophagy-lysosomal degradation pathway may be a response to increased macronutrient recycling to maintain the energy demands of "stressed" neurons in AD brain. While this may be the case in early AD, the progression of AD may ultimately result in increasing neuron damage concomitant with a disruption of lysosomal function, as has been shown previously by the addition of exogenous Aβ to cultured neurons (55; 56). Recent evidence suggests that increased CB in AD brain may actually serve a protective role. CB was shown to be anti-amyloidogenic, as indicated by the CB-induced decrease in the more "amyloidogenic" $A\beta_{1-42}$, and by an increase in plaque deposition in CB-deficient mice (57).

3.3. The pathological accumulation of AVs in AD

The accumulation of AVs in AD was recently confirmed by Nixon and colleagues upon the analysis of cortical biopsies obtained from AD patients (58; 59). Ultrastructural analyses indicated robust increases in AVs in dystrophic neurites, in particular those with terminal fields in proximity to Aβ-containing senile plaques. AVs were also apparent in dendrites that were not markedly dystrophic. In addition, the perikarya of AD cortical neurons showed a greater than 20-fold increase in AVs concomitant with a decrease in numbers of mitochondria. Interestingly, the mitochondrial marker lipoic acid was shown recently to co-localize to dense, lipofuscin-positive granules in AD brain, which further implicates an attempt to recycle potentially damaged mitochondria by macroautophagy (60). Furthermore, AD neurons exhibiting paired helical filaments appeared more likely to exhibit AVs in the perikarya compared to neurons lacking paired helical filaments (58).

Dystrophic neurites contained abundant AVs that were either immature (double-membraned) or mature (single-membraned), and contained organelles and osmiophilic, electron-dense debris. The presence of multivesicular bodies, which fuse with AVs, and multilamellar bodies, which are also formed during macroautophagy (61) were also noticed in dystrophic neurites. Some neurites contained AVs that were homogeneous in nature whereas other neurites contained multiple species of AVs. The presence of immature AVs in some dystrophic neurites, and late-stage autolysosomes in others suggests that autophagy is differentially but significantly affected in AD neuronal subpopulations. Western blot analysis also indicated an increase in LC3-II immunoreactivity specific to human AD brain (59), confirming results of ultrastructural analyses indicating an increase in the population of immature AVs.

Recent data indicates a decrease in levels of activated (phosphorylated) mTOR in cells treated with Aβ, in the cortices of mice harboring the PS1/APP mice, and in lymphocytes of AD patients (62), which suggests a mechanism by which macroautophagy may be induced in AD. However, a different study reported a robust increase in mTOR phosphorylation in neurons of AD brain (63), suggesting an inhibition of macroautophagy in AD. It is not entirely clear how levels of mTOR affects AD pathogenesis, but recent evidence indicates that treatment with rapamycin induces Aβ production concomitant with AV accumulation (59), Further studies are warranted to determine the role of mTOR activation in AD onset and progression and if the regulation of mTOR activation in AD brain is specific to distinct neuron populations.

The PS1/APP transgenic mouse model of AD, which expresses mutant human PS1 and the Swedish variant of Aβ and leads to the deposition of Aβ after 10 weeks (64) has also been shown recently to accumulate AVs (59). By 9 months of age, PS1/APP transgenic mice

displayed a greater than 20-fold increase in populations of immature and mature AVs versus age-matched, non-transgenic controls. There was massive accumulation of AVs in dystrophic neurites similar to human AD brain, but normal-appearing dendrites also displayed a modest increase in AVs. A striking observation was that a significant increase in AVs was observed in younger PS1/APP mice prior to Aβ deposition or the onset of other markers of neuropathology. Together these findings suggest that altered neuritic macroautophagy, consisting of both its induction in damaged neurites and its inhibition due to decreased vacuolar recycling (35; 65), may contribute to the neuropathological phenotype of AD.

3.4. The autophagy-lysosomal degradation pathway and intracellular Aβ

Growing evidence suggests that alterations in the autophagy-lysosomal degradation pathway may lead to an increased production and accumulation of intracellular Aβ. Both *in vivo* and *in vitro* treatment with the lysosomotropic agent chloroquine, known to cause an inhibition of macroautophagy (27; 66) was shown to accumulate levels of intracellular Aβ (67; 68). In addition, the stimulation of macroautophagy by amino acid deprivation or treatment with rapamycin was shown to increase levels of Aβ production and secretion, concomitant with an increase in AV formation (59). Together these studies emphasize the potential for both stimulation as well as inhibition of macroautophagy to contribute to the onset and progression of the AD phenotype. The "triple transgenic" mouse model for AD, which harbors mutant tau, APP and PS1 human mutations, was shown recently to exhibit an increase in intraneuronal Aβ accumulation that correlated with the onset of behavioral deficits (69), further implicating the role of altered intracellular Aβ processing in AD.

In AD brain, brains of transgenic mice and cultured cells, Aβ has been localized to different populations of intracellular vesicles including AVs (59), endosomes (70) and mutivesicular bodies (71; 72). The accumulation of Aβ in AVs was also shown to coincide with increased PS1 and nicastrin, which form parts of the β-secretase complex, as well as robust levels of β-secretase activity (59), suggesting that AVs possess the ability to generate intracellular Aβ from APP. These results strongly suggest that the accumulation of AVs in AD may provide a mechanism for aberrant intracellular production of Aβ, but further studies are required to associate intracellular Aβ production in AVs with extracellular deposition in plaques.

A causal relationship between Aβ, lysosome dysfunction and neuron death in AD is suggested by a variety of studies. Treatment of neurons with $A\beta_{1-42}$ induced lysosome dysfunction (56; 73) and activation of CD (74), suggesting that intracellular Aβ may provide a feed-forward inhibition of lysosome function. Lysosomal dysfunction may result from Aβ-induced oxidative damage and/or accumulation in lysosomes (73; 75). Oxidative stress has been shown to accumulate Aβ in lysosomes as a result of nascent autophagosome formation, since the inhibition of macroautophagy prevented the oxidative stress-induced increase in lysosomal Aβ (76). Oxidative stress has been shown to induce lysosome damage and precede activation of the mitochondrial or intrinsic apoptotic pathway (77-80). Further studies are needed to delineate the precise connections between aberrant intracellular production of Aβ with lysosome dysfunction and neuronal demise in AD.

3.5. Granulovacuolar degeneration in AD

One of the earliest descriptions of aberrant autophagy in AD may be that of granulovacuolar degeneration (GVD) by Simchowicz in 1911(81). GVD is one of the pathological hallmarks of AD and is defined by the cytosolic appearance of one or more translucent vacuoles that are 3-5 microns in diameter and contain an electron-dense, granular core (82). The localization of GVD in AD is relatively selective for hippocampal pyramidal neurons (83). The reason for this selectivity is currently unknown but may be due in part to the distribution and/or regulation of proteins specific to the hippocampus. For instance, there is isoform-specific expression of casein kinase-1 family of phosphotransferases in GVD vs. neurofibrillary tangles (84; 85), suggesting that the accumulation of distinct phosphoproteins may promote hippocampal specificity to GVD formation.

Many structural proteins are known to localize to GVD, including neurofilament (86), tau (87; 88), tubulin (89) and ubiquitin (90), which supports a role for the autophagy-lysosomal degradation pathway in GVD-laden neurons. Electron microscopic analysis of GVD in hippocampal neurons indicated the presence of double-membranes (91), a convincing argument for the presence of AVs. Interestingly, there has been a paucity of reports indicating the direct co-localization of lysosomal markers within GVD, although the lysosomal membrane marker Lamp-1 was shown recently to be up-regulated in AD hippocampal neurons that preferentially exhibit GVD (92). GVD may represent a population of AVs that have not yet fused with lysosomes, and their accumulation in hippocampal neurons suggests a type of lysosome dysfunction relatively specific to hippocampal pyramidal neurons that precludes their degradation.

Immunohistochemical analysis of AD brain has revealed intense cleaved caspase-3 immunoreactivity in neurons with features of GVD (93-95). Interestingly, these cleaved caspase-3 immunoreactive neurons do not exhibit nuclear apoptotic morphology, although in one study there was an increase in TUNEL reactivity co-localized to GVD-positive neurons suggesting some form of DNA damage (95). It is not exactly clear how to interpret the GVD-specific localization of cleaved caspase-3. Activation of caspase-3 may possibly occur within GVD, although enzymes with the potential to activate caspase-3 have yet to be localized to GVD. Another issue is whether GVD serves as a cell-survival response that sequesters apoptosis activation from ultimately killing the cell or if the putatively aberrant autophagy and lysosome function that occurs in GVD ultimately leads to caspase-3 dependent apoptosis. These issues are further compounded by the

Table 1. Markers localized aberrantly to GVD in AD brain

Marker	References
Neurofilament	86
Tau	87-88
Tubulin	89
Ubiquitin	90
Cleaved Caspase-3	93-95
Casesin Kinase-1	84-85
Lamp-1 (in neurons with GVD)	92
JNK MAPK	96
c-Jun	96
GSK 3-β	97
Phospho-Smad 2	98

asynchronous nature of apoptotic cell death that occurs over a period of years in AD (3), in addition to the terminal nature of the post-mortem human brain that precludes determination of the time course of neuron death.

Other potential death markers have been localized to GVD in AD hippocampal neurons. For instance, activated forms of c-Jun transcription factor, c-Jun-N-terminal mitogen activated protein kinase (JNK MAPK) and glycogen synthesis kinase 3-beta (GSK 3-beta) have all been localized to GVD (96; 97) which may play a role in the phosphorylation of structural proteins in AD. In addition, phospho-Smad2 has also been localized ectopically to GVD apart from its normal nuclear localization, which may indicate a defect in TGF-β signaling pathway which is thought to be neuroprotective in AD (98). A compiled list of markers found to localize to GVD, or at least to neurons exhibiting GVD (such as Lamp-1) can be found in Table 1. Future studies will be important to determine the importance of GVD for hippocampal neuron death vs. survival in AD and whether the accumulation of specific proteins in GVD is mediated by a disruption in the autophagy-lysosomal degradation pathway.

3.6. Calpain activation in AD and the link to macroautophagy and neuron death

Calpains are important modulators of intracellular calcium signaling. When activated by intracellular calcium, they have been shown to regulate both apoptotic and non-apoptotic neuron death via their enzymatic cleavage of select intracellular proteins (99). Increased calpain activation has been documented early in the course of AD and has been proposed to play a role in cytoskeletal changes leading to the accumulation of neurofibrillary tangles (100; 101). Calpain activation has also been implicated in Aβ-induced death of cultured neurons (102; 103). Calpain activation-induced apoptosis has been proposed to be mediated in part by the cleavage of pro-apoptotic Bax (104), which has been shown to accelerate apoptosis (105; 106), and also by a decrease in levels of anti-apoptotic Bcl-2 (107). Conversely, calpain activation is also associated with an inhibition of apoptosis and the promotion of caspase-independent neuron death (108; 109), which may suggest stimulus- and cell-type specific roles of calpains in regulating neuron death.

Recent evidence suggests a direct link between macroautophagy and calpain-induced cell death. Calpain activation was shown to cleave Atg5, which allows for its translocation to mitochondria where it binds to Bcl_{XL} and triggers the intrinsic apoptotic pathway (110). Conversely, a protective role for calpains may lie in their ability to stimulate macroautophagy, since calpain-deficient cells were unable to initiate stimulus-induced enhancement of macroautophagy, an effect that was concomitant with robust enhancement of apoptotic death (111). Stimuli that trigger the increased mobilization of intracellular calcium have been shown recently to stimulate Beclin and Atg7-dependent macroautophagy by inactivating mTOR (112), which provides indirect evidence for the role of calpain activation in promoting macroautophagy. Calpain-induced neuron death has also been shown to activate ERK MAPK, which has been documented in AD brain (113) and has been shown to stimulate Atg6/Beclin-independent macroautophagy *in vitro* (9). Taken together, these findings suggest that calpain activation may have different consequences over the course of AD progression. In early AD, calpains may stimulate macroautophagy and promote neuron survival; however, as the disease progresses, excessive calpain stimulation of macroautophagy may lead to both caspase-dependent and -independent neuron death. Future studies are warranted to further delineate the relationship of calpain activation to macroautophagy in AD.

4. AUTOPHAGY AND LYSOSOME FUNCTION IN PD

4.1. Overview of PD

PD is the most common age-related movement disorder and second most common human neurodegenerative disease. The clinical symptoms are tremor, rigidity, bradykinesia and loss of posture. The two major diagnostic neuropathological features of PD are loss of dopaminergic neurons and the appearance of characteristic intraneuronal inclusions called Lewy bodies (LB). The most prominent component of LB is alpha-synuclein (α-syn), whose normal function is in synaptic vesicle release. Five to ten percent of PD cases are inherited, some of these from dominant genetic mutations in α-syn, or recessive genetic mutations in Parkin, DJ-1, PINK1, LRRK2, UCHL1, ATP13A2 and other unidentified genes (114; 114). Some of these mutations cause early-onset Parkinsonism and dopaminergic neuron death, without obvious LB formation in postmortem brains. The mechanisms underlying the development of sporadic PD are unclear (114).

4.2. PD neuropathology

PD neuropathology includes progressive loss of nigrostriatal dopaminergic neurons with both apoptotic and autophagic cell death morphologies, and accumulation of LB that consist largely of ubiquitin cores and α-syn halos in affected neurons (115). Although α-syn and abnormal ubiquitinated protein aggregates are consistent features of LBs, how these proteins accumulate and whether they represent neurotoxic or neuroprotective structures are still unclear. Although α-syn gene duplication and triplication

are known causes of familial PD, the vast majority of PD cases have no evidence for α-syn overproduction suggesting that altered degradation of α-syn may be significant in PD pathogenesis. Both the proteasome and lysosome pathways can degrade α-syn *in vitro*. A53T mutant but not wildtype α-syn expression in neuronal cells was shown to induce non-apoptotic cell death, accumulation of ubiquitinated proteins and AVs, and reduction of acidic organelles (116). Ubiquitin accumulation can occur in response to either proteasome inhibition or macroautophagy defects. Increased numbers of AVs have been observed in dopaminergic neurons of PD brain concomitant with an increase in neurons exhibiting apoptotic morphology (117; 118), suggesting that macroautophagy may be altered in PD brain and may lead to the induction of apoptotic neuron death. Many factors, such as nutrient or trophic factor deprivation, an increase in protein aggregation, excitotoxic stimuli, or hypoxia-ischemia can induce autophagic stress (see review by Boland and Nixon) (35), and these factors may all contribute to the progression of PD.

4.3. Autophagic stress and PD

Two hypotheses have been proposed to explain the role of dysregulated autophagy in PD pathogenesis. Excess autophagy initiation may result in accumulation of AVs if it exceeds the capability of the cell to dispose of the newly generated AVs (119; 120). Alternatively, normal levels of autophagy initiation, coupled with an inhibition of autophagy completion may result in undigested materials in the AVs and the delayed recycling of AVs, leading to their accumulation.

Although direct evidence is still lacking, it is conceivable that loss of function of Parkin, an E3 ligase, may result in deficiency of clearing misfolded or mutated proteins either by directly affecting autophagosomes formation, or indirectly affecting macroautophagy due to proteasome deficits (121). LRRK2 may affect the posttranslational modification of α-syn or components of the proteasome or macroautophagy pathways, and thus may alter the half-life and/or function of α-syn (122).

Mitochondria dysfunction has also been observed in PD patients, and recent studies have identified a role for macroautophagy in the digestion of mitochonrdria, so called "mitophagy" (7-9). DJ-1 and PINK1 are mitochondrial associated proteins and it is conceivable that mutations in these proteins affect intracellular energy levels or increase oxidative stress, to indirectly influence the initiation or effectiveness of autophagy. Defective mitochondria are ultimately cleared by the lysosome. If damaged mitochondria overload the autophagy pathway, completion of autophagy may be ineffective, and lead to the formation of inclusion bodies, a pathologic feature of PD.

Transgenic mice with neuron specific deficiencies in Atg5 or Atg7 exhibit progressive neurodegeneration and an accumulation of intraneuronal ubiquitin inclusions (15; 16). These findings suggest that basal neuronal autophagy is required for clearance of ubiquitinated proteins and is essential for neuron survival. It will be interesting to examine whether specific proteins such as α-syn, accumulate in the brains of these mice, and whether Atg5 or Atg7 deficiency exacerbates α-synucleinopathy phenotypes in various mouse models that over-express wildtype or mutant α-syn. So far, limited research has been done to determine whether mutations or polymorphisms of Atg genes are associated with human diseases. Recently, a variant of Atg16L1 was identified as a risk locus for Crohn disease (123; 124) but whether Atg polymorphisms or mutations are risk factors for PD has not been examined.

4.4. Altered lysosome function in human PD

Lysosomal dysfunction has been noted in PD patients. Hereditary Parkinsonism with dementia was shown to be caused by mutations of ATP13A2, a lysosomal ATPase. Mutations of ATP13A2 result in its retention in the endoplasmic reticulum, degradation by the proteasome and thus a loss of its function (125). ATP13A2 is expressed at relatively high levels in both human and mouse brain. Interestingly, mouse midbrain dopaminergic neurons were found to express significantly higher levels of ATP13A2 compared to other brain areas, and surviving substantia nigra neurons in PD patients express elevated ATP13A2 mRNA (125). The identification of endogenous ATP13A2 substrates and whether loss of ATP13A2 function results in lysosomal dysfunction and/or abnormal protein accumulation are unknown(125) but may prove particularly informative in regards to PD pathogenesis.

In addition to macroautophagy, altered CMA may contribute to PD pathogenesis. CMA is initiated by chaperone molecules binding to KFERQ-consensus sequence-containing cytosolic proteins and delivery to the lysosomes via Lamp-2a receptors (126). Wildtype α-syn has a pentapeptide sequence that can serve as a CMA recognition motif and can be translocated to the lysosome via CMA. The PD pathogenic α-syn A53T and A30P mutations effectively inhibit CMA (127), and CMA is reduced during normal aging (128). The blockade of CMA has been shown to induce an up-regulation of macroautophagy (33). However, when protein accumulation exceeds the capacity of macroautophagy, the ability of either autophagic pathway to effectively clear accumulated proteins becomes compromised, leading to a feed-forward exacerbation of protein accumulation that may contribute greatly to neuronal demise (129).

The lysosomes are where CMA and macroautophagy converge, and where long-lived proteins and damaged organelles are degraded (130; 131). The major proteases expressed in the brain include cysteine proteases cathepsin F (CF) (132), cathepsin B (CB) and cathepsin L (CL), as well as aspartate proteases CD and cathepsin E (CE) (133-139; 139-142). Expression, activation and inhibition of these cathepsins are differentially regulated, and individual cathepsins often have non-redundant functions in normal and diseased states (143). CD is predominantly lysosomal (144), and ceramide generated by the endolysosomal acid sphingomyelinase can activate CD, which in turn is responsible for the proteolytic

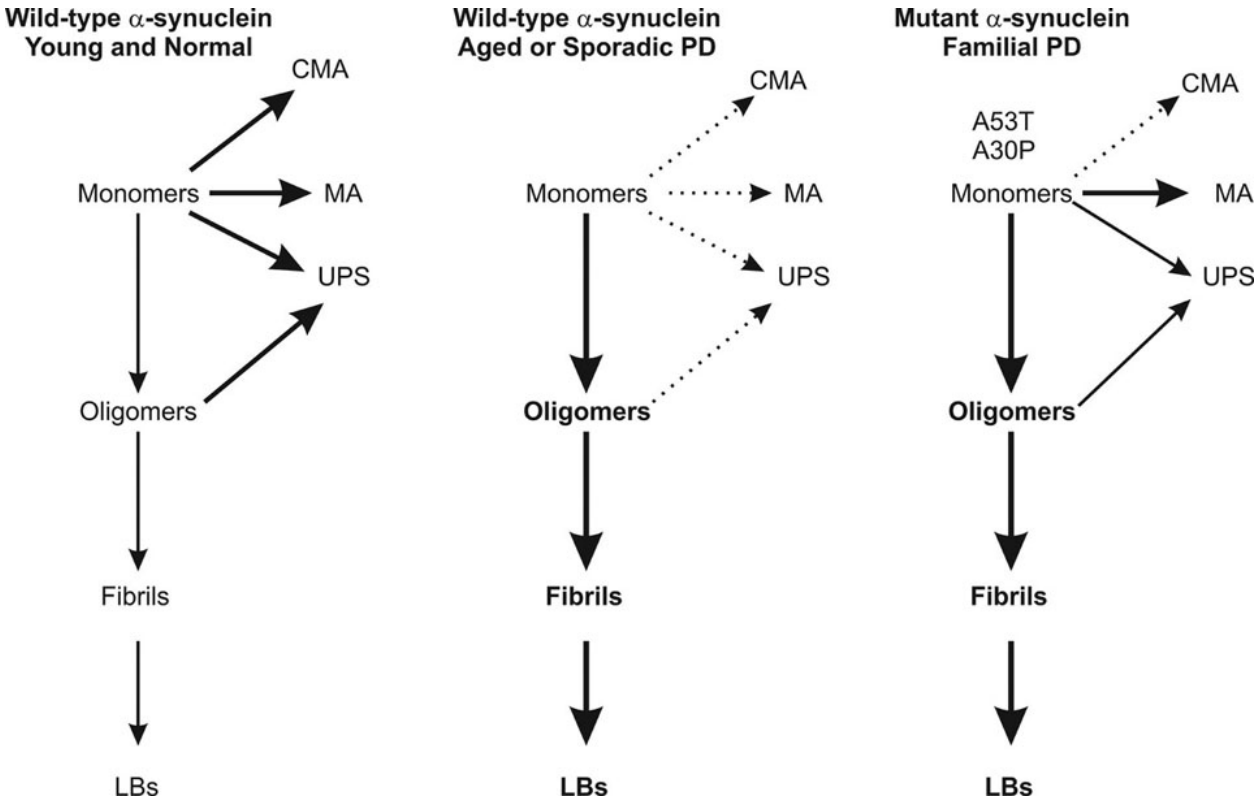

Figure 2. In young/healthy individuals, α-syn accumulation may be cleared via macroautophagy (MA), chaperone mediated autophagy (CMA) or the ubiquitin proteasome pathway (UPS). In older individuals or in sporadic PD, protein degradation pathways are compromised leading to an increased accumulation of α-syn. In experimental models of familial PD, mutations in α-syn (A53T and A30P) have been shown to result in the inhibition of CMA and in the compensatory over-stimulation of MA.

activation of other lysosomal proteins (145). During aging, certain cathepsin activities are reduced (134; 139; 146-149) but limited data are available regarding which cathepsin digests α-syn. *In vitro*, CD was shown to cleave α-syn at tyrosine 125 (150). Evidence regarding whether cathepsin polymorphisms are risk factors for PD is still scarce (130; 151).

Taken together, results from previous studies suggest that age and type of PD can have a major influence on the manner and degree of α-syn clearance (Figure 2). In young and/or genetically normal individuals, α-syn is cleared by lysosomal-mediated macroautophagy and CMA, as well as by the UPS pathway (152). In age-dependent sporadic PD, it is possible that the accumulation in α-syn may arise from an age-related decline in the general function of macroautophagy, CMA and the UPS. In some forms of familial PD, mutant α-syn may cause an exacerbation of macroautophagy as a result of an initial CMA blockade, as has been shown experimentally (33; 153). This would suggest that macroautophagy may be up-regulated in familial PD, at least early in disease onset, as a response of neurons to an inhibition of CMA. However, such a compensatory up-regulation of macroautophagy in individuals harboring α-syn mutations has not been reported. Enhancing macroautophagy by mTOR-dependent and mTOR-independent autophagy enhancers may aid in the clearance of A53T and A30T mutant α-syn (30; 31; 120; 154; 155).

4.5. Oxidative stress and PD

Mitochondria are targets of oxidative stress, and environmental and pharmacological neurotoxins are associated with the induction of mitochondrial oxidative stress and the selective degeneration of dopaminergic neurotoxins. Agents that lead to PD-like pathogenesis include the class of mitochondrial complex I inhibitors, which is represented by 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), rotenone, and paraquat (1, 1'-dimethyl-4, 4'-bipyridinium dichloride). These compounds readily cross the blood-brain barrier and are thought to disrupt the electron transport chain in the mitochondria of affected dopaminergic neurons, in turn generating oxidative stress-induced damage. Recent evidence also suggests that these factors may directly or indirectly alter autophagy.

The neurotoxin MPTP is converted to MPP+ in the nervous system by monoamine oxidase B (MAO-B), which in turn inhibits complex one of the mitochondrial electron transport chain, leading to a decrease in cellular ATP levels, a loss of mitochondrial membrane potential, an increase of reactive oxygen species, and ultimately dopaminergic neuron death (156). This oxidative stress induced macroautophagy may underlie environmental

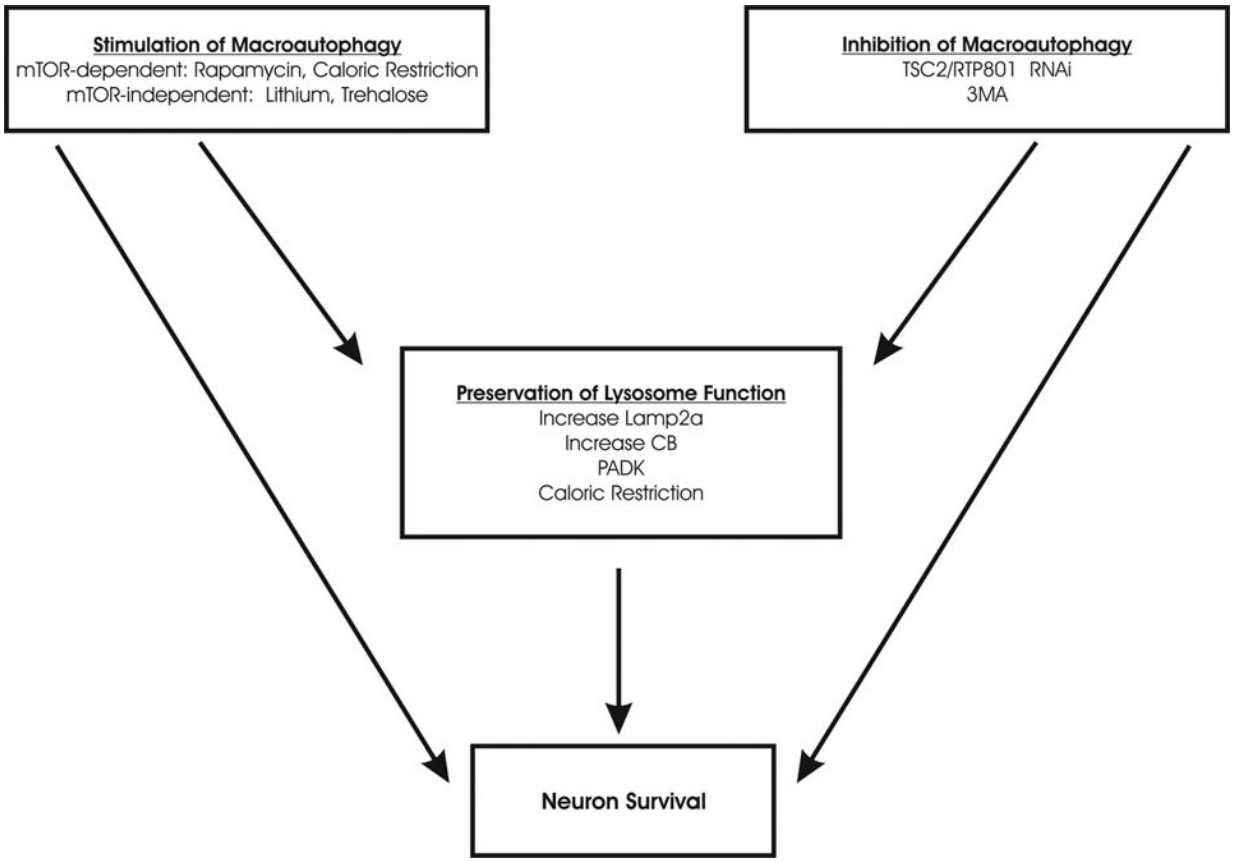

Figure 3. Potential therapies for AD and PD are divided into classes based on their potential for reducing neuron loss by causing either a stimulation or inhibition in macroautophagy, or by directly influencing lysosome function.

toxin-induction of Parkinsonism, since MPTP exposure in humans and non-human primates leads to classical PD symptoms (157). Interestingly, MPP+ has been shown to induce robust Atg6/Beclin-independent and ERK-dependent activation of macroautophagy (9), suggesting that the induction of macroautophagy may be implicated in the death of dopaminergic neurons in PD.

Rotenone is an insecticide which has also been shown to inhibit mitochondrial complex I activity (158). Rotenone induces mitochondrial fission, and mitochondrial fission has been shown to induce autophagy (159). Unlike MPTP, rotenone has been shown to induce the accumulation of ubiquitin- and α-syn-containing inclusion bodies (160). Although rotenone induces motor deficits in experimental animal models, these behavioral changes did not always correlate to the induction of dopaminergic neuron death (161).

Paraquat is one of the most commonly used herbicides and is structurally similar to the known dopaminergic neurotoxin MPP+ (1-methyl-4-phenyl-pyridine). The use of paraquat has been implicated as an etiologic factor in the development of PD. Low concentrations of paraquat induce an accumulation of AVs, and an accumulation of LC3-GFP in human neuroblastoma SH-SY5Y cells, a dopaminergic cell line. Paraquat increases the degradation of long-lived proteins, which is blocked by the autophagy inhibitor 3-methyladenine and is regulated by mammalian target of rapamycin (mTOR) signaling. Paraquat-induced cell death has been shown to be apoptosis-dependent and was attenuated by caspase inhibitors. The inhibition of autophagy was shown to accelerate paraquat-induced cell death (162-170), which suggests that the induction of macroautophagy was a protective response of cells to the paraquat death stimulus.

5. TARGETING THE AUTOPHAGY-LYSOSOMAL DEGRADATION PATHWAY IN THERAPIES FOR AD AND PD

Currently prescribed therapies for AD and PD offer little hope for long-term symptom management and at best offer only a slight delay in disease progression. Decreases in lysosome function, macroautophagy and CMA are all associated with normal aging, and their documented alterations in AD and PD suggest that such age-related alterations may play a significant role in the onset and progression of these diseases. There are several candidate therapies that modulate (either directly or indirectly) the autophagy-lysosomal degradation pathway which may lead ultimately to a decrease in neuron loss, which are described below and are outlined in Figure 3.

5.1. Therapies designed to enhance macroautophagy

Since a decrease in macroautophagy has been observed in aging, combined with evidence suggesting that macroautophagy is inhibited in late-stage AD (35; 153), therapies designed ultimately to enhance or maintain macroautophagy should be considered as potential treatment options. Therapies that have been shown to stimulate macroautophagy can be divided into two categories: those that are mTOR-dependent, and those that are mTOR-independent. Treatment with rapamycin is a well-established means of inducing macroautophagy and was shown previously to attenuate neuron death in experimental models of Huntington's disease (171). Rapamycin was shown subsequently to aid in the clearance of mutant A53T and A30P a-syn concomitant with an increase in macroautophagy, suggesting its potential utility in treating familial PD. However, rapamycin has been suggested to exacerbate damage in models of AD. For instance, treatment with rapamycin was shown recently to increase Aβ production concomitant with AV accumulation (59) and exacerbate Aβ-mediated neurotoxicity (172), suggesting that the stimulation of macroautophagy in AD may actually promote disease pathogenesis.

Lithium is a widely prescribed anti-depressant that induces autophagy by inhibiting inositol monophosphatase (IMPase), leading to a depletion in free inositol and reduced levels of myo-inositol-1,4,5-triphosphate (IP3) (154; 155). This pathway has been shown to stimulate macroautophagy in an mTOR-independent manner (154), thus further implicating the existence of multiple pathways that regulate the induction of macroautophagy. The induction of macroautophagy by lithium was shown to enhance the clearance of autophagy substrates, including mutant forms of α-syn that cause autosomal dominant forms of familial PD(155), suggesting its potential utility in treating PD.

Trehalose is a disaccharide found in many non-mammalian species. Recently, trehalose was reported to be an mTOR-independent activator of autophagy. Trehalose-induced autophagy enhanced the clearance of autophagy substrates including the A30P and A53T mutants of α-syn. Treatment with trehalose and rapamycin together exerted an additive effect on the clearance of mutant α-syn, likely because of increased autophagic activity (120).

Caloric restriction has been proposed to attenuate the deleterious effects of aging, possibly through the maintenance of basal macroautophagy which normally declines with aging (10; 173; 174). In the liver, the enhancement of macroautophagy is thought to occur via a general reduction in plasma insulin levels, which negatively regulates macroautophagy (175). Whether macroautophagy is altered in the brain following caloric restriction has yet to be determined. The regulation of brain glucose levels is a tightly regulated process due to the exquisite energy demands of neurons (35) and as such many beneficial effects of caloric restriction may be limited to the periphery. Nevertheless, several "calorie mimetics" have been proposed (176) that may also result in an enhancement of macroautophagy and cell viability and as such should be tested for their efficacy in altering the changes in macroautophagy associated with normal aging brain and with age-related neurodegenerative disease.

5.2. Inhibition of macroautophagy as a therapeutic rationale

The over-stimulation of macroautophagy has been correlated previously with an induction of cell death (177; 178). Although an induction of macroautophagy has been hypothesized to serve an initial protective role in AD, it is feasible that long-term over-stimulation of macroautophagy may actually induce neuron death in age-related neurodegenerative disease. As such, agents that attenuate the induction of macroautophagy may serve useful in the early treatment of AD or PD. RTP801 is a stress-response gene that is up-regulated by death stimuli including hypoxia and DNA damage (179; 180). The over-expression of RTP801 was shown previously to phosphorylate and inactivate TSC2, resulting in the phosphorylation and inactivation of mTOR (180). Recent evidence has indicated that levels of RTP801 are elevated in neuromelanin-containing substantia nigral neurons but not cerebellar neurons of PD brain (119), suggesting that RTP801 may play a select role in dopaminergic neurodegeneration. In *in vitro* studies, the death of neuronal PC12 cells by the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) occurred concomitant with a significant elevation of RTP801 and a significant inactivation of mTOR, and that RTP801 over-expression also induced death of PC12 cells to a degree equal to that of 6-OHDA (119). Treatment of cells with shRNAs for RTP801 or TSC2, with which RTP801 interacts to block mTOR activation, was shown to significantly attenuate 6-OHDA-induced cell death(119). To date, whether RTP801 regulates the induction of macroautophagy as a result of its effects on mTOR has not been investigated. Together these findings suggest that at least in the 6-OHDA model of PD, TSC2 and mTOR inactivation may induce neuron death that depends in part on an induction of macroautophagy. Furthermore, the generation of therapies (RNAi-mediated or pharmacological) that prevent either the up-regulation of RTP801 or the inactivation of TSC2/mTOR may prove beneficial in the treatment of PD.

Treatment with 3-methyl adenine (3MA), which inhibits class III PI3-K and thus inhibits the stimulation of macroautophagy, has been shown recently to prevent the rapamycin-induced accumulation in Aβ *in vitro* (59), which further implicates the therapeutic potential for inhibiting the aberrant stimulation of macroautophagy in AD. It will be important in the future design of AD and PD therapeutics to determine the relative contribution of macroautophagy induction vs. inhibition towards the onset and progression of these diseases.

5.3. Therapies that preserve lysosome function

While the preservation of lysosome function may be an indirect consequence of enhanced macroautophagy, recent evidence suggests that the direct modulation of lysosome function may preserve lysosomal membrane integrity, perhaps by attenuating oxidative stress-induced damage, and may attenuate neuron death in age-related

neurodegenerative disease. Previous studies have shown an increase in CB levels and activity in AD brain (40; 42; 50; 51), suggesting that the increase in endogenous CB in AD may be a protective mechanism of neurons to enhance lysosome function. This possibility has been recently substantiated by the finding that the lentiviral-mediated delivery CB reduces Aβ burden in aged APP transgenic mice and was shown to cleave Aβ to produce less amyloidogenic fragments (57). In addition, CB deficiency in mice was shown in this study to increase Aβ burden (57), further implicating a protective role of CB in treating AD.

Age-related declines in Lamp-2a have been recently described that correlate with an age-related decrease in CMA (181). While a role for Lamp-2a has not yet been described in age-related neurodegenerative disease, mutant α-syn has been shown to inhibit CMA function in a model of familial PD (127), suggesting that therapies resulting in an increased expression of Lamp-2a may counteract any putative decline in CMA in subsets of PD patients.

N-Cbz-L-phenylalanyl-L-alanyl-diazomethylketone (PADK) is a compound that has been shown to enhance lysosome function by increasing levels of lysosomal hydrolases (74; 182). PADK was shown to attenuate both the increase in tau deposition and the decrease in synaptic integrity associated with treatment with the lysosomotropic agent chloroquine, effects that are very similar to that of Aβ treatment (74; 182). Caloric restriction or calorie restriction mimetics (176) may also be useful in preserving lysosome function, since a decrease in accumulation of the undigestable oxidized lipopigment lipofuscin was reported in brains of mice following caloric restriction (183), which may be a direct result of a decrease in oxidative stress-induced damage in neurons that is known to increase with aging. Taken together, therapies which preserve lysosome function may attenuate age-related declines in macroautophagy and CMA and may also attenuate alterations in the autophagy-lysosomal degradation pathway observed in age-related neurodegenerative disease.

6. CONCLUSIONS

Increased numbers of AVs have been observed in selectively degenerating neuronal subpopulations in both AD and PD concomitant with alterations in lysosome function. Genetic and environmental factors have been shown to cause autophagic stress that may play an important role in AD and PD pathogenesis. Regulating the autophagy-lysosomal degradation pathway has the potential for enhancing protein clearance and providing neuroprotection in both AD and PD.

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