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REVIEW

Promoting Autophagic Clearance: Viable Therapeutic Targets in Alzheimer's Disease

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Abstract Many neurodegenerative disorders are characterized by the aberrant accumulation of aggregate-prone proteins. Alzheimer's disease (AD) is associated with the buildup of β-amyloid peptides and tau, which aggregate into extracellular plaques and neurofibrillary tangles, respectively. Multiple studies have linked dysfunctional intracellular degradation mechanisms with AD pathogenesis. One such pathway is the autophagy–lysosomal system, which involves the delivery of large protein aggregates/inclusions and organelles to lysosomes through the formation, trafficking, and degradation of double-membrane structures known as autophagosomes. Converging data suggest that promoting autophagic degradation, either by inducing autophagosome formation or enhancing lysosomal digestion, provides viable therapeutic strategies. In this review, we discuss compounds that can augment autophagic clearance and may ameliorate disease-related pathology in cell and mouse models of AD. Canonical autophagy induction is associated with multiple signaling cascades; on the one hand, the best characterized is mammalian target of rapamycin (mTOR). Accordingly, multiple mTOR-dependent and mTOR-independent drugs that stimulate autophagy have been tested in preclinical models. On the other hand, there is a growing list of drugs that can enhance the later stages of autophagic flux by stabilizing microtubule-mediated trafficking, promoting lysosomal fusion, or bolstering lysosomal enzyme function. Although altering the different stages of autophagy provides many potential targets for AD therapeutic interventions, it is important to consider how autophagy drugs might also disturb the delicate balance between autophagosome formation and lysosomal degradation.

Keywords Autophagy · Alzheimer's disease · Neurodegeneration · Therapeutics · Tau · Amyloid (Aβ) · Flux · Lysosomes

Introduction

Alzheimer's disease (AD), like many neurodegenerative diseases, is largely characterized by the aberrant accumulation of endogenous proteins, resulting in the formation of cytotoxic aggregates and inclusions. Multiple lines of evidence have shown that intracellular degradation mechanisms can be activated to remove pathological forms of these proteins, and thus serve as viable druggable targets. One such pathway is the ubiquitin-proteasome system, which breaks down short-lived and soluble proteins. While the ubiquitin-proteasome system degrades disease-linked proteins associated with neurodegenerative disease [1, 2], the narrow pore of the proteasomal barrel may impede clearance of larger protein oligomers and aggregates [3]. In contrast, the autophagy–lysosome system degrades long-lived and large protein complexes and organelles through a multistep process that requires a fine balance between initial induction and end-stage degradation. Dysfunction at either end of the pathway has been linked to AD pathogenesis [4, 5], thus providing multiple targets for pharmacological intervention. This review focuses on the different stages of autophagic clearance as targets for AD therapeutics, which are also relevant to other proteinopathies associated with neurodegenerative disorders.

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Autophagic Process and Machinery

Autophagy is a highly conserved pathway that delivers intracellular cytoplasmic components to lysosomes. Three distinct forms have been identified based on their mode of delivery to lysosomes: macroautophagy, chaperone-mediated autophagy, and microautophagy [6–9]. Macroautophagy (herein referred to as autophagy) involves the formation, trafficking, maturation, and subsequent degradation of double-membrane structures known as autophagosomes. The cumulative process is referred to as autophagic flux and is essential for neuronal and brain health. Disruption of any of these highly regulated steps will result in incomplete autophagic digestion and impaired autophagic flux, and represents distinct targets for drug development. Following induction, an isolation membrane (also known as a phagophore) elongates to engulf damaged organelles and proteins, and encloses to form an autophagosome. Autophagosomes can fuse directly with lysosomes to form autolysosomes, or, alternatively, with late endosomes/multivesicular bodies to form amphisomes. These acidified structures fuse with lysosomes, where their cargo and inner membrane are digested by lysosomal hydrolases [10] (Fig. 1). To date, 36 autophagy-related genes that are essential for autophagosome biogenesis have been identified in yeast [11], and many have known mammalian orthologs [12, 13].

Regulation of Autophagy

Mammalian target of rapamycin (mTOR) is a ubiquitously expressed protein kinase at the center of a complex signaling network that regulates mRNA translation [14], ribosome biogenesis [15], metabolism [16], and autophagy [17]. mTOR is part of 2 core complexes: the mTOR complex 1 (mTORC1), which includes regulatory-associated protein of mTOR, and the mTOR complex 2 (mTORC2) [18]. mTORC1 plays a major role in autophagy regulation through interactions with serine/threonine kinase autophagy-related protein (Atg)1 or its mammalian ortholog, Unc-51 like kinase 1 (ULK1). ULK1 forms a protein complex with Atg13 and FAK-family interacting protein of 200 kDa (FIP200). Under basal conditions, mTORC1 binds directly to the ULK1 complex to suppress its activity [19–21]. However, amino acid deprivation or pharmacological inhibition of mTOR initiates ULK1–FIP200–Atg13 dissociation [19, 22], and enhances its interaction with the energy sensing kinase adenosine monophosphate-activated protein kinase (AMPK) [23, 24]. AMPK, in turn, activates ULK1 [24], and also directly inhibits mTOR through phosphorylation of tuberous sclerosis complex 2 and regulatory-associated protein of mTOR [25–27]. Therefore, both mTOR inhibitors and AMPK activators can be used to regulate positively ULK1 [28–31].

Nucleation

Until recently, little was known about how the ULK1–FIP200–Atg13 complex was involved in the initiation of autophagosome formation. Beclin 1, the mammalian ortholog of Atg6, is essential for isolation membrane nucleation. It forms a core complex with Atg14L, (activating molecule in Beclin 1-regulated autophagy (AMBRA1), p150, and hVps34/class III phosphatidylinositol 3-kinase (PI3K), which is tethered to the cytoskeleton through the association between AMBRA1 and dynein motor complex. ULK1 phosphorylation of AMBRA1, releases the Beclin 1/PI3K complex so that it can be translocated to the endoplasmic reticulum (ER), which is thought to be the site of autophagosome formation [32–34] (Fig. 1). Atg14L directs the Beclin 1/PI3K complex to autophagosomes by identifying curved membrane structures with a high content of phosphatidylinositol 3-phosphate and is also involved in the maintenance of membrane curvature [35]. While Beclin 1 interaction with *UVRAG* induces autophagy [36], RUN domain and cysteine-rich domain containing Beclin 1-interacting protein binds with Beclin 1 to inhibit autophagy [37].

The ULK1–FIP200–Atg13 complex plays an essential role in recruiting membranes to phagophore assembly sites in yeast or preautophagosomal structures in mammals. In yeast, Atg1 mediates the trafficking of Atg9 [38], which is an integral membrane protein critical for autophagosome formation that cycles between the phagophore assembly sites and cytosol. In mammals, Atg9 cycles between the trans-Golgi network and late endosomes [38], and requires Atg2 and tryptophan–aspartic acid (WD)-repeat protein interacting with phosphoinositides, as well as the Beclin 1/PI3K complex, for its normal function [39]. Tryptophan–aspartic acid (WD)-repeat protein interacting with phosphoinositides and Atg2 bind to phosphatidylinositol 3-phosphate on phagophores and autophagosomal membranes [40], and are thought to mediate the conversion of omegasomes (phosphatidylinositol 3-phosphate-enriched ER membrane structures) to autophagosomes [41].

Elongation, Closure, and Cargo Recruitment

Autophagosomal membrane elongation involves two ubiquitin-like conjugation reactions. Atg12 and Atg8 are both conjugated by E1-like activating enzyme, Atg7, but are processed by 2 different E2-like conjugating enzymes (Atg10 and Atg3, respectively). Atg12 is conjugated with Atg5 [42], and noncovalently binds with Atg16L to form a complex that is localized to elongating isolation membranes [43, 44]. Small GTPase protein, Rab5 facilitates Atg12–Atg5 conjugation [45, 46].

Yeast Atg8 has several mammalian orthologs, including microtubule-associated protein 1A/1B-light chain 3 (LC3),

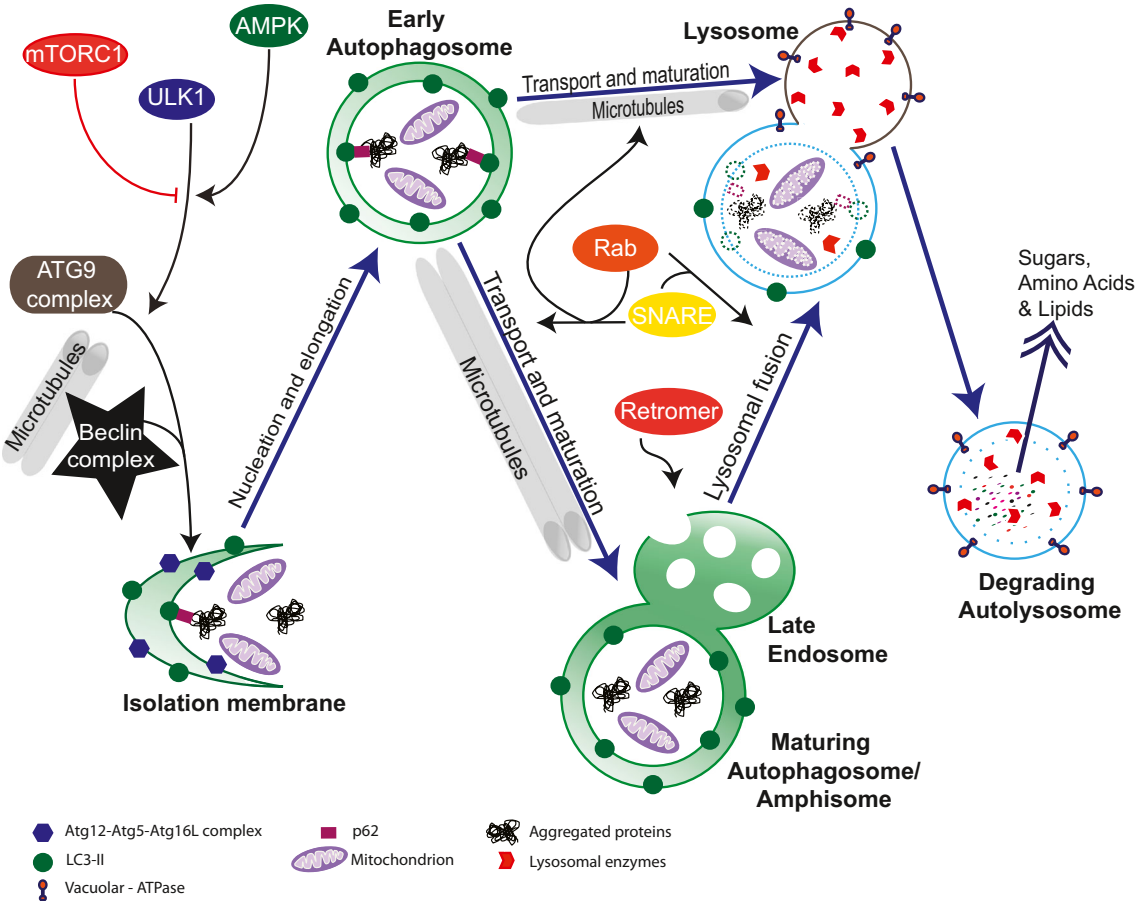

Fig. 1 Key molecular players in autophagic flux. Autophagy induction is mediated by mammalian target of rapamycin (mTOR) complex (mTORC)1, adenosine monophosphate-activated protein kinase (AMPK), and Unc-51 like kinase 1 (ULK1), which interact with autophagy-related protein (ATG)9 and Beclin 1 complexes during nucleation of the isolation membrane. Isolation membrane elongation is dependent upon the Atg12–Atg5•Atg16L complex and microtubule-associated protein 1A/1B-light chain 3 (LC3)-II. The scaffolding protein p62 recruits autophagic protein substrates to LC3-bound autophagosomal membranes. The structure then encloses to form an autophagosome. During maturation, autophagosomes can directly fuse with lysosomes or with endosomes and multivesicular bodies to form amphisomes, which is regulated by Rab, solube N-ethylmaleamide-sensitive factor attachment protein receptor (SNARE), and retromer proteins. Autophagic cargo (including organelles and protein aggregates) and the inner membranes of autophagosomes are digested by lysosomal enzymes during lysosomal fusion, which is mediated by the SNARE and Rab proteins. The acidic environment of lysosomes is maintained by vacuolar-ATPase

which is cleaved at the C-terminal and retrieved from nonautophagosomal membranes by Atg4 to form cytosolic LC3-I [47]. LC3-I conjugation to phosphatidylethanolamine is facilitated by the unique protein-lipid E3-like activity of the Atg12–Atg5•Atg16L complex to form lipidated LC3-II [48–51]. Lipidated LC3-II association with isolation membrane is required for elongation and closure of autophagosomal membrane, and remains bound to the autophagosome until it fuses with lysosomes [52], whereas the Atg12–Atg5•Atg16L complex dissociates upon autophagosomal closure [53] (Fig. 1).

Sequestosome1/p62 is a multifunctional scaffolding protein commonly found in ubiquitinated inclusion bodies and rapidly accumulates when autophagy is suppressed [54]. p62 has multiple protein–protein interaction motifs and binds both polyubiquitinated proteins and LC3 to recruit protein cargo to autophagosomes [55, 56]. As aggregated forms are degraded by autophagy [57], p62 also serves a useful marker for autophagic flux. Additionally, p62 interacts with polyubiquitinated forms of AD-associated protein tau and accumulates in other related tauopathies [58, 59].

Maturation of Autophagic Vacuoles

Maturation of autophagosomes involves fusion with early/late endosomes or with multivesicular bodies [7, 60–65]. Amongst the key players responsible for these fusion events are microtubules [66–70], soluble *N*-ethylmaleamide-sensitive factor attachment protein receptor (SNARE) proteins [71, 72], and ultraviolet radiation resistance-associated gene protein [73]. Additionally, the small GTPase Rab family of proteins, especially Rab7, has been implicated in maturation and fusion of

amphisomes with lysosomes [74, 75]. Rab11 has been shown to promote late endosome–autophagosome fusion by interacting with Hook protein [64], which normally anchors late endosomes tightly to microtubules [67]. In addition to these, other Rab proteins have been implicated in autophagy [76, 77]. Rab protein inactivation by specific GTPase-activating proteins may provide a potential drug target that could increase Rab function, thereby enhancing autophagic maturation.

Retromer is a conserved protein sorting and trafficking complex that contains an assembly of proteins that associate with endosomes and regulate protein transport from endosomes to the trans-Golgi network and to cell membrane [78–80]. A study in *Caenorhabditis elegans* indicated a role for a Beclin 1 ortholog in retromer function [81], suggesting crosstalk between the endosomal and autophagy systems. Furthermore, mutations in vacuolar protein sorting 35, part of the retromer trimer complex, disrupt Atg9 trafficking and ultimately impair autophagy [82].

Axonal Transport

Mammalian autophagosomes are formed in various regions of the cytoplasm and, upon maturation, are transported retrogradely to lysosomes, which are primarily located in the cell body or juxta-nuclear region. Microtubules and the dynein motor complex mediate retrograde transport and have been directly implicated in autophagosomal transport and fusion of autophagic/endocytic vesicles with lysosomes [83–85]. Live imaging studies in primary dorsal root ganglion neurons revealed that punctate green fluorescent protein–LC3 structures form in distal neurites, where they initially undergo bidirectional transport, but are eventually transported predominantly in the retrograde direction, which is associated with autophagosome maturation [86]. A recent study suggests that competitive binding of kinesin and dynein contributes to the initial bidirectional movement of autophagic structures; however, when scaffolding protein c-Jun NH2-terminal kinase-interacting protein-1 binds to LC3, the kinesin motor complex is inhibited, thus allowing for sustained dynein-mediated transport of autophagosomes in the retrograde direction [66]. Rab7 has also been implicated in the transport of autophagosomes along microtubules via its interaction with FYVE and coiled-coil domain containing 1 [87]. As autophagic vesicles in neurons are often transported across long distances from distal axons to the cell bodies where they fuse with lysosomes, drugs that promote transport may be effective in alleviating impaired autophagic flux in neurodegenerative disease.

Lysosomal Fusion and Degradation

Autolysosomal fusion depends on at least 3 independent factors: transport of autophagosomes/amphisomes to lysosomes, acidic lysosomal pH, and lysosomal membrane proteins (Fig. 1). Membrane protein complexes—class C core vacuole/endosome tethering factor, SNARE, and homotypic fusion and vacuole protein sorting, which is thought to be modulated by Rab7 [88]—act as tethers that facilitate fusion between autophagic vesicles and lysosomes [89, 90]. SNAREs are localized on both mature autophagosomes/amphisomes and lysosomes, and act as a bridge between the 2 structures to initiate fusion [72, 91]. While the majority of SNARE proteins are localized to endosomes and synaptic vesicles, syntaxin17 has recently been identified as an autophagosome-specific SNARE [92, 93].

The acidic pH of lysosomes is critical for their function and is maintained largely by vacuolar-type H^+-ATPase, which pumps protons into the lysosomal lumen [94, 95]. Homeostatic autophagic flux requires proper lysosomal acidity and is completely halted by vacuolar-type H^+-ATPase inhibitor bafilomycin A1 [96]. Similarly, a deficiency of lysosomal cathepsin enzymes disrupts autophagic flux causing the accumulation of autophagosomes and undigested autophagic cargo [97, 98]. Additionally, the loss of lysosomal associated membrane protein-2, which is involved in the selective uptake of proteins by the lysosome [99], leads to the accumulation of autophagic vacuoles [100]. As the final effectors of the autophagic machinery, lysosomal dysfunction leads to the blockage of the whole system, as evidenced in the lysosomal storage diseases [101].

AD Pathogenesis and Link to Autophagy

AD is characterized pathologically by the formation of extracellular plaques consisting of insoluble aggregated amyloid-β (Aβ) peptides and neurofibrillary tangles (NFTs) containing aggregated microtubule-associated tau. β-amyloid precursor protein (APP) is a transmembrane protein that is cleaved by α-, β-, and γ-secretases to form amyloidogenic and nonamyloidogenic peptides [102]. Familial mutations in APP and γ-secretase components presenilin-1 (PS1) or presenilin-2, cause enhanced production of Aβ peptides, which are prone to self-aggregation and the formation of oligomers, fibrils, and plaques [103]. Tau normally binds to and stabilizes microtubules; however, genetic tau mutations or hyperphosphorylation leads to detachment from microtubules and misfolding, followed by formation of pretangle aggregates and eventual deposition into filamentous inclusions (NFTs) [104]. AD pathology is also closely associated with inflammatory responses, including microglial clustering in

and around dense core Aβ plaques [105], elevated levels of proinflammatory cytokines [106], and microglial activation that precedes NFT formation [107]. Additionally, genetic variants of triggering receptor expressed on myeloid cells-2 and CD33, which encode microglial receptors that regulate phagocytosis and inflammatory response, are significant risk factors in developing AD [108, 109]. As the disease progresses, there is widespread synaptic and neuritic degeneration, and subsequent neuronal cell death.

A growing body of evidence points to defective autophagic clearance as one of the disease-causing culprits of AD pathogenesis. Autophagy plays a vital neuroprotective role in central nervous system neurons, as it facilitates the removal of aggregated ubiquitinated protein inclusions and is essential for the prevention of neurodegeneration [110, 111]. Dysfunctional autophagy has been linked to many chronic proteinopathies, including frontotemporal dementia (FTD), amyotrophic lateral sclerosis, Parkinson's disease (PD), Huntington's disease (HD), and AD [1, 112–119].

In response to increased levels of cytosolic proteins and/or aggregates, autophagic induction or degradation may be heightened in affected neurons. In both patients with AD and the PS1/APP mouse model, there is a massive proliferation of autophagosomes and autolysosomes in dystrophic neurites [114, 120], which are enriched in Aβ and γ-secretase subunits [120, 121]. Whether the marked increase of these structures reflects elevated autophagosome formation or incomplete lysosomal/autolysosomal digestion is still under debate; however, multiple studies point to the latter [4, 122–124]. Strong induction of autophagy in primary cortical neurons leads to robust accumulation of mature autophagic vesicles, rather than early autophagosomes [122, 125]. Lysosomal protease cathepsin D is highly upregulated in affected regions of AD brains [126], suggesting a compensatory mechanism for insufficient lysosomal processing or degradation. Impaired lysosomal degradation may also be connected to early-onset familial AD mutations. PS1 is required for lysosomal acidification, and fibroblasts from mutated PS1 from patients with familial AD have defective autophagic–lysosomal proteolysis, along with impaired autolysosome acidification and cathepsin activation [4]. These studies suggest that promoting lysosomal clearance would reverse AD pathogenesis and improve cognitive function. For example, enhancing proteolysis in CRND8 transgenic mice through the deletion of cathepsin inhibitor, cystatin B, ameliorates Aβ plaque load, and learning and memory deficits [127].

While it is clear that improper lysosomal fusion and degradation are closely associated with AD neuropathology and enhancing lysosomal function reverses disease progression in mice, there is growing evidence that autophagy induction is also a viable therapeutic target that can ameliorate the pathological features associated with AD. Autophagosome formation progressively declines during normal aging [128], and may contribute to toxic protein accumulation in sporadic cases of age-related neurodegenerative diseases. Mice with central nervous system-specific deletion of essential autophagy genes, *Atg5* or *Atg7*, display progressive neurodegeneration and pervasive polyubiquitinated protein inclusions [110, 111]. Genetic ablation of *Atg7* has also been shown to exacerbate Aβ pathology. APP transgenic mice crossed with conditional knockout mice with forebrain-specific *Atg7* deletion display increased intracellular Aβ accumulation in neurons [129], while mice with microglial-specific *Atg7* ablation accumulate higher levels of Aβ following stereotaxic injection of fibril Aβ [130]. Similarly, the loss of Beclin 1 in the APP transgenic mouse model promotes Aβ deposition, and can be rescued by Beclin 1 viral gene transfer [5]. Postmortem examination of brains from patients with AD reveals reduced levels of Beclin 1, which is involved in regulating APP processing and turnover [5, 131], while microglia isolated from postmortem AD brains display lower levels of Beclin 1, which is associated with defective microglial phagocytosis of Aβ in APP transgenic mice [132]. These studies suggest that promoting levels of proteins associated with autophagy may have dual beneficial effects in AD models by enhancing autophagic degradation of Aβ in neurons and phagocytosis and degradation by microglia. This may also be relevant in microglial clearance of circulating Aβ peptides or tau released from neurons, as well as antibodies targeting these substrates.

Impaired autophagy increases levels of intracellular Aβ in APP mice, but also reduces extracellular deposits of Aβ, suggesting that while autophagy-stimulating compounds may reduce toxic intracellular Aβ levels, they may also exacerbate secretion of extracellular Aβ over time [129]. Together, these studies indicate an important role for autophagy in maintaining homeostatic levels of Aβ, and suggest that therapies targeted at autophagy may reduce Aβ-induced toxicity, but have detrimental effects if the level of induction exceeds lysosomal clearance of autophagic vacuoles. This is an important element in developing autophagy drugs and assays for the screening of new compounds.

Autophagy Therapeutics in Disease-related Protein Clearance

The goal of most preclinical AD research programs is to focus on discovering compounds that reduce tauopathy and Aβ accumulation, and reverse cognitive impairment. To this end, numerous transgenic animal models of AD have been generated to express different familial mutations of APP, PS1, tau, and combinations of these mutations. These models recapitulate aspects of AD neuropathology, behavioral deficits, and disease time course to varying degrees; therefore, selecting the appropriate mouse model to test potential therapeutics warrants careful consideration. Additionally,

conflicting results within the same mouse models may be due to the route of administration, duration of treatment, and disease stage at onset of treatment. These factors should be considered in evaluating the data in the studies presented below. To date, multiple compounds have been identified in the context of AD and other neurodegenerative diseases that stimulate autophagic clearance by promoting induction, trafficking/maturation, or lysosomal fusion (Fig. 2).

Targeting Canonical Autophagy Induction Through mTOR Signaling

Rapamycin

Multiple studies indicate that inhibition of mTOR increases lifespan in yeast, *C. elegans*, and *Drosophila* [133–135], and evidence suggests this effect is conserved in mammals [136–138]. Rapamycin, a US Food and Drug Administration (FDA)-approved antifungal antibiotic [139], anticancer cytostatic agent [140], and immunosuppressant [141, 142], inhibits mTOR signaling by forming a drug–receptor complex with FKBP12, which binds with the mTORC1 complex [143]. In addition to enhancing longevity [144, 145], rapamycin treatment has been shown, through autophagic induction, to clear aggregate-prone forms of disease-related proteins in cell and animal models of PD [1], HD [2, 119], and AD [146, 147].

Rapamycin was initially identified as an autophagy inducer capable of clearing aggregate-prone forms of huntingtin and α-synuclein [1, 148]. Rapamycin treatment induces clearance of wild-type and mutant R406W tau, reduces eye toxicity, and increases lifespan in a *Drosophila* model of AD, and promotes clearance of nonmicrotubule-bound aggregate-prone tau in COS-7 cells [119]. In 7PA2 cells (Chinese hamster ovary cells that stably express mutant APP), Aβ levels are reduced after rapamycin treatment [149]. These findings were extended to the 3×TgAD mouse model of AD (a triple transgenic mouse that displays both Aβ plaques and tau tangles) [150]. Rapamycin leads to a reduction in both phospho-tau (Thr181) and Aβ levels in the CA1 region of hippocampus

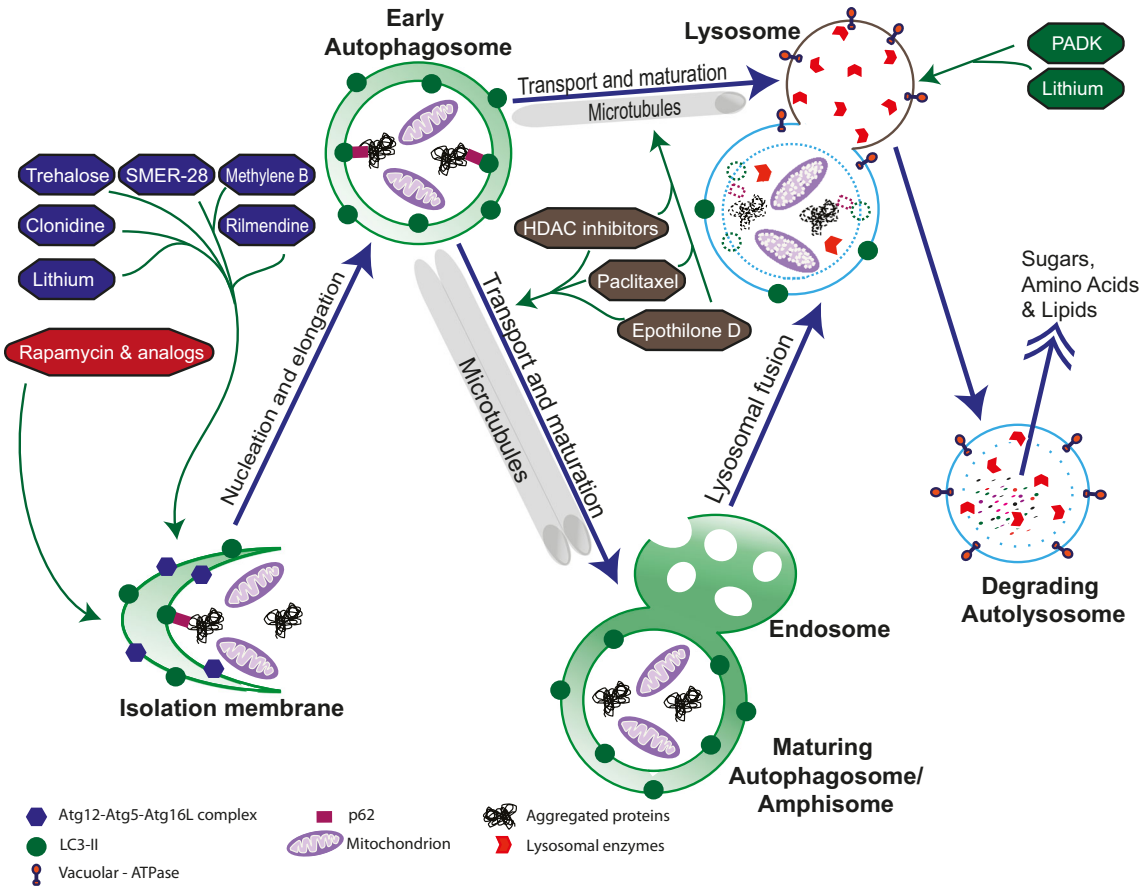

Fig. 2 Autophagy-enhancing drugs and their targets. Compounds that stimulate autophagosome biogenesis include canonical mammalian target of rapamycin (mTOR)-dependent inhibitors (red) and multiple mTOR-independent autophagy inducers (blue), which likely act through different mechanisms. Compounds that promote maturation, trafficking, and degradation include microtubule stabilizers that aid in transport (gray) and enhancers of lysosomal fusion (green). SMER-28 = small molecule enhancer of autophagy; PADK = Z-Phe-Ala-diazomethylketone; HDAC = histone deacetylase; Atg = autophagy-related protein; LC3 = microtubule-associated protein 1A/1B-light chain 3

and reversal of early learning and memory deficits in 3× TgAD mice [149]. Interestingly, mTOR kinase activity increases in the hippocampus and cortex of these mice with age, suggesting a reduction in autophagic activity over time [149]. These studies further support the notion that induction of autophagy may be beneficial in clearance of aggregate-prone proteins/peptides. In another study, 3×TgAD mice treated with rapamycin early in life (starting at 2 months) exhibited reduced phospho-tau (at the Ser212, Thr214, and Thr231 residues) and Aβ pathology, and showed preserved learning and memory function without causing overt side effects [147]. Studies in hAPP-J20 mice, which have high hippocampal levels of $A\beta_{1\text{-}42}$ peptide and synaptic degeneration [151], confirmed that rapamycin boosted autophagy, which improved behavioral deficits and also decreased $A\beta_{42}$ levels, but not $A\beta_{40}$ [146].

Rapamycin Analogs

Although rapamycin crosses the blood–brain barrier (BBB), it has poor water solubility and stability in solution [152]. Rapamycin analog, cell cycle inhibitor 779 (also known as temsirolimus), is an FDA-approved cancer drug, with improved pharmacological properties comparable to rapamycin. Like rapamycin, temsirolimus upregulates autophagy through inhibition of the mTOR pathway, and has been shown to ameliorate motor dysfunction and neuropathology in mouse models of 2 polyglutamine disorders [148, 153]. Two recent studies suggest a neuroprotective role for temisirolimus in AD models. Temsirolimus administration in APP/PS1 mice reduces Aβ plaque load and alleviates spatial learning and memory impairments through its activation of autophagy [154]. Likewise, the same group showed the beneficial effects of temsirolimus on tauopathy. Temsirolimus treatment in okadaic acid-treated SH-SY5Y cells (which induces tau hyperphosphorylation) and P301S tau transgenic mice reduces levels of hyperphosphorylated tau, while rescuing spatial learning deficits in mice through autophagy induction [155].

The Benefits and Risks of mTOR-dependent Autophagy Induction

Despite the promising effects of drugs that enhance autophagy through inhibition of mTOR, long-term administration of compounds that impede mTOR activity may have detrimental effects. The mTOR signaling cascade is required for protein synthesis associated with synaptic plasticity and memory formation in hippocampus (reviewed in [156]); therefore, the reversal of cognitive dysfunction observed in mice treated with mTOR inhibitors may be transitory, while long-term administration required in chronic disorders like AD may eventually exacerbate memory loss over time owing to untoward effects from inhibiting protein synthesis. Additionally, long-term use of rapamycin and its analogs may cause a broad array of side effects, including, but not limited to, increased infections, skin disorders, swelling of the lower extremities, reduced male fertility, hyperlipidemia, insulin resistance, and diabetes mellitus [157–161].

mTOR-independent Autophagy Inducers

Small-molecule Screening

Initial screens for small-molecule autophagy enhancers that activate autophagy in an mTOR-independent manner identified several compounds that reduced mutant forms of huntingtin and A53T α-synuclein aggregates in cell models, and were neuroprotective in *Drosophila* models of HD [162]. Small-molecule enhancer of rapamycin-28, an autophagy inducer with minimal cytotoxic effects, reduces Aβ and APP/C-terminal fragment accumulation in N2a-APP neuroblastoma cells [163]. FDA-approved hypertensive drugs, clonidine and the related drug rilmendine, were identified as autophagy enhancers in a screen of compounds that cleared aggregate-prone forms of α-synuclein and huntingin in PC12 cells [164]. While both drugs bind α_2-adrenoceptors and I_1 imidazoline receptors (I_1R), rilmendine has a much greater specificity for I_1R, and therefore fewer potential side effects. Rilmendine rescues motor dysfunction and decreases soluble mutant huntingtin levels, but not aggregates, in a transgenic mouse model of HD; however, owing to high dosing, treated mice experienced adverse effects that would limit its therapeutic potential. Nevertheless, converging data support the view that compounds that induce autophagic clearance can ameliorate disease pathogenesis and restore physiological function (Fig. 2).

Methylene Blue

In a screen of compounds that inhibit heparin-induced tau aggregation into filaments without disrupting microtubule interactions, several phenothiazines were identified with IC_{50} (half maximal inhibitory concentration) values in the low micromolar range [165]. Phenothiazines are clinically used as neuropsychotic agents. They readily cross the BBB and are generally well tolerated without major side effects. Methylthioninium chloride, also known as methylene blue (MB), is a non-neuroleptic phenothiazine that has been shown to reduce aggregation of truncated tau and Aβ oligomerization *in vitro* [166, 167], and acts as a memory-enhancing drug [168]. As MB has pleiotropic effects, including modulation of cyclic guanosine monophosphate signaling and synaptic neurotransmission, as well as inhibition of chaperone protein heat shock protein 70 (which promotes degradation) [169], it is unclear exactly how MB mediates its aggregate-inhibiting

properties. However, a study in a mouse hippocampal cell line demonstrated that MB induces autophagy [170].

Studies from our laboratory have shown that MB reduces aggregated and phosphorylated tau in the JNPL3 mouse model (which expresses the P301L tau mutation) following treatment in *ex vivo* organotypic brain slice culture or administration *in vivo* by oral gavage [171]. MB increased levels of autophagy markers LC3, Beclin 1, and cathepsin D, suggesting that MB-induced clearance of tau is associated with autophagy activation [171]. While focal infusion of MB, by osmotic minipump, improved cognitive function and reduced tau levels in rTg4510 mice (another transgenic line expressing the P301L tau mutation), only long-term, high-dose administration of MB (*ad libitum*) beginning before the formation of NFTs led to the reduction of soluble tau and reversal of spatial learning impairments [172]. These effects were not observed following long-term, low-dose treatments at the same age, and tau NFT pathology was not reversed in either dosing regimen. In another study, MB treatment in 17-month-old rTg4510 mice (when mice already display substantial hippocampal and cortical neurofibrillary tangles) did not reduce existing NFT pathology [173]. In the triple transgenic 3×TgAD model, where mice develop both plaques and tangles, MB significantly decreases soluble Aβ, but does not reduce tau phosphorylation or mislocalization [174]. The conflicting results of these studies may be attributed to the use of various mouse models (under the control of different promoters), time points of intervention (where late stage intervention is less promising than earlier time points), brain bioavailability [172], as well as dosing regimen (routes of administration and dosing schedules).

MB may have multiple targets, as a recent study suggests that AMPK mediates MB-induced neuroprotection, independent of mTOR signaling [170]. MB (also known as Rember) may act as a potent tau aggregation inhibitor and has been shown to slow cognitive decline in patients with mild-to-moderate AD in a phase 2 trial [175]. Rember (TauRx, Singapore) is currently in phase 3 clinical trials in patients with behavioral-variant FTD [176].

Inhibitors of Myo-inositol-1,4,5,-Triphosphate and Glycogen Synthase Kinase-3β Signaling

Autophagy can be activated through several known mTOR-independent mechanisms. One such pathway involves the reduction of myo-inositol-1,4,5,-triphosphate (IP_3) levels to induce autophagy. IP_3 is a second messenger that mediates the release of calcium from the ER through its receptor, IP_3R. A recent study suggests that the IP_3R negatively regulates autophagy via interactions with the Beclin 1 complex [177]. The IP_3R antagonist xestospongin B disrupts the IP_3R–Beclin 1 interaction, thereby inducing autophagy [177]. Lithium, a major treatment for bipolar affective disorder, inhibits inositol monophosphatase, which reduces IP_3 signaling and thus upregulates autophagy. Lithium treatment in cell models of HD and PD reduced the accumulation of aggregate-prone forms of huntingtin and α-synuclein, respectively [178]. Lithium also inhibits glycogen synthase kinase (GSK)-3β (a kinase that hyperphosphorylates tau leading to NFT formation), which indirectly suppresses autophagy through its activation of mTOR [179]. Owing to its opposing effects on autophagy, Sarkar et al. [179] have proposed that lithium, in combination with rapamycin, is an effective brain-penetrant therapy to induce autophagy, and suggest that lower doses for each drug can be used in combination to induce autophagy while potentially reducing harmful side effects. Although this combinatorial therapy has only been tested in *Drosophila* models of HD, lithium may also provide beneficial effects in ameliorating tauopathy. In addition, GSK-3β inhibition directly improves lysosomal function by increasing biogenesis and acidification, and increasing APP and C-terminal fragment degradation [180, 181]. NP12, a specific GSK-3β inhibitor, reduces neuronal loss, astrocyte activity, and Aβ/tau pathology, and improves cognitive function in mice carrying $APP_{Swedish}$ mutations K670N and M671L and tau mutations G272V, P301L, and R406W [182].

Trehalose

Trehalose is a naturally occurring nonreducing disaccharide, consisting of 2 glucose molecules, that is produced endogenously in bacteria, fungi, plants, and invertebrates as a response to environmental stressors [183]. Trehalose treatment in vertebrates and mammalian-derived cell lines confers protection against oxidative damage, prevents protein aggregation, and promotes protein interactions by acting as a molecular chaperone [184]. Trehalose was initially identified as being neuroprotective through chemical screening in mutant huntingtin models and its inhibition of oligomeric $A\beta_{40}$ aggregation [185, 186], and has since been shown to reduce disease-related protein aggregates associated with several neurodegenerative diseases *in vitro* through its induction of autophagy [162, 187].

In animal AD models, trehalose protects against Aβ and tau accumulation. Direct injection of trehalose into the lateral ventricles of APP/PS1 mice rescues deficits in spatial memory and learning, and reduces Aβ plaque load [188]. In transgenic mutant P301S tau models, *ad libitum* administration of trehalose reduces sarkosyl-insoluble tau aggregates and rescues cell death in the cortex and brainstem, but does not prevent motor deficits [189]. p62 levels are also reduced following trehalose treatment, indicating that autophagic induction is associated with tau removal. Data from our laboratory reveal similar effects in rTg4510 and JNPL3 transgenic tau mice treated with trehalose. Treated mice exhibit enhanced levels of

autophagy markers, decreased tau aggregation, and improved performance in motor and cognitive behavioral tests [190].

Trehalose decreases the levels of endogenous tau in primary cortical neurons and reduces aggregates of tau with an FTD-17 mutation in N2a neuroblastoma cells, presumably by autophagy [191]. In addition to promoting autophagy, trehalose directly prevents tau aggregation *in vitro* [191], likely through its ability to stabilize proteins in their native form, thereby suppressing aggregation [192]. Therefore, trehalose may be a viable preventative therapy owing to its dual neuroprotective mechanisms.

Although multiple studies have demonstrated that trehalose confers beneficial effects on protein aggregation, behavior, and cell survival in an mTOR-independent manner [193], and leads to a dose- and time-dependent increase in the number of autophagosomes and autophagic markers [194], the specific autophagy-related molecular mechanisms have yet to be identified. A recent study in the mutant superoxide dismutase (SOD)1(G93A) mouse model of ALS suggests that trehalose treatment enhances autophagic flux. *Ad libitum* administration of trehalose reduced p62, ubiquitin, and SOD1 accumulation, while alleviating impaired autophagic flux [195]. While SOD1(G93A) mice exhibited elevated levels of Atg5, LC3-II, and increased numbers of autophagosomes compared with wild-type mice, transgenic mice treated with trehalose showed no significant change in autophagic induction markers compared with sucrose-treated control mice, suggesting that trehalose may not induce autophagy, but possibly enhances clearance (lower autophagosomes to autolysosomes ratio). Alternatively, these results may indicate that trehalose treatment combined with neuropathological conditions leads to more efficient lysosomal clearance. More studies in AD models will be necessary to pinpoint the mechanisms underlying the beneficial effects of trehalose in alleviating tauopathies and Aβ accumulation.

Trehalose is found in plants such as kelp and aloe, and is currently used as a dietary supplement and sugar substitute in food. Its druggable properties (e.g., trehalose is nontoxic at high concentrations, tolerated by humans, and readily bioavailable in the periphery and the brain) make it a suitable candidate for chronic treatment in neurodegenerative diseases. However, as trehalose is a sugar, chronic administration would require monitoring for diabetic-like complications. Furthermore, the chemical structure of trehalose does not lend itself to modification as a lead compound. Therefore, uncovering the mechanism of action for trehalose-mediated clearance of tau through autophagy is of particular interest. Identifying the signaling pathways that govern these effects also holds therapeutic potential as drug targets.

Compounds Promoting Autophagosome Maturation, Transport, and Fusion

Microtubule stabilizers have great potential as an adjuvant therapy to autophagy inducers. Histone deacetylase inhibitors, such as sodium valproate, sodium butyrate, and suberoylanilide hydroxamic acid, have been shown to reduce memory deficits in the $APP_{Swedish}$/PS1ΔE9 mouse model of AD [196, 197], and have recently been shown to activate autophagy in rabbit cardiomyocytes [198]. Autophagy induction by histone deacetylase inhibitors has been attributed to microtubule stabilization due to increased tubulin acetylation [199–201]. Paclitaxel, another microtubule stabilizer, ameliorates motor impairment while improving fast axonal transport and axonal morphology in tau transgenic mice [202]. Conflicting reports, however, have emerged regarding the role of paclitaxel in autophagy induction [203, 204]. Through its stabilization of microtubules, paclitaxel may be a viable therapy for enhancing autophagic transport and fusion. This drug, however, requires further investigation as the bioavailability of paclitaxel in brain is suboptimal [205, 206]. Microtubule stabilizer, Epothilone D readily crosses the BBB [206], and has recently been shown to increase axonal stability, function, and transport, reduce tau pathology, preserve hippocampal morphology, and prevent cognitive deficits in PS19, 3×Tg, and rTg4510 animal models of AD [207–209]. Epothilone D (also known as BMS-241027) was tested in phase 1 clinical trials for safety and tolerability; however, further studies were discontinued based on unfavorable study outcomes (ClinicalTrials.gov identifier NCT01492374). Dictyostatin, triazolopyrimidines, and phenylpyrimidines have recently been identified as brain-penetrant, microtubule-stabilizing agents [210, 211], though their effects on autophagic flux have not yet been validated.

Compounds that modulate lysosomal fusion include potential cystatin B and C antagonists [127], lysosomal cathepsin B modulators [212], and GSK-3β inhibitors, such as lithium, valproate, and NP12 [180, 181]. Genetic deletion of cystatin B and C has been shown to improve neurological deficits and restore autophagic dysfunction in the TgCRND8 and hAPP-J20 mouse models of AD [127, 213]. However, conflicting reports indicate that cystatin C is neuroprotective in several neurodegenerative diseases, and these effects may be mediated by inducing autophagy [214–217]. Future studies should examine the effects of cystatins in AD [218], and whether neuroprotection is attenuated in the absence of autophagy. Cathepsin B is a lysosomal/endosomal enzyme with neuroprotective properties based on its protease activity, and in particular, its $Aβ_{1-42}$ cleavage function [219]. Z-Phe-Ala-diazomethylketone, a positive modulator of cathepsin B activity, reduces Aβ levels in 10–11-month-old APP mice with Swedish and Indiana mutations ($APP_{Swe/Ind}$) and in 20–22-month-old $APP_{swedish}$/PS1ΔE9 mice, protects against

synaptic degeneration, and mitigates behavioral deficits [212]. Similarly, cathepsin D, another lysosomal/endosomal enzyme, has been implicated in clearance of Lewy bodies in PD [220]. One way to pharmacologically enhance cathepsin B and D activity is by administering pharmacological chaperones similar to the ones used in lysosomal storage diseases [221]. To date, no known molecule has been tested for this property.

Conclusions

Enhancing autophagic clearance of toxic protein aggregates through mTOR-dependent or mTOR-independent compounds ameliorates protein aggregation, neuron survival, and behavior. However, while converging evidence suggests that augmenting autophagy is a promising therapeutic intervention [162, 222], it may also overwhelm the lysosomal machinery, leading to increased numbers of autophagosomes and undigested autolysosomes that can impair axonal trafficking and lead to dystrophic axons. In a mouse model of excitotoxicity, constitutive activation of a glutamate receptor induced the buildup of green fluorescent protein–LC3-labelled autophagosomes in dystrophic axons [223]. These data suggest that excessive autophagosome formation may eventually form roadblocks to normal retrograde transport, causing axonal swellings. However, in diseases like AD, inducing autophagy may exacerbate already impaired lysosomal clearance [125]. Therefore, it is important to consider the downstream effects of drug therapies targeted at each stage of autophagy in different diseases, and how they might disturb the delicate balance between autophagosome formation and lysosomal degradation. We propose that a more viable therapeutic strategy for stimulating autophagic clearance of disease-related proteins in AD and other neurodegenerative diseases incorporates not only drugs that increase autophagic induction, but also those that target later steps in the autophagy–lysosome system, thus improving autophagic flux (Fig. 2). Although the right combination of drugs may differ based on the disease type, stage of progression, underlying cause (i.e., genetic *vs* idiopathic), and a variety of other factors, it is clear that modulation of neuronal autophagy may be a promising therapeutic intervention to attenuate AD pathogenesis and rescue cognitive impairment.

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