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·Review·

Dysfunctional autophagy in Alzheimer's disease: pathogenic roles and therapeutic implications

Jun-Hua Liang, Jian-Ping Jia
Department of Neurology, Xuan Wu Hospital of the Capital Medical University, Beijing 100053, China
Corresponding author: Jian-Ping Jia. E-mail: jiajp@vip.126.com

Neuronal autophagy is essential for neuronal survival and the maintenance of neuronal homeostasis. Increasing evidence has implicated autophagic dysfunction in the pathogenesis of Alzheimer's disease (AD). The mechanisms underlying autophagic failure in AD involve several steps, from autophagosome formation to degradation. The effect of modulating autophagy is context-dependent. Stimulation of autophagy is not always beneficial. During the implementation of therapies that modulate autophagy, the nature of the autophagic defect, the timing of intervention, and the optimal level and duration of modulation should be fully considered.

Keywords: Alzheimer's disease; autophagy; presenilin; axon; lysosome; animal model

Introduction

The term "autophagy" refers to a catabolic process in which cellular components, organelles, and misfolded proteins are degraded in lysosomes and recycled[1, 2]. Based on the mode of substrate delivery to lysosomes, autophagy is classified into three forms in most mammalian cells: chaperone-mediated autophagy, microautophagy, and macroautophagy[3, 4]. Macroautophagy (hereafter referred to as autophagy) is believed to be the main pathway. This form of autophagy involves the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome for degradation[2]. Autophagy is initiated by the formation of double-membrane vesicles, called autophagosomes, which sequester cellular components in a non-degradative compartment. Autophagosomes are then transported along microtubules to the perinuclear region (which contains the highest concentration of lysosomes) where they fuse with lysosomes to form autolysosomes, in which the captured materials, together with the inner membrane, are degraded by lysosomal enzymes[2]. Autophagy is responsible for the quality control of essential cellular components by clearance of damaged proteins and organelles. It can also be induced in cells under stress to aid in adjusting to changes in the environment[2, 5, 6].

Autophagy plays a role in both health and disease. Increasing evidence has implicated autophagic dysfunctions in the pathogenesis of several neurodegenerative diseases, particularly Alzheimer's disease (AD). This review presents recent advances in understanding the physiological functions and the unique characteristics of neuronal autophagy, focusing on the mechanisms underlying defective autophagy in AD. Explorations of the regulation of autophagy in AD experimental models and their implications for the future therapeutic use of autophagic modulation in AD patients are also discussed.

Characteristics and Function of Autophagy in the Nervous System

Characteristics of Neuronal Autophagy

Scarcity of autophagosomes in healthy neurons

Autophagosomes are maintained at minimal numbers in healthy neurons. Previous studies using mice expressing green fluorescent protein-tagged microtubule-associated protein 1 light chain 3 (GFP-LC3) as an autophagosome marker showed that GFP-LC3-labeled autophagosomes do not occur in the brain, even after 48 h of food withdrawal[7]. This was further confirmed in another study which showed that normally cultured cortical neurons rarely contain

autophagosomes, as identified by LC3 labeling or by ultrastructural analysis[8]. An electron microscopic study of brain biopsy specimens also confirmed the scarcity of autophagosomes in healthy neurons[9].

High efficiency of autophagosome clearance The scarcity of autophagic vacuoles (AVs) in healthy neurons initially led to the assumption that baseline autophagy in neurons is relatively inactive, but this now seems unlikely. In fact, the processes of autophagosome formation, maturation, and digestion within lysosomes in normal neurons are constituently active and highly efficient. This has been highlighted by an insightful study which showed that blocking autophagosome clearance in cultured primary neurons by inhibiting lysosomal proteolysis causes rapid and marked AV accumulation without altering the induction of autophagy[8].

Then, the question arose as to how to interpret the seemingly contradictory scenario in which neuronal autophagy is highly active but autophagosomes are rarely detected. To answer this conundrum, a key point is that the presence of autophagosomes depends on both their rates of production and clearance by lysosomes[10]. Thus, the appearance of autophagosomes *per se* is not a measure of functional autophagy. Autophagic flux − the net rate of autophagosome content degradation through the autophagic pathway[11] − reflects the efficiency of the process. The efficient clearance of autophagosomes by basal autophagy accounts for the low levels of autophagosomes in healthy neurons.

Role of Basal Neuronal Autophagy

Essential role of autophagy in neuronal homeostasis and survival The post-mitotic nature of neurons predisposes them to the accumulation of unfavorable proteins and damaged organelles that are diluted through cell division in replicating cells. Neurons require active basal autophagy as a means of quality control for protection, especially under disease conditions, for the removal of misfolded proteins and damaged organelles. The importance of constitutive autophagy for neuronal protection has been highlighted by two landmark studies that showed that mice deficient in either of the essential autophagy genes, autophagy-related 5 (Atg5) and autophagy-related 7 (Atg7), specifically in the central nervous system, exhibit progressive neuronal degeneration and massive neuronal loss within the first few months of life[12, 13]. Moreover, in Atg5-deficient or Atg7-deficient neurons, diffuse abnormal intracellular proteins accumulate, and then a massive number of aggregates and inclusions develop. A number of additional studies in a variety of organisms have demonstrated that suppression of the autophagic pathway results in intraneuronal protein aggregates and neuronal degeneration[14, 15]. These studies provide strong evidence that continuous autophagy is normally responsible for clearing abnormal intracytoplasmic contents, which otherwise form protein aggregates, disrupt neuronal function, and ultimately lead to neurodegeneration.

Role of basal autophagy in the maintenance of axonal homeostasis Neurons are typically composed of a soma, a dendritic tree, and an axon. As a highly specialized neuronal compartment, the axon performs many autonomous functions distant from the soma[16]. The fact that the axon transfers proteins and organelles *via* long-distance axoplasmic transport requires that the synthesis and anterograde transport of proteins and membrane be well-balanced by their clearance[17]. The products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport[17]. Accumulating evidence has revealed that, under normal conditions, autophagy is of particular importance for the maintenance of local axon homeostasis and protection against axonal degeneration[12, 13].

Autophagy is uniquely regulated within the axonal compartment, independent of the dendrites and soma. In earlier studies, Hollenbeck observed real-time autophagosome formation in the distal region of the axon, suggesting the local biogenesis of autophagosomes in axons[17]. Recent studies involving live imaging showed that autophagosomes are normally produced in the distal ends of axons and undergo retrograde transport to the soma for the completion of degradation[16, 18].

Autophagy is required for normal axon terminal membrane trafficking and turnover. Aberrant membrane structures accumulate in axons when autophagy is suppressed in the mouse brain[19, 20]. In the above-mentioned study by Hollenbeck, it was shown that the basal level of autophagy might be a key mechanism for remodeling neurite and growth-cone structure during neurite extension[17]. Autophagy might also work as an adaptive response to remodel axon terminal structures

for regeneration after axonal injury[21]. Suppression of basal autophagy in the central nervous system causes neurodegeneration, accompanied by severe axonal swelling[12, 13]. Specific ablation of the essential autophagy gene Atg7 in Purkinje cells results in cell-autonomous axonal dystrophy and degeneration[19], implying the indispensable role of autophagy in housekeeping functions in axon terminals and in protection against axonal degeneration.

Role of neuronal autophagy in synapse development

Synapses are regions of high energy-demand and protein-turnover; the morphological and functional modifications of synapses depend on coordinated protein synthesis and degradation[22-27]. It is now clear that neuronal autophagy plays a crucial role in the development and remodeling of synapses, which is required for learning and memory[24, 25]. Evidence shows that autophagy promotes synapse growth in the *Drosophila* neuromuscular junction by downregulating Highwire[28]. Overexpression of Atg1 is sufficient to induce high levels of autophagy and subsequent enhancement of synaptic growth, while suppressing autophagy through mutations of genes essential for autophagy results in the reduction of synapse size. In *Caenorhabditis. elegans*, endocytosed gamma-amino butyric acid A receptors at inhibitory synapses are targeted to autophagosomes. By functioning as a degradative pathway for these receptors, autophagy serves as a mechanism to control the balance of neuronal excitation and inhibition[29].

Neuropathological Evidence Linking Autophagic Pathways to AD

Intraneuronal neurofibrillary tangles (NFTs) formed by aggregated hyperphosphorylated tau, and extracellular neuritic plaques mainly composed of aggregates of β-amyloid peptide (Aβ), are the neuropathological hallmarks of AD. As discussed above, abnormal autophagic proteolysis can lead to a build-up of waste proteins or unwanted aggregates. Electron microscopic studies of the AD brain by Nixon *et al.* showed that amyloid plaque-associated dystrophic neurites display a massive accumulation of AVs, providing the first pathological evidence for the extensive involvement of autophagy in the pathogenesis of AD[9]. Findings from the brains of $PS1_{M146L}$/APP_{751SL} mice that overexpress familial AD-related mutant human PS1 and APP genes suggest that the dystrophic neurites have excessive accumulation of AVs[30]. The abnormal accumulation of AVs has also been observed in several other animal models of AD, including APPswe/$PS1_{M146L}$ mice and TgCRND8 mice overexpressing mutant human APP695[31, 32]. In contrast to the rarely-observed AVs in normal brains, the abundance of AVs in the brains of AD patients and AD animal models indicates that defective autophagic lysosomal proteolysis may underlie the pathogenic protein accumulation in AD.

Where Is the Autophagic Pathway Defective in AD?

The abnormal accumulation of autophagosomes in neurons constituted the first clue of deficits in autophagy. However, the precise mechanisms underlying autophagic dysfunction in AD are not fully understood. As described earlier in this review, the number of autophagosomes in a cell depends on the balance between AV formation and degradation. The build-up of AVs in neurological disorders may reflect stimulation of the induction of autophagy, impairment of later digestive steps in the autophagic pathway, or a slow rate of autophagosome formation combined with insufficient lysosomal fusion and digestion[33] (Fig. 1). Determining the defective step(s) along the autophagic pathway in AD is critical for understanding the pathogenic significance of autophagy in AD and developing potential therapeutic interventions based on the modulation of autophagy.

Altered Autophagy Initiation in AD

Evidence suggests that abnormalities of autophagy at the level of induction or autophagosome formation contribute to the pathogenesis of AD. Although autophagosomes are numerous in the AD brain and in the $PS1_{M146L}$/APP_{751SL} mouse model of AD, this does not necessarily mean that autophagy initiation is upregulated. In contrast, the expression of beclin-1, an essential initiator of autophagy, is decreased in AD patients compared with healthy individuals[34, 35]. Caspase 3-mediated cleavage of beclin-1 occurs in AD brains; the loss of beclin-1 is believed to be caused by the increased activity of this enzyme in AD patients[36]. The deletion of beclin-1 in an APP transgenic mouse model disrupts autophagy, and increases intracellular Aβ accumulation[34]. Conversely, a genome-wide research indicated that autophagy is

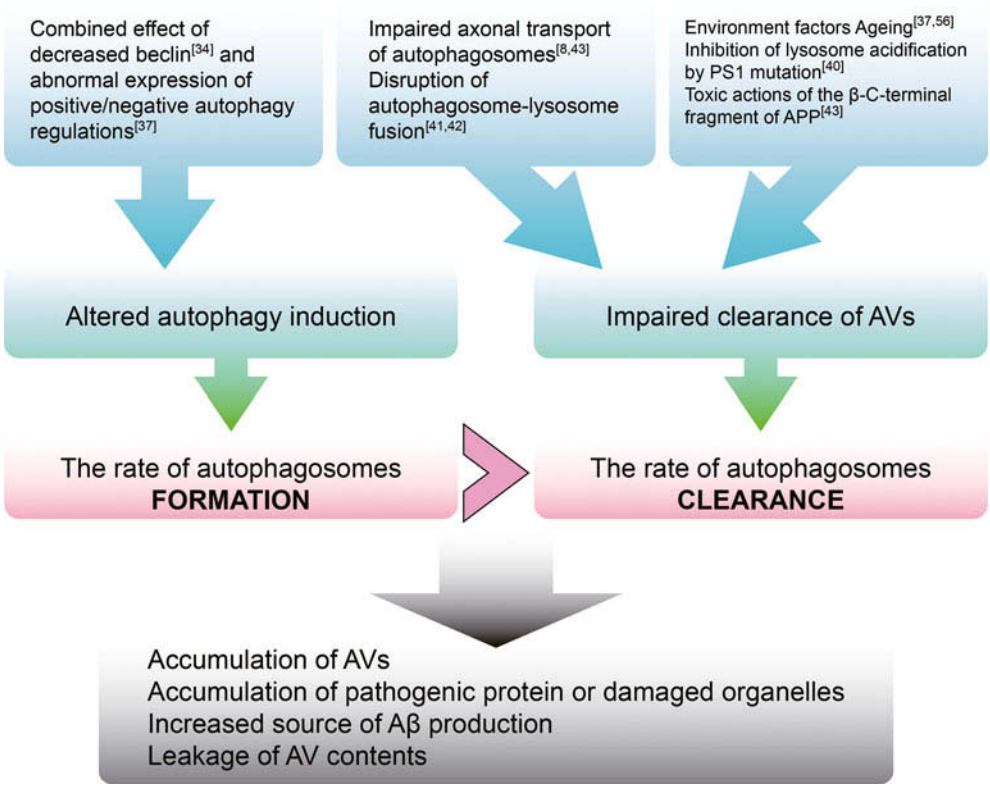

Fig. 1. Model of autophagic failure in AD. The presence of autophagic vacuoles (AVs) in cells depends on both their rate of formation and the rate of lysosomal clearance, which are influenced by many factors in AD. Defective autophagy in AD leads to accumulation of pathogenic protein or damaged organelles and increased production of Aβ.

specifically up-regulated in AD, due both to reactive oxygen species-dependent activation of the type III PI3 kinase (a critical kinase for the initiation of autophagy) and to the transcriptional up-regulation of positive regulators of autophagy[37]. These apparently conflicting findings require further clarification.

Defective Lysosomal Clearance of Autophagic Substrates in AD

The final step of autophagy is the digestion of sequestered materials in autolysosomes. In addition to the defects in earlier stages of autophagy, accumulating evidence has suggested a critical role of lysosomal proteolytic failure in the development of AD-related neurodegeneration. The AVs accumulating in the AD brain are electron-dense autolysosomes and autophagosomes filled with undigested or incompletely-digested "waste" proteins[9]. AV and lysosome fractions isolated from the brains of TgCRND8 mice contain abnormally high levels of LC3-II, ubiquitinated proteins, and Aβ[38]. The morphology of accumulated AVs in the AD brain and in the transgenic mouse model of AD resembles that induced by blocking lysosomal proteolysis by deleting specific cathepsins or administering lysosomal protease inhibitors[8, 39]. For example, selectively blocking cathepsin-mediated proteolysis within autolysosomes with cysteine-protease and aspartyl-protease inhibitors in neurons causes a marked accumulation of electron-dense double-membrane-limited AVs containing incompletely degraded LC3-II[8]. The above evidence strongly points to the disruption of substrate proteolysis within autolysosomes as the principal mechanism underlying autophagic dysfunction in AD. This possibility has been further underscored by the recent finding that presenilin 1 might be involved in the defective proteolysis of autophagic substrates in patients with AD. Presenilin 1 plays a critical role in lysosomal acidification and autophagosome–lysosome fusion[40-42]. Fibroblasts from patients with familial

AD-linked PS1 mutations have abnormal accumulation of AVs, with the turnover of long-lived proteins being markedly impaired. Therefore, familial AD-linked PS1 mutations may have a loss-of-function effect on lysosomal proteolysis, leading to AV accumulation and impaired autophagic substrate turnover in AD[40].

Defective Autophagosome Transport

In normal neurites, immature AVs are transported retrogradely toward the soma for further degradation. The significant build-up of AVs within dystrophic neurites in the AD brain suggests that their transport might be impeded. A selective transport deficit of autophagy-related compartments has been reported in mouse and cell models of AD[43]. The possible role of defective axonal transport of AVs in AD pathogenesis is further suggested by the finding that inhibiting autophagosome delivery to lysosomes induces a rapid accumulation of AVs in neurites with morphology similar to that seen in the AD brain[8]. Further studies are required to clarify the molecular defects underlying the transport failure of AVs.

To accurately identify and quantify the autophagic dysfunction in AD, specific protein markers and diagnostic methods are needed. Transmission electron microscopy is currently the "gold standard" for monitoring autophagy in tissue. Immunohistochemical staining and immunoblotting against autophagy-specific biomarkers such as LC3 have been widely used to assess autophagy[11]. Other approaches include the use of weakly basic dyes which accumulate in the acidic autophagosome-lysosome compartment, and forced expression of GFP-LC3 to detect AVs under the microscope as fluorescent dots[11]. To date, there are no methods to monitor autophagy *in vivo*. Exploring biomarkers that can be widely applied in clinical settings to assess the level of autophagy in AD is critical for improved understanding of the autophagic dysfunction in AD and for the successful development of therapies based on the modulation of autophagy.

Modulation of Autophagy as a Potential Therapy for AD

Since the initial observations that autophagic dysfunction may contribute to the accumulation of proteins in the AD brain, there has been a growing interest in manipulating autophagy as a potential therapeutic target for AD (Table 1).

Therapeutic Induction of Autophagy

Pharmacological induction of autophagy has proved effective in reducing neuronal aggregates and slowing the progression of neurological symptoms in several mouse models of AD. The mammalian target of rapamycin (mTOR) is a critical negative regulator of autophagy[44]. In the platelet-derived growth factor promoter expressing amyloid precursor protein transgenic AD mouse model, long-term inhibition of mTOR by rapamycin prevents AD-like cognitive deficits and lowers the levels of Aβ42, a major toxic species in AD[46, 48]. As expected from the inhibition of mTOR, the induction of autophagy increases in the neurons of rapamycin-treated transgenic mice, suggesting that the reduction of Aβ is due in part to this increase[46]. In 3×Tg AD mice, inducing autophagy *via* rapamycin significantly reduces amyloid plaques, NFTs, and cognitive deficits[45]. Induction of autophagy by administration of a lentiviral vector expressing beclin-1 reduces both intracellular and extracellular amyloid pathology in APP transgenic mice[34]. These findings and related studies[49] have led to the reasonable proposition that targeting the induction of autophagy can have potential therapeutic benefits in AD.

However, conflicting data from recent studies in AD models raise caution about the applicability of the induction of autophagy as a generalized treatment strategy for AD. For example, inhibition rather than stimulation of autophagy has been suggested to alleviate Aβ42-induced cell death[47, 50]. Rapamycin treatment of flies expressing Aβ1-42 results in a significantly shortened lifespan, indicating that enhancement of autophagy may enhance Aβ1-42 neurotoxicity[47]. Thus, it appears that although basal autophagy is required for neuronal survival, the benefit of enhanced induction of autophagy is context-dependent. The timing of intervention in the progression of the disease should be taken into account when implementing autophagy induction as a therapeutic approach. This is illustrated by the findings that increasing the induction of autophagy prior to the development of AD-like pathology in 3×Tg-AD mice reduces the levels of soluble Aβ and tau and amyloid plaques, whereas induction after the formation of mature plaques and tangles has no effect on AD-like pathology or cognitive deficits[45]. In addition, differences in models may account for the conflicting results on the role of autophagy modulation. For example, the inhibition of autophagy by neuron-specific expression of Atg5RNAi

Table 1. Effects of autophagy modulation in experimental models of AD

Experimental model	Intervention	Timing	Duration	Effect on autophagy	Result
3×Tg-AD mice (Tg human APP_{Swe}/Tg human tau_{P301L}/ $PS1_{M146V}$ knock-in)	Food containing rapamycin (2.24 mg/ kg)	Starting at 6 months of age	10 weeks	Induction	Reduced amyloid plaques, NFTs and cognitive deficits[44].
3×Tg-AD mice	Food containing rapamycin (2.24 mg/ kg)	Starting at 2 months of age	16 months	Induction	Reduced amyloid plaques, NFTs and cognitive deficits[45].
3×Tg-AD mice	Food containing rapamycin (2.24 mg/ kg)	Starting at 15 months of age	3 months	Induction	No effects on AD-like pathology and cognitive deficits[45].
PDAPP Tg mice (hAPP(J20))	Food containing rapamycin (2.24 mg/ kg)	Starting at 4 months of age	13 weeks	Induction	Alleviated cognitive deficits and decreased soluble Aβ42[46].
Aβ42-expressing flies	Food containing 1 mmol/L rapamycin	NA	NA	Induction	Shortened lifespan[47].
Aβ40-expressing flies	Food containing 1 mmol/L rapamycin	NA	NA	Induction	No changes in lifespan[47].
Aβ42-expressing flies	Neuron-specific Atg5RNAi expression	NA	NA	Downregulation	Increased lifespan[47].
Aβ40-expressing flies	Neuron-specific Atg5RNAi expression	NA	NA	Downregulation	Shortened lifespan[47].
TgCRND8 mice over-expressing mutant human APP695	Genetically deleting cystatin B	NA	NA	Enhanced lysosomal proteolysis	Reduced amyloid pathologies and memory deficits[38].
PDAPP Tg mice (hAPP(J20))	Administration of a lentiviral vector expressing beclin 1	NA	NA	Induction	Reduced amyloid pathology[34].

Aβ, amyloid β; APP, amyloid precursor protein; Atg5, autophagy-related gene 5; NFTs, neurofibrillary tangles; PS1, presenilin 1; Tg, transgenic.

extends the lifespan of Aβ1-42 flies while having deleterious effects in flies without Aβ1-42 expression, suggesting that Aβ1-42 expression may shift protective neuronal autophagy to a pathogenic condition[47].

Then why would blocking autophagy be beneficial, given its protective role? The main reason is that enhanced autophagy does not necessarily lead to productive autophagy, i.e., an increase in autophagic flux. If the lysosomal clearance of autophagosomes is halted, the activation of autophagy results in the harmful accumulation of intermediate AVs. Nixon and colleagues have shown that autophagosomes in AD brains may be a major reservoir of Aβ[51]. Enhancement of new autophagosome formation without a parallel increase in autophagic flux may actually increase Aβ production and leakage of the catabolic contents of AVs[52].

Targeting Defective Lysosomal Proteolysis

The optimal modification of autophagic failure should enhance autophagosome clearance by the lysosome. In the TgCRND8 AD mouse model that exhibits defective proteolytic clearance of autophagic substrates, enhancement of lysosomal activity by genetic ablation of cystatin B (an endogenous inhibitor of lysosomal cysteine proteases) enhances the clearance of the autophagic substrates, and ameliorates amyloid pathologies and memory deficits in the animals[38]. These findings, together with other related studies[53], suggest that restoring normal lysosomal proteolysis might be the key to developing novel therapeutic interventions against AD, although pharmacological compounds with such effects are not yet available.

Combination Therapy

Successful autophagy-based therapy should take full consideration of both the upstream and downstream autophagic deficits in AD. It is tempting to speculate that autophagy might be partially defective in AD patients at both the autophagosome-induction and autophagosome-degradation stages. In such cases, moderately increased levels of autophagic induction in combination with therapies to promote the successful completion of autophagic degradation might be a promising intervention strategy. However, targeting the induction of autophagy and defective lysosomal proteolysis at the same time is challenging and remains to be investigated. Another potential combination strategy is to simultaneously use two pharmacological autophagy-inducers acting on different regulatory pathways. For example, use of the mTOR-dependent autophagy enhancer rapamycin and the mTOR-independent autophagy enhancer lithium in combination causes greater upregulation of autophagy and yields greater efficiency in clearing protein aggregates[54, 55]. Compared with either treatment alone, using two drugs in combination may enable reduction of the dose of each treatment, which might decrease the likelihood of adverse effects.

Considerations for Therapeutic Use of Autophagy Modulation in AD

Therapeutic use of autophagy modulation in AD would require several important considerations. The first is the specific step or steps along the autophagy pathway that may be defective in AD. The development of autophagy-regulating therapeutics should be based on the nature of the autophagic defect. For instance, if autophagosome degradation is impaired, enhancement of the induction of autophagy should not be used before the autophagic efficiency is restored to avoid exaggerating the AV accumulation. The second consideration is the disease stage in which it would be used. Early in the pathological process of AD, increasing the induction of autophagy may be a valid therapeutic strategy. However, once the neuropathology is well-established, increasing autophagic induction alone may be ineffective, as more mature stable aggregates may be too large to be degraded by autophagy. A final consideration is the optimal level and duration of autophagic modulation therapy. Overactive autophagy could be as deleterious as defective autophagy. A greater understanding of the precise modulation of the autophagic pathways will be important in this regard.

Conclusion

Converging evidence has firmly established the importance of autophagic dysfunction in the pathogenesis of AD. A more complete understanding of the regulatory pathways of neuronal autophagy and further clarification of the pathogenic mechanisms of dysfunctional autophagy in AD will help the development of therapeutic interventions. We also need new agents to target specific steps in the autophagic pathway to achieve more accurate modulation of autophagy. Future research should also make efforts to address the precise determinants of the outcome of autophagic modulation.

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