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Corresponding Author: Hyewhon Rhim

Authors: Md. Ataur Rahman[1], Hyewhon Rhim[1,*]

Institution: [1]Center for Neuroscience, Korea Institute of Science and Technology, Seoul, Korea,
[2]Department of Neuroscience, Korea University of Science and Technology, Daejeon, Korea,

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Title: Therapeutic implication of autophagy in neurodegenerative diseases

Author's name: Md. Ataur Rahman[1] and Hyewhon Rhim[1, 2*]

Affiliation: [1]Center for Neuroscience, Korea Institute of Science and Technology, Seoul, Korea; [2]Department of Neuroscience, Korea University of Science and Technology, Daejeon, Korea.

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[*]**Corresponding Author's Information:** Center for Neuroscience, Brain Science Institute, Korea Institute of Science and Technology (KIST), 5 Hwarang-ro 14-gil, Seongbuk-gu, Seoul 02792, Korea. Tel.:+82-2-958-5923, Fax: +82-2- 958-6937. E-mail: hrhim@kist.re.kr

ABSTRACT

Autophagy, a catabolic process to maintain intracellular homeostasis, has been recently focus in numerous human disease conditions, such as aging, cancer, development, immunity, longevity, and neurodegeneration. However, sustaining autophagy is essential for cell survival and dysregulate autophagy is anticipated to speed up neurodegeneration progression; although, the actual molecular mechanism is not yet fully understood. In contrast, emerging evidence suggests that basal autophagy is necessary for removal of misfolded aggregation proteins and damaged cellular organelles through lysosomal mediated degradation. Physiologically, neurodegenerative disorders are related to the accumulation of amyloid β peptide and α-synuclein protein aggregation in Alzheimer disease and Parkinson disease, respectively. Even though autophagy could impact several facets of human biology and disease, however it functions as a clearance for toxic protein in the brain contributes us novel insight into the pathophysiological understanding of neurodegenerative disorder. In particular, several studies demonstrate that natural compounds or small molecule autophagy enhancer stimuluses autophagy which is essential in clearance amyloid β and α-synuclein deposits. Therefore, this review briefly deliberate the recent implication of autophagy in neurodegenerative disorder control and emphasize the opportunities and potential therapeutic application of autophagy.

INTRODUCTION

Autophagy is a cellular process, pathological and physiological conditions, that leads to removal of damaged/injured organelles and aggregated proteins (1). In more general terms, basal autophagy is important for removing misfolded proteins and damaged organelles, and therefore, plays a crucial role in maintaining cellular homeostasis. Because, autophagy participates in a variety of normal physiological processes such as glucose homeostasis (2), lipid metabolism (3), aging (4), and, many more. However, failure of autophagy caused cellular dysfunctions which are unable to remove defective proteins or damaged organelles. In contrast, knockdown/inhibition of autophagy often causes premature aging in *Caenorhabditis elegans* (5). Interestingly, research shows in *Drosophila melanogaster,* lifespan extends after enhanced autophagy (6). Several other studies shows that deletion/mutation of the autophagy-related gene (Atg) provokes an imbalance of body homeostasis resulting in obesity (7), insulin resistance (8), diabetes (9), and some of neurodegeneration diseases (10). In addition, neurodegenerative disease occurs when aggregated proteins are not accurately degraded by autophagy, for example, accumulation of aggregated forms of amyloid-β (Aβ) peptide (11) and tau protein (12) in Alzheimer Disease (AD), α-synuclein in Parkinson Disease (PD) (13), and that of huntingtin in Huntington Disease (HD) (14). Therefore, autophagy pathway could be a potential therapeutic target for the treatment of neurodegenerative diseases. Indeed, neuronal cells are easily damaged through impaired autophagy among all types of cells in physiological system (15), due to lacking of autophagy have a difficulty to maintain synaptic activity (16) and axonal homeostasis (17). Several studies have shown a relationship between neurodegeneration and autophagy signaling pathway (18). This review would like to emphasize to understanding the basic molecular mechanisms and regulation of autophagy in neurodegenerative diseases control.

MOLECULAR MECHANISMS OF AUTOPHAGY

Autophagy occurs throughout different consecutive steps such as sequestration, deliver to lysosomes (autolysosome), degradation and utilization of toxic components, and collectively every step might exert diverse role during autophagy. Importantly, formation of phagophore is initiated during the early stages of autophagy that is likely derived from numerous sources, including endoplasmic reticulum (19), endoplasmic reticulum-golgi intermediate compartment (20), plasma membrane (21), recycling endosomes (22), golgi complex (23), and lipid droplets (24). In fact, multiple autophagy-related gene is involved in membrane rearrangements and autophagosome formation that consequent attached to the lysosomes. Generally, there are two ubiquitin-like conjugation reactions involve during autophagy where firstly, Atg12 conjugation to Atg5. Another Atg16L1 binds to conjugate Atg12-Atg5 to form a complex which is essential for phagophore maturation (25). However, second conjugation of Atg8 and LC3 is involved to complete autolysosome formation in that process LC3 is cleaved through Atg4 to obtain cytosolic LC3-I. Finally, LC3-I is coupled to phosphatidylethanolamine (PE) on macromolecular assembly of phagophore to appear microtubule-associated protein light chain 3 (LC3-II) proteins (Figure 1). It has been found that LC3-II localizes to the autophagosomal membrane, which is most important marker of autophagy in mammal cells (26). Whereas, several adaptor proteins such as neighbor of BRCA1 gene 1 (NBR1), nuclear dot protein 52 (NDP52), and p62/sequestosome 1 protein directly binds to LC3-II (27, 28). Subsequently, misfolded protein (29) or abnormal cellular organelles as well as mitochondria (30), peroxisome (31), endoplasmic reticulum (32) are engulfed by double-membrane autophagosome. Strikingly, the

autophagosomes are finally combined with lysosome that forms an autolysosome where the internal substrates are degraded to produce nutrients and metabolites (33).

CLEARENCE OF AGGREGATE-PRONE PROTEINS THROUGH AUTOPHAGY IN NEURODEGENERATION

Recently, several studies revealed that intracellular protein aggregation and misfoldings are common phenomenon of many neurodegenerative diseases such as AD, PD, HD, and spinocerebellar ataxias (SCA) (34). Till to the date, there is no efficient therapeutic approaches that cure or prevent the neurodegeneration diseases in humans. Thus, it is urgently needed to understand the molecular mechanism and factors that regulating the expression levels of misfolding and aggregate-prone proteins during neurodegeneration. Usually, there are many methods to treat neurodegenerative diseases that might be to enhance degradation of prone-proteins. However, among of them autophagy-lysosome and ubiquitin-proteasome pathways are most possible routes for misfolding protein clearance (Figure 2). Although the ubiquitin proteasome system primarily degrades small-molecule proteins, but its accurate mechanism is not clear whether it is a possible therapeutic target or not the clearance of aggregate-prone proteins. In addition, autophagic degradation of aggregate-prone proteins are related with decreasing protein aggregation and toxicity. Therefore, enhancing autophagy might be a promising therapeutic approach for neurodegenerative diseases in there the aggregate-prone proteins are used as autophagy substrates such as tau (35), α-synuclein (36), mutant huntingtin (14), and mutant ataxin 3 (35) for their clearance in neurodegeneration control.

Autophagy in Alzheimer disease

AD is a multifactorial, irreversible, progressive and most common type of dementia in elderly people worldwide which gradually demolishes memory and thoughts, and ultimately the capability to carry out the cognitive function (37). Recently, it has been shown that the main pathological feather of AD comprises intracellular neurofibrillary tangles containing hyperphosphorylated tau protein, self-aggregating extracellular Aβ plaques, weakening of cholinergic function, and eventually autophagy dysfunction (38, 39). However, Aβ peptide derived from the successive splitting of amyloid precursor protein (APP) through β-site APP cleavage enzyme 1 (BACE1) and γ-secretase. Accumulation of this peptide causes cognitive deficits and neuronal dysfunction in the cortex and hippocampus of AD pathogenesis (40). Particularly, Aβ may also be produced in autophagosomes, which seem to comprise APP and presenilin-1 enzyme that participate in the cleavage of APP to Aβ (41). Additionally, autophagy condition might play an important role in Aβ secretion into extracellular space where it forms plaques accumulation. Numerous study shows that deletion of Atg7 in transgenic mice model of APP results in fewer Aβ extracellular secretion as well as plaque formation, therefore autophagy up regulation would simply be degraded Aβ secretion (42). Furthermore, numeral of research have been suggested that autophagy is involved in the removal of Aβ under physiological states, therefore maintaining Aβ homeostasis in a healthy brain is essential (43). Conversely, it has been indicated that the autophagy activation is decreased in AD patient's brains and animal models (44). More importantly, autophagy reduction activity promotes accumulation of Aβ in the brain and consequently contributes AD pathogenesis (45). Therefore, targeting Aβ and tau protein should be considered as an important therapeutic approach against AD pathogenesis via upregulation of autophagy process.

Most importantly, class III PI3K/beclin-1 signaling is a constructive and significant regulator in autophagy (46). For that reason, it seems that modulation of autophagy represents a feasible method for Aβ clearance in the brain (45). Moreover, substantial efforts have been made to recognize safe and effective pharmacological stimulation of autophagy for AD treatment (47). In practical, the existing outcomes have provided biochemical, epidemiological, neuropathological, and genetic evidence and motivated the design of therapeutic approaches against AD. Incidentally, most anti-AD research group has been emphasizing on discovering a BACE1, a rate-limiting enzyme in Aβ generation, enzymatic inhibitor (48). To understand the molecular basis of AD in autophagy, recent studies have been shown that elevated protein levels of BACE1 found in AD patients as well as mouse models suggesting the down-regulation of BACE1 expression might be considered as a novel target for AD treatment (49, 50). Even though such improvement, however, an effective therapy to halt the development of neural damage in AD patients is still missing. Meanwhile, medications for AD approved by the FDA such as one NMDA receptor antagonist and four acetylcholinesterase (AChE) inhibitors (51). However, combined therapy with NMDA receptor antagonist along with AChE inhibitors could facilitate greater efficacy. Otherwise, multifunctional agents are as well found to be possible drug candidates for AD (52). For that reason, research for multi-target compounds has been constantly grown interests in AD treatment (53).

For treatment purpose, autophagy enhancers treated in various AD mouse models have produced useful possessions related with a decrease in Aβ levels. For example, carbamazepine, an mTOR-independent autophagy stimulator, improves memory deficits by enhancing autophagy via decreasing Aβ formation in APP/PS1 double-transgenic mice (54). Notably, Aβ plaques formation diminishes by rapamycin in triple-transgenic PS1/APP/Tau mice and improves

cognitive defection through inducing autophagy (55). Additionally, lithium treatment also suppresses in aged APP/PS1 mutant mice pathology via GSK3β inhibition disturbing tau phosphorylation (56). Interestingly, latrepirdine, an antihistamine drug, shows to increase autophagy by reducing Aβ peptide through inactivating the mTOR complex 1 which recovers cognition function in AD mice model (57). Correspondingly, trehalose, a disaccharide sugar, reduces aggregation of insoluble tau which enhances neuronal brainstem survival in cerebral cortex via influences autophagy (58). Moreover, rapamycin and lithium decreases aggregation of p62 in cortical and hyperphosphorylated tau protein (59, 60). Taken together, all of this evidence indicates that autophagy stimulation is a promising therapeutic approach for tauopathies as well as Aβ clearance in AD mouse models.

Autophagy in Parkinson disease

PD is the progressive disorder of nervous system and second most familiar neurodegeneration in humans. PD is a long-term degenerative brain disorder that mostly affects motor function of the central nervous system, happened by progressive degeneration of dopamine neurons in an area called substantia nigra in the brain, clumps of α-synuclein protein known as Lewy Bodies (61). In addition, dementia becomes more common in the advanced phases of the disease, while depression and anxiety are also general occurring in more than a third of people with PD (62). However, PD is related with A53T and A30P point mutations of α-synuclein or leucine-rich repeat kinase 2 mutations or sometimes gene multiplication. Besides, PTEN-induced kinase 1 or Protein deglycase mutations in parkin are also caused on recessive early-onset PD (63). The pathological feature of early-onset PD is Lewy body formation of α-synuclein aggregation (64). It has been found that α-synuclein point mutants are sometimes

autophagy substrates and its excessive expression does not distress autophagy (65). For that reason, autophagy influences by lithium, trehalose, carbamazepine, calpastatin, and rilmenidine promotes the removal of mutant α-synuclein (66). In contrast, wild-type α-synuclein overexpression reduces autophagosome synthesis in transgenic mice and mammalian cells through down regulation of Ras-related protein Rab-1A (65). Additionally, overexpression of α-synuclein has been proposed to prevent autophagy in rat midbrain by repossessing transcription factor EB into combinations and affecting its cytoplasmic retention, but transcription factor EB overexpression activates autophagy and prevents neurotoxicity of α-synuclein-induction (67). Hence, autophagy activator protein beclin-1 ameliorates neuronal pathology in lentiviral overexpression transgenic mice model through decrease α-synuclein accumulation (36). Even though the effectiveness of autophagy stimulation *in vivo* attempts further consideration, however, absence of strong neurodegeneration phenotypes together with dopaminergic neuronal susceptibility in numerous transgenic PD model has prevented treatment trials in PD (68).

In preclinical approach of PD, autophagy has been increased by several potential autophagy enhancers such as rapamycin, lithium, metformin and trehalose, routinely used in a laboratory setting to increase clearance of α-synuclein and thereby protecting neuronal cells (69). Till to the date, most comprehensively tested autophagy-enhancer is rapamycin, isolated from bacterial strain of *Streptomyces hygroscopicus*. In particular, rapamycin blocks the upstream signaling factor mTOR by binding to its intracellular small protein FK506 binding protein 12 receptor, thus disrupting mTOR function (70). However, rapamycin has capable to decrease α-synuclein accumulation and block α-synuclein induce neurodegeneration in wild-type and A53T α-synuclein expressing PC12 cells (71) as well as α-synuclein overexpressing mice (72) and rats (67). In addition, rapamycin decreases neuronal cell death induce by 6-OHDA (73), rotenone

(74), MPTP mouse model of PD (74). Furthermore, rapamycin exclusively improve motor function in A53T α-synuclein overexpressing mice (75) and also show neuroprotective activity in *Drosophila melanogaster* mutated PTEN-induced putative kinase 1 as well as parkin (76), although it has been accompanying with a widespread side-effects (77). By compare, another preclinical trial drug lithium which induce autophagy as an mTOR-independent manner and affects together *in vivo* as well as *in vitro* models of neuroprotection (78). Nevertheless, preclinical model of PD, lithium inhibits aggregation of α-synuclein in PC12 cells expressing A53T and A30P α-synuclein (79). In addition, lithium also prevents against rotenone-induction neurotoxicity and cell death through the stimulation of autophagy (80). Overall taken together, those preclinical studies via rapamycin in addition with lithium shows valuable effects on diverse model of PD-related pathological progressions, such as α-synuclein aggregation, which is associated with autophagy stimulation. Therefore, these pioneering studies have been demonstrated that prospective role of autophagy-enhancing approaches in experimental settings into the physiological function of autophagy in PD-related pathology.

Autophagy in other neurodegenerative diseases

Huntington disease (HD), an autosomal dominant mutation, is a progressive neuronal genetic disorder that impacts muscle coordination as a results formation of cognitive decline and dementia, thereby unsteady movements, dropping in mental and emotional abilities, and finally psychiatric and behavioral problems (81). In pathologically, polyglutamine disorder are a group of neurodegenerative diseases caused by expansion of cytosine-adenine-guanine repeats encoding, therefore rendering the protein aggregation-prone and pathogenic (82). For treatment

of HD with CCI-779, an rapamycin analog drug, improve pathology in mice model of HD by diminishing aggregation of mutant huntingtin protein via the stimulation of autophagy pathway (14). Interestingly, numerous mTOR-independent autophagy activators, for example Ca^{2+} channel blockers (loperamide, verapamil, amiodarone), inositol-lowering agents (lithium), calpain inhibitor (calpastatin), and NOS inhibitor (L-NAME) improve removal of huntingtin as well as release toxicity in cellular HD models (66). Recently, it is describe that a natural compound quaternary ammonium salt berberine, isoquinoline alkaloids, has been found that to prevent mutant Htt protein accumulation, alleviate HD symptoms, as well as activate autophagy in HD cell and mouse models (83). However, another natural product onjisaponin B, derivative from Radix Polygalae, has found to control autophagy in PC12 cells through Atg7 as well as AMPK-mTOR signaling pathway to enhance clearance of the Htt mutant protein and α-synuclein mutant (84). Therefore, it has been suggested that modulation of autophagy in HD might be a favorable approaches to treat this neurodegeneration.

Spinocerebellar ataxia (SCA), progressive genetic neuronal disease, is described by means of gradually growing problems connected with muscle stiffness, speech and swallowing difficulties which leads to involuntary eye movements. In addition, SCA is caused by aggregation of mutant ataxin-3 therefore it impair learning and cognition function (85). However, modulation of autophagy is involved in the removal of the abnormal ataxin-3 protein, and hereafter impaired autophagy may affect the aggregation of the mutant ataxin-3 protein that initiating toxicity to cells as well following cell death. Accordingly, targeting SCA3 through directing autophagy could become a novel therapeutic strategy (86). Likewise, trehalose, a natural alpha-linked disaccharide, is an mTOR-independent autophagy inducer could be used to control ataxin-3 protein in SCA. However, recently it has been found that trehalose analogs, such

as lactulose and melibiose, is significantly decreased aggregation of the abnormal ataxin-3 protein in a cell model which is associated with the stimulation of autophagy along with a reduction in free radical production (87). Therefore, autophagy induction could be used as a therapeutic strategy to control SCA.

Amyotrophic lateral sclerosis (ALS), a rare group of neurological disorder, is effect of gradually degeneration and death of nerve cells mainly motor neurons which control for voluntary muscle function resulting in difficulty in speaking, walking, swallowing, chewing, and finally breathing (88). To control this disease, it is necessary to induce autophagosomes through a decreasing mTOR complex 1 using Cu-Zn superoxide dismutase mutant mice model (89). However, p62 could bind to superoxide dismutase mutation in an ubiquitin-independently to target its removal by autophagy (90). Conversely, mTOR-independent autophagy stimulation by lithium eases removal of mutant superoxide dismutase and hinder the disease start. However, treatment with trehalose has been found to be more beneficial in superoxide dismutase mutant mice through up-regulation of mTOR-independent autophagy in ALS (91). Therefore, autophagy stimulation is very essential issue in *in vivo* studies of ALS treatment.

THERAPEUTIC IMPLICATIONS OF AUTOPHAGY IN NEURODEGENERATION

As a therapeutic purpose, it has been indicated that upregulation of autophagy through mTOR complex 1-mediated pathway might be targeted to removal of aggregate protein molecules and decrease cytotoxicity of mouse, zebrafish, and *Drosophila* become well studies (92). However, tauopathies, α-synucleinopathies, SC 3, HD, and familial prion disease models has been implicated to treat neurodegenerative disease through this strategy. In particular, mTOR-independent autophagy inducers rapamycin analogue such as rilmenidine and trehalose

drugs has been used in these diseases (92). On the other hand, autophagy inhibitor increases the toxicity of these protein that leads to enhance of the relevant protein during neurodegeneration (92). Recently, it has been shown that phosphatidylinositol binding clathrin assembly protein, also known as PICALM, act as equally autophagosome formation and autophagosome degradation process in AD genome-wide association studies (GWAS) hit. Hence, altered PICALM activity *in vitro* as well as *in vivo* lead to increase toxicity and accumulation of tau in AD pathogenesis (93). Moreover, pharmacologically inhibition of mTOR complex 1 is more reliable target in autophagy control in neurodegeneration (94). It is also mention that rapamycin and its chemically synthesized analogues such as CCI-779 are widely used potential activator of autophagy in yeast and mammalian cells in neurons as well as *in vivo* in mouse brain. Eventually, widespread preclinical animal model studies are required to induce autophagy in neurodegenerative disease.

Most recent observation found that inhibition or reduction of histone deacetylase 6 has been revealed to rescue memory in 5XFAD AD mouse models and AD patients, which has further therapeutic possibilities for AD as well as other neurodegenerative diseases (95). In addition, it has also been found that transplanted protein-induced pluripotent stem cells (iPSCs) derived from skin of mouse fibroblasts differentiates into glial cells and reduction of plaque depositions which alleviated the cognitive dysfunction in 5XFAD transgenic AD mouse model, suggesting a favorable therapeutic application for AD (96). Similarly, insulin-degrading enzyme, major proteases of Aβ, secreted from astrocyte through autophagy-based alternative secretory pathway in AD conditions (97). Furthermore, statin, a class of lipid-lowering medications, induces autophagy in astrocytes culture through AMPK-mTOR mediated pathway and it has been suggested that autophagy is essential in insulin-degrading enzyme secretion, thus

modulation of autophagy could provide a possible therapeutic approach in Aβ pathology by increasing clearance of extracellular Aβ (98). Hence, accumulation of Aβ peptide participates to the pathological condition of AD, while inhibiting Aβ production or increasing Aβ removal may be implicated in slowing the improvement of AD (40). In particular, the promotion of Aβ clearance is currently considered to be an additional therapeutic approach for AD. Thereby, autophagy has been found to be an important role in the clearance of Aβ under physiological conditions, for that reason it is essential to maintain Aβ homeostasis in the healthy brain (43). Most importantly, our current research is considerable effort directed to identify safe and more effective pharmacological inducers of autophagy in neurodegenerative diseases. Therefore, to target of AD drug development natural compound-mediated enhancement of autophagy might represent a sustainable strategy to Aβ clearance. To assess this question, alternatively natural compound could be a potential drug candidate which facilities to enhance autophagy in AD patients. As a result, recently it has been found that several alkaloids, such as oxoisoaporphine, significantly decreased Aβ secretion in neuroblastoma cells overexpresses in human APPsw Swedish mutant (99). Furthermore, another latest report suggests that chronic administration of isoquinoline alkaloid, berberine, significantly decreases Aβ deposits in addition to promotes Aβ clearance as a result improving cognitive impairments in 3XTg AD mice by enhancing the activity of autophagy via the class III PI3K/beclin-1 pathway (100).

For the treatment of PD, several natural compound recently have been gained attention to clearance of α-synuclein in animal and cell model. Specifically, recent studies have been indicated that a natural compound, curcumin, derived from the curry spice of turmeric shows low toxicity in normal cells and also significantly decrease the accumulation of α-synuclein in A53T cell via downregulation of the mTOR signaling by increasing autophagy (101). Interestingly,

oxyresveratrol, a natural antioxidant, increases mTOR dependent autophagy in human neuroblastoma cell model independent of apoptosis (102). In addition, 18α-glycyrrhetinic acid, well known gap-junction inhibitor, induces autophagy through upregulation of beclin-1, Atg5, Atg7 and LC3 as well as downregulation of p62 in neuroblastoma cells (103). Therefore, these review has an important observation that the pharmacological targeting of autophagy enhancer using natural compound holds a favorable potential implication for the development therapies in PD.

CONCLUSION

Even though a variety of autophagy-related proteins participate and control in autophagy pathway (104), however several studies have been performed to explore autophagy regulation through the active ingredients of plants. Although numerous fundamental queries are essential to be further addressed before many novel agents could be useful in a clinical approaches, thus the research of interest in autophagy is developing rapidly and clinically applicable might be anticipated as soon as possible. Furthermore, it is very important to characterization of dysfunctional autophagy in diverse stages of genetic and molecular subtypes in neurodegeneration. It is also necessary to study with the active clinical translation of downstream autophagy regulation which proposes an exciting new era for the development of therapeutic strategies. Consequently, additional studies are required on physiological roles of modulation of brain autophagy process in neurodegenerative diseases. Finally, we would like to screen new natural compounds that modulate autophagy and identify main targets key molecular

mechanisms underlying pathophysiological roles of neurodegeneration with concern for potential therapeutic drugs target.

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Figure legends

Figure 1. Regulation of autophagy signaling pathway. Autophagy may initiate deprivation of nutrients or growth factors which activate AMPK and/or inhibition of mTORC1 lead to stimulation of ULK complex (FIP200 and ATG13). Beclin-1 become phosphorylates leading to VPS34 activation and then initiation of phagophore formation. VPS34 complex function comprises a regulatory subunit like VPS15 (p150) and Beclin-1, connection with other regulatory factors e.g. AMBRA, ATG14, UVRAG, and BIF-1. Atg5–Atg12 conjugation involves Atg7 and Atg10 that form a complex, Atg12-atg5-Atg16 which influences the formation of phagophore. Atg5 and Atg12 forms a complex with Atg16, which act E3-like function towards LC3-PE assemble (LC3-II) that has a role to initiation of phagophore formation. LC3-II is a particular autophagy indicator marker which is eventually disrupted by autolysosome. Autophagosome maturation also are involving fusions with lysosomes that is mediated by Rab7, ESCRT and SNARE proteins which eventually leading to cargo degradation and recycling of nutrients and metabolites.

Figure 2. Modulation of autophagy and intersections between autophagy and disease-associated genes as a therapeutic approach for neurodegenerative disorders. Impaired autophagy is involved in many pathogenesis of neurodevelopmental and neurodegenerative diseases. Different cytosolic mutant and aggregate proteins are linked with neurodegenerative which may predominantly remove through autophagy. Pharmacologically stimulation of small molecule autophagy enhancer may initiate autophagy via mTOR dependent/independent pathways that reduced mutant protein-associated aggregates and toxicity leading to cytoprotection and rescue against neurodegeneration. Several genes connected with neurodegenerative disorders have now been implicated in autophagy function. These genes performance at a number of diverse steps throughout the autophagic method, from early steps of autophagosome development through autolysosome formation. Their proposed sites of action are designated, along with the neurodegenerative disease with which they are related.

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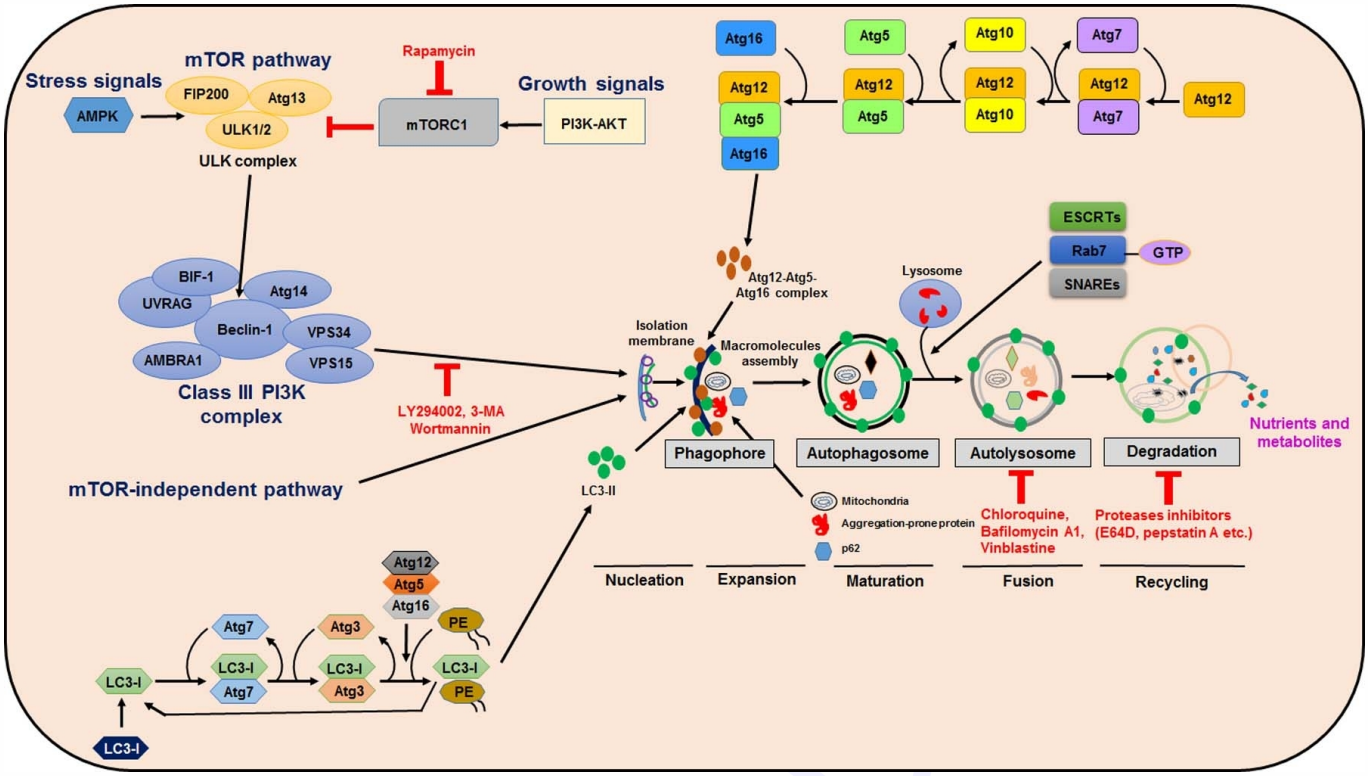

Fig. 1

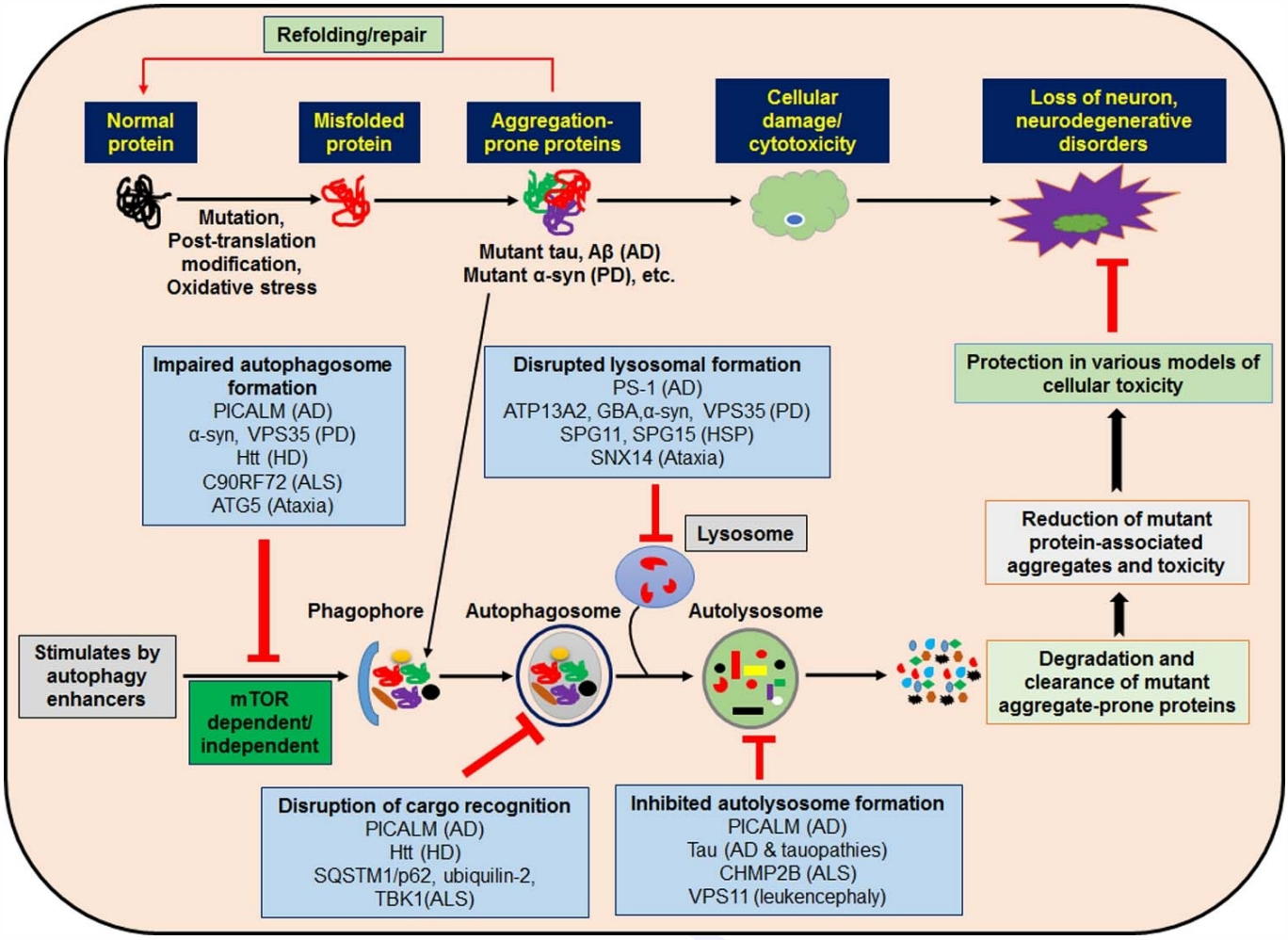

Fig. 2