Autophagy in Alzheimer's disease pathogenesis: therapeutic potential and future perspectives

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Abstract

Alzheimer's disease (AD) is a complex neurodegenerative disease in the elderly. It is the most common cause of dementia in human. AD is characterized by accumulation of abnormal protein aggregates including amyloid plaques (composed of beta-amyloid (Aβ) peptides) and neurofibrillary tangles (formed by hyper-phosphorylated tau protein). Besides, synaptic plasticity, neuroinflammation, calcium signaling etc. are found to be dysfunctional as well in AD patients. Autophagy is an evolutionarily conserved lysosome-dependent cellular event in eukaryotes. It is closely linked to the modulation of protein metabolism, through which damaged organelles and mis-folded proteins are degraded and then recycled to maintain protein homeostasis. Accumulating evidence has showed that impaired autophagy contributes to AD pathogenesis. In the present review, we highlight the role of autophagy, including bulk and selective autophagy, in regulating metabolic circuits in AD pathogenesis. We also discuss the potential and future perspectives of autophagy-inducing strategy in AD therapeutics.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that causes the most common (approximately 60-80%) type of human dementia cases (Crous-Bou,

Minguillon, Gramunt & Molinuevo, 2017). It is estimated that more than 35 million individuals worldwide are living with AD and the number may rise to 65.7 million in a decade and 115.4 million in 2050 (Gulland, 2012). Despite the continuous research on AD since its first description in 1906, the molecular basis of AD pathogenesis is still not fully understood and there are no effective interventions to block or revere AD progression.

There are two critical hallmark features in AD patients' brains: the extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Senile plaques consist of short peptides called beta-amyloid (Aβ) and NFTs are formed by hyperphosphorylated microtubule associated protein tau (MAPT) (Suh & Checler, 2002). In addition to the abnormal protein aggregates, synapse loss, mitochondrial dysfunction, neuroinflammation activation, insulin signaling disturbance, disrupted calcium homeostasis, cholesterol metabolism defect and so on, have been observed as well in AD patients and rodent animal models (Querfurth & LaFerla, 2010).

The etiology of AD is extremely complex and it is believed to be the comprehensive results of multiple factors including age, family history, genetic background, education, brain injury and other risk factors (2020). According to the age of onset, AD is usually categorized into early-onset AD (EOAD, onset < 65 years) and late-onset AD (LOAD, onset ≥ 65 years). EOAD is caused by the pathogenic mutations in three genes including *APP*, *PSEN1* and *PSEN2*, and is inherited in an autosomal dominant manner (Hardy, 2017; Rogaev et al., 1995; Sherrington et al., 1995). Though EOAD is more aggressive, it just accounts less than 3% in all AD cases (Sun, Xie, Tang, Li & Shen, 2017). More than 90% of all AD cases are LOAD. The genetic components of LOAD are much more complex than EOAD. *APOE* ε4 has been viewed as the strongest LOAD risk gene (Farrer et al., 1997; Saunders et al., 1993). In addition to *APOE*, more than 20 risk genes for LOAD, including *ADAM10, PICALM*, *TREM2*, *CLU*, *SORL1*, *CR1*, *BIN1*, *CD33* and so on, have been identified recently through genome-wide association studies (GWAS) (Jansen et al., 2019; Lambert et al., 2013). These risk genes are involved in multiple signaling pathways, providing more perspectives to understand AD pathogenesis.

In recent years, it has been demonstrated that dysfunctional autophagy is closely linked

with AD. Originally, immature autophagosome accumulation and dystrophic neurites were observed in the brains from AD patients via electron microscopy analysis (Nixon et al., 2005). Consistently, accumulated autophagosomes were detected as well in both neuronal dendrites and soma in rodent AD models (Yu et al., 2005). Interestingly, it is found that the abnormal autophagosome accumulation occurs prior to the formation of amyloid plaques (Yu et al., 2005). Besides, the expression of several autophagy-related proteins was reported to be down-regulated with the progression of AD (Rubinsztein et al., 2005). These findings strongly suggest that autophagy is defective in AD and compromised autophagy contributes to AD pathogenesis.

2. Overview of autophagy

Autophagy is a conserved catabolic process to degrade defective proteins or organelles in lysosomes, followed by recycling the basic components in eukaryotic cells. According to the distinct mechanisms through which the autophagic cargos are delivered to lysosomes, autophagy is usually classified into three different types: macroautophagy, chaperon-mediated autophagy (CMA) and microautophagy (Nixon, 2013) (Figure 1).

2.1 Macroautophagy

Macroautophagy is the dominant type of autophagy and has been extensively studied, so it is commonly referred to as “autophagy” for short. Autophagy is a highly dynamic and tightly regulated cellular event in eukaryotic cells (Ohsumi, 2014; Takeshige, Baba, Tsuboi, Noda & Ohsumi, 1992; Tsukada & Ohsumi, 1993). The basal level of autophagy is low under physiological condition, however, it can be rapidly induced by multiple stimuli such as energy deprivation (Kim, Kundu, Viollet & Guan, 2011), nutrient starvation (Kuma et al., 2004), misfolded proteins (Kraft, Peter & Hofmann, 2010), damaged organelles (Glick, Barth & Macleod, 2010), infection, inflammation (Deretic, Saitoh & Akira, 2013) and other stressors (Kroemer & White, 2010). The key event of autophagy is the formation of autophagosome, which is mediated by a variety of proteins called ATGs (autophagy-related genes) (Lamb, Yoshimori & Tooze, 2013) (Figure 2). The whole autophagy process is termed “autophagic flux”, and based on the status of autophagosome, it is generally divided into four stages: initiation, autophagosome formation, maturation (or fusion) and degradation (Kiriyama &

Nochi, 2015). Each step is tightly regulated by multiple molecules.

2.1.1 Autophagy initiation

Autophagy initiation is mediated by the ULK (UNC-51-like kinases) complex. ULKs are serine/threonine kinases, including ULK1 (ATG1, homolog in yeast) and ULK2 (ULK1/2). The ULK complex consists of the core component ULK1 or ULK2, and other interacting proteins including ATG13, FIP200 and ATG101 (Figure 2). In response to autophagy inducers, the ULK complex is activated, thus promotes the activation of its downstream effector, VPS34 complex, which mediate the autophagy nucleation process (Backer, 2016). The VPS34 complex is comprised of VPS34, Beclin-1 (ATG6 in yeast), VPS15 and ATG14L (Ohashi, Tremel & Williams, 2019) in mammals, and it is activated through the phosphorylation of ATG14L and/or Beclin-1 by ULK1 (Park et al., 2016; Park et al., 2018; Russell et al., 2013). VPS34 complex acts as a lipid kinase termed class III PI3K (phosphatidylinositol-3-phosphate kinase), which phosphorylates PtdIns (phosphatidylinositol) to generate PI3P (phosphatidylinositol 3-phosphate). PI3P serves as a scaffold to recruit PI3P-binding molecules such as the PX-BAR protein SNX18 (Knaevelsrud et al., 2013), WIPI (Axe et al., 2008), and FYVE domain-containing proteins including DFCP1 and Alfy (Marat & Haucke, 2016). PI3P and its binding proteins initiate the formation of an isolated pre-autophagosomal structure known as phagophore (Figure 2). It has been reported that PI3P produced at endoplasmic reticulum (ER) mediates the formation of membrane structures called omegasomes where phagophores begin to be synthesized (Ktistakis & Tooze, 2016). However, in addition to the ER, membranes required for phagophore expansion are likely to originate from multiple sources such as plasma membrane and Golgi apparatus (Lahiri, Hawkins & Klionsky, 2019). Recently, two independent group revealed that RAB11A-positive recycling endosomes contribute to phagophore formation as well in starvation or viral infection induced autophagy (Kuroki, Osari, Nagata & Kawaguchi, 2018; Puri et al., 2018). Moreover, it is proposed that PI3P-WIPI2-RAB11A signaling cascade enables recycling endosomes, instead of the ER, as the primary sources for phagophore initiation (Puri et al., 2018). In summary, autophagy is initiated through the sequential activation of ULK complex, VPS34 complex and the subsequent production of

PI3P (Figure 2).

The initiation stage is sophisticatedly regulated by various molecules. In starvation-induced autophagy, mTORC1 serves as one of the key upstream regulators of the ULK complex (Laplante & Sabatini, 2013). In nutrient-rich conditions full of amino acids and growth factors, mTORC1 is activated through the modulation of two GTPase, Rheb and Rag (Kim & Guan, 2019). Active mTORC1 phosphorylates ULK1, ULK2 and/or ATG13 to suppress ULK complex activation. Additionally, TFEB, the master transcription factor of a variety of lysosomal and autophagy related genes, is suppressed by the activated mTORC1 (Martina, Chen, Gucek & Puertollano, 2012; Pena-Llopis et al., 2011; Settembre et al., 2012). Taken together, autophagy initiation is blocked. Upon starvation, however, mTORC1 is inactivated followed by the activation of ULK complex through a series of dephosphorylation and phosphorylation (Perluigi, Di Domenico & Butterfield, 2015), and subsequently autophagy is induced. Another upstream regulatory factor of ULK complex is AMPK, which is a kinase and a well-characterized energy sensor. AMPK can promote autophagy through phosphorylating ULK1. Interestingly, AMPK and mTORC1 are likely to compete on ULK1 phosphorylation, as mTORC1-mediated ULK1 phosphorylation blocks its further phosphorylation by AMPK (Kim, Kundu, Viollet & Guan, 2011). In addition, AMPK is demonstrated to activate autophagy through directly suppressing mTORC1 activity or activating mTORC1 inhibitor TSC2 (Gwinn et al., 2008; Inoki et al., 2006) (Figure 2).

Autophagy induction is tightly modulated via the VPS34 complex as well. Beclin-1 is the key subunit in the complex, and various molecules regulate autophagy through interacting with Beclin-1. It has been demonstrated that UVRAG (Liang et al., 2006), AMBRA1 (Fimia, Corazzari, Antonioli & Piacentini, 2013) and Bif-1 (Takahashi et al., 2007) stimulate VPS34 complex activity and enhance autophagy through association with Beclin-1. Additionally, besides its role in ULK complex activity and mTORC1 activation, AMPK is found to promote autophagy through Beclin-1 phosphorylation at Thr388 (Zhang et al., 2016). For the negative regulators, Bcl-2 (Pattingre et al., 2005) and Bcl-xL (Maiuri et al., 2007) bind to Beclin-1 to suppress VPS34 complex, thus autophagy is compromised. Accordingly, mutations in Bcl-2 (G145A) or Bcl-xL (G138A) that disrupt the interaction with Beclin-1 rescue the suppressed

autophagy (Sinha & Levine, 2008). Notably, the small GTPase Rab5 is found to be indispensable for autophagy initiation through modulating VPS34 complex (Jean & Kiger, 2014). Rab5 is viewed as one of the core molecules that mediate endocytosis (Langemeyer, Frohlich & Ungermann, 2018). However, it has been demonstrated that Rab5 is required for autophagosome formation through interacting with VPS34 and Beclin-1 in both HD (Huntington's disease) cell and Drosophila models (Ravikumar, Imarisio, Sarkar, O'Kane & Rubinsztein, 2008). Consistently, in growth factor withdrawal induced autophagy, Rab5 was shown to interact with class IA PI3K subunit p110β, which causes Rab5 activation through enhancing the transformation from GDP-bound state (Rab5-GDP) to GTP-bound state (Rab5-GTP). Then VPS34 complex was activated and autophagy was induced (Dou et al., 2013). Similar functions for Rab5 were reported in hepatitis C virus NS4B-induced autophagy (Su, Chao, Huang, Weng, Jeng & Lai, 2011).

2.1.2 Autophagosome formation

Autophagosome formation is the key event for the whole autophagic flux, which is mediated by two ubiquitin-like conjugation systems in eukaryotic cells. After autophagy is initiated and phagophore is synthesized, phagophore then expands and finally it seals to form an isolated compartment termed autophagosome. Firstly, ATG7 acts as the E1-like enzyme, ATG10 as the E2-like enzyme, to catalyze the conjugation of ATG12 to ATG5. ATG16L is then recruited to the ATG5-ATG12 subcomplex to form ATG5-ATG12-ATG16L multimeric protein complex (Mizushima et al., 2003). The other conjugation system is the lipidation of MAP1LC3/LC3 (ATG8 in yeast). Full-length LC3 is firstly cleaved by the protease ATG4 at its carboxyl terminus to generate the cytosolic LC3 type I (LC3I). The cleavage exposes the C-terminal glycine residue where phosphatidylethanolamine (PE) is conjugated to LC3I. This process is known as LC3 lipidation and the lipidated LC3 is called LC3 type II (LC3II) (Tanida, Ueno & Kominami, 2004). LC3 lipidation is mediated in an ubiquitin-like conjugation manner, in which ATG7 serves as the E1-like enzyme as well, ATG3 as the E2-like enzyme, and ATG5-ATG12-ATG16L complex as the E3-like ligase (Romanov et al., 2012). Thus, LC3II is covalently attached to the phagophore membrane where proteins containing LC3-interacting region (LIR) are recruited. Subsequently, phagophore elongates

until it seals at some point to form a vesicle termed autophagosome.

2.1.3 Autophagosome maturation and degradation

Once autophagosome is formed, LC3II bound to the exterior membrane of autophagosome (cytosolic phase) is immediately removed by the ATG4 proteinases (Kauffman et al., 2018). Usually, autophagosomes are then transported to the perinuclear region and fused with proximal lysosome to form autolysosomes (Figure 2). It has been demonstrated that the fusion step is mediated by several tethering proteins including SNAREs and the HOPS complex (Jiang et al., 2014; Wang et al., 2016). In an alternative way, autophagosomes may firstly fuse with late endosomes to temporarily form amphisomes, and amphisomes then undergo fusion with lysosomes to make autolysosomes (Galluzzi et al., 2017). Autophagosomes (or amphisomes) and autophagy cargos are degraded by resident lysosomal hydrolases, and the generated small molecules such as amino acids are reused by the cells.

2.1.4 Selective autophagy

Originally, it is thought that autophagy cargos are sequestered in a bulk and non-selective way (bulk autophagy) induced by stimuli such as nutrient deprivation. However, accumulating evidence has demonstrated that autophagosomes selectively recognize specific cargos through adaptor proteins. This form of autophagy is known as selective autophagy (Figure 3). Based on the type of cargos, selective autophagy that degrades protein aggregates is termed as aggrephagy, damaged mitochondria as mitophagy, peroxisomes as pexophagy, cytosolic pathogens as xenophagy, and so forth (Kirkin & Rogov, 2019) (Figure 3). Typically, the aberrant proteins are firstly labeled with ubiquitins, and then the ubiquitinated cargos are recognized by the ubiquitin-binding domain (UBD) of adapter proteins such as p62/SQSTM1, NBR1, NDP52, OPTN, TAX1BP1, TOLLIP and RPN10 (Menzies et al., 2017). These adaptor proteins also contain LIR (LC3-interacting region) motifs through which they associate with LC3, thus the adaptor proteins link the ubiquitinated cargos with autophagosomes. Currently, the adaptor proteins are usually referred to as selective autophagy receptors (SARs) (Chu, 2019; Kirkin & Rogov, 2019) (Figure 3). Selective autophagy has recently gained more attention due to its therapeutic potential for neurodegenerative diseases and aging.

2.2 Chaperon-Mediated Autophagy

In chaperon-mediated autophagy (CMA), the cargos are also selective. Only the substrate proteins containing a KFERQ-like motif can be recognized by chaperon protein complex (Olson, Terlecky & Dice, 1991) (Figure 1). In contrast to selective autophagy, the targeted cargos are delivered to lysosomal surface without formation intermediate vesicles such as autophagosome or autolysosome. Instead, the KFERQ-containing substrates are recognized by chaperon protein HSPA8 (also known as Hsc70) together with associated co-chaperons. Subsequently, the cargo-HSPA8 complex is transported to lysosomal membrane where the complex interacts with the cytosolic domain of the lysosomal transmembrane protein LAMP2A (Cuervo & Dice, 1996) , an isoform of another lysosomal membrane protein LAMP2. Before the docking of cargo-HSPA8 complex, LAMP2A exists either in monomeric or homodimeric state. Once the substrate complex binds to LAMP2A, HSPA8 is released from the complex and LAMP2A quickly undergoes oligomerization to form a pore-like complex. The substrates are firstly unfolded and then translocated into lysosomal lumen where they are degraded. And the LAMP2A oligomer undergoes disassembly to be monomer or dimer for next round of delivery (Bandyopadhyay, Kaushik, Varticovski & Cuervo, 2008; Bandyopadhyay, Sridhar, Kaushik, Kiffin & Cuervo, 2010) (Figure 1). Although macroautophagy is believed to be the major type of autophagy in mammals, CMA-mediated protein degradation may be largely underestimated. Recent findings suggest that CMA pathway plays a critical role in degrading intracellular proteins (Kaushik & Cuervo, 2018). If certain gene is down-regulated at the protein level, the possibility that it might be degraded through CMA pathway should not be overlooked.

2.3 Microautophagy

Microautophagy is the most straightforward type of autophagy in which the cytoplasmic cargos are directly engulfed by the lysosome or vacuole through membrane invagination. Ultimately, a vesicle surrounding the cargos is formed, then both of the vesicle and cargos are degraded by lysosomal hydrolases (Li, Li & Bao, 2012) (Figure 1). Microautophagy can randomly select the substrates or specifically degrade some cargos such as peroxisomes. The peroxisome selective microautophagy is termed micropexophagy (Oku & Sakai, 2016).

3. Autophagy in Alzheimer's disease pathogenesis

Alzheimer's disease (AD) is characterized with amyloid deposition due to aberrant Aβ metabolism, and neurofibrillary tangles consisting of phosphorylated tau. Besides, synaptic loss, overactive neuroinflammation, impaired mitochondria, dysfunctional calcium and insulin signaling and so on, are also observed in AD pathologies (Querfurth & LaFerla, 2010). The mammalian nervous system, especially for neurons, depends heavily (if not solely) on autophagy to maintain the protein homeostasis (Chung, Hernandez, Sproul & Yu, 2019). In addition, the unique features of neuronal cells confer vulnerability to dysfunctional autophagy. For instance, axonal autophagosomes in neurons have to move to the cell body to fuse with lysosomes, because lysosomes are rarely distributed in distal axons (Cheng, Zhou, Lin, Cai & Sheng, 2015; Maday, Wallace & Holzbaur, 2012).

Accumulating evidence has indicated that impaired autophagy contributes to AD pathogenesis. Initially, it is found that autophagosomes and autolysosomes are accumulated in AD patients' brains through electron microscopy (Nixon et al., 2005). The core subunits of VPS34 complex, Beclin-1 and VPS34 have been demonstrated to be significantly reduced with AD progression (Jaeger, Pickford, Sun, Lucin, Masliah & Wyss-Coray, 2010; Lucin et al., 2013; Pickford et al., 2008; Rohn, Wirawan, Brown, Harris, Masliah & Vandenabeele, 2011). In line with these findings, PI3P production, which is mediated by the VPS34 complex, was shown to be down-regulated as well in AD patients' brains (Morel et al., 2013). So, it is believed that the accumulation of autophagic structures including autophagosomes and autolysosomes are due to the blockage of autophagic flux (Wolfe, Lee, Kumar, Lee, Orenstein & Nixon, 2013). However, Ralph A. Nixon's group recently reported that the expression of genes required for autophagosome formation and some lysosomal genes were up-regulated at AD early stages when they were studying the CA1 neurons in hippocampus from AD patients (Bordi et al., 2016). The findings suggest that the enhanced autophagy at the early stage of AD is protective in response to stress, but autophagic flux is ultimately compromised with AD progression (Chung, Hernandez, Sproul & Yu, 2019). Taken together, growing evidence has indicated that dysfunctional autophagy is closely correlated with AD pathogenesis.

3.1 Autophagy in Aβ metabolism

Autophagy is involved in Aβ metabolism probably through regulating both of its

generation and clearance. Aβ originates from the processing of its precursor protein APP, which is sequentially cleaved by β-secretase (BACE1) and γ-secretase (Selkoe & Hardy, 2016). It has been demonstrated that a compound induced and ATG5-dependent autophagy enhances the degradation of APP itself (Cavieres et al., 2015). In addition, APP and the four subunits of γ-secretase complex were observed residing in autophagosomes, suggesting that at least some Aβ peptides are produced through autophagic pathway (Di Meco, Curtis, Lauretti & Pratico, 2020). Moreover, it has been revealed that autophagy is required not just for Aβ production, but also Aβ secretion. As mentioned before, ATG7 acts as the E1-like enzyme for the ubiquitin-like conjugation in autophagosome formation. Nilsson *et al.* established the ATG7 KO mouse strain and implemented its cross with AD mouse model (Nilsson et al., 2013; Nilsson et al., 2015). It is shown that extracellular Aβ plaque formation was drastically decreased in ATG7 KO, autophagy-deficient AD mice. On the other hand, intraneuronal Aβ was markedly accumulated, indicating that Aβ secretion was compromised after autophagy is impaired (Nilsson et al., 2013; Nilsson et al., 2015).

Not just the production, autophagy is also found to modulate Aβ clearance. When AD model mice were treated with rapamycin, a specific inhibitor of mTOR, thus autophagy was enhanced, it is observed that both intracellular Aβ and extracellular amyloid deposition in brains were markedly reduced, and the animals' cognition was significantly improved as well (Caccamo, Majumder, Richardson, Strong & Oddo, 2010; Majumder, Richardson, Strong & Oddo, 2011). In addition to small molecules, consistent results were obtained in genetically engineered animal models. Recently, Rocchi and the coauthors knock in a point mutation (F121A) in gene coding Beclin-1 in mice. The mutation disrupts the interaction between Beclin-1 and Bcl-2, resulting in the stimulation of basal autophagy. The established mice (Beclin-1 F121A) were crossed with AD model mice, and the results demonstrated that amyloid plaques were significantly decreased and cognitive impairment was prevented in AD model mice carrying the Beclin-1mutation (Rocchi et al., 2017). In line with this study, in Beclin-1 knock out (KO) AD mice, both intraneuronal Aβ and extracellular Aβ aggregation were accumulated compared to the control mice (Pickford et al., 2008). Autophagy seems to affect Aβ clearance at multiple steps. Cathepsin B is a critical lysosomal protease required for

the degradation of autophagic substrates. It has been shown that genetic ablation of Cathepsin B worsened AD pathologies in AD model mice, including the elevated abundance of Aβ42 and more amyloid deposition. Conversely, when cathepsin B was overexpressed through lentiviral transduction, amyloid plaques were reduced even in the aged AD mice (Mueller-Steiner et al., 2006).

In addition to bulk autophagy, selective autophagy is also found to function in Aβ metabolism. As discussed earlier, the type of selective autophagy that degrades aggregated proteins is called aggrephagy (Kirkin & Rogov, 2019). p62/SQSTM1, NBR1 and OPTN have been identified as the selective autophagy receptors specific for aggrephagy (Malampati et al., 2020). Usually, the ubiquitinated Aβ is degraded by the ubiquitin-proteasome system (UPS) (Hong, Huang & Jiang, 2014). However, the extent of ubiquitination of Aβ has been demonstrated to be correlated with its stability. The longer of the Aβ ubiquitin chain, the more instable of Aβ. The polyubiquitinated Aβ tends to aggregate to form insoluble Aβ fibrils or plaques (Morimoto et al., 2015), which are resistant to UPS-mediated degradation (Verhoef, Lindsten, Masucci & Dantuma, 2002). Alternatively, the Aβ fibrils are selectively recognized by the autophagy receptors p62/SQSTM1 and NBR1 and undergo degradation by aggrephagy (Morimoto et al., 2015).

3.2 Autophagy in tauopathies

Autophagy plays a critical role in tau pathology as well other than Aβ metabolism. Initially, in vitro data showed that once autophagic flux was blocked, tau clearance was compromised and insoluble tau aggregates were significantly accumulated (Hamano et al., 2008). In the brains of familial AD patients, it is found that hyperphosphorylated tau showed colocalization with the autophagosome marker LC3 and the autophagy receptor p62/SQSTM1, while the overlapping was not observed in control subjects without neurodegenerative diseases (Piras, Collin, Gruninger, Graff & Ronnback, 2016). Consistently, LC3 and p62/SQSTM1 immunoreactivity was observed associated with tau aggregates in a tau model cell line (Guo et al., 2016). These studies indicate that tau tauopathies are likely to be altered by autophagy as well. Indeed, in ATG7 conditional KO (cKO) mice, the phosphorylation of tau was markedly up-regulated, which is possibly due to the accumulation

of GSK3β, one of the major tau kinases, in brains of ATG7 cKO mice (Inoue et al., 2012).

In line with its role in Aβ metabolism, autophagy induction is shown to alleviate tauopathies. The mouse model carrying tau mutant treated with mTOR inhibitor rapamycin was demonstrated to significantly decrease phosphorylated tau levels. Conversely, TSC2 (a mTOR negative regulator) KO mice, in which mTOR signaling was constitutively activated, displayed elevated tau levels as well as tau phosphorylation (Caccamo et al., 2013). Most recently, a study obtained several mTOR inhibitors that are more potent than rapamycin, and the authors applies these compounds to neurons differentiated form human neural progenitor cells (NPCs) with tau mutant. The results showed that these identified compounds drastically reduced tau phosphorylation and insoluble tau (Silva et al., 2020), which provides more evidence that stimulated autophagy ameliorates tauopathies. mTOR is not the only molecule in autophagic pathway targeting tau related pathology. It has been observed that, in addition to p62/SQSTM1, the autophagy receptor NDP52 recognizes phosphorylated tau as well in brains of AD model mice (Kim et al., 2014). And when NDP52 was up-regulated by a compound from tea extract, the clearance of phosphorylated tau by autophagy was demonstrated to be enhanced in cultured neurons (Chesser, Ganeshan, Yang & Johnson, 2016). Likewise, elevated expression of NDP52 by its upstream transcription factor Nrf2 was shown to promote the degradation of phosphorylated tau (Jo, Gundemir, Pritchard, Jin, Rahman & Johnson, 2014; Malampati et al., 2020). In addition to mTOR and autophagy receptors, TFEB, one of the core regulators in autophagy, is also viewed as a critical factor in tau pathologies. More studies have demonstrated that up-regulation of TFEB in tau mouse models markedly reduced soluble phosphorylated tau and insoluble tau aggregates, and the cognitive functions of the mouse models were thus shown to be improved (Polito et al., 2014; Wang, Wang, Carrera, Xu & Lakshmana, 2016). The obtained evidence indicates that multiple autophagy related proteins are potently involved in tauopathies.

3.3 Autophagy in synaptic function

Synaptic dysfunction is another characteristic feature of AD pathology in addition to Aβ deposition and tau aggregation. Synapses are neuron specific structures serving as the basic units for communications from presynaptic neurons to postsynaptic neurons. It has been

observed that the number of synapse is reduced, together with cognitive impairment, at the early stage of AD pathogenesis (Chen, Fu & Ip, 2019). Recently, accumulating evidence proposes that functional autophagy is required for synaptic functions including neurotransmission and synaptic plasticity (Lieberman & Sulzer, 2020). Synaptojanin-1 is a presynaptic lipid phosphatase that is involved in the endocytosis of synaptic vesicles. It is reported that late endosomes and autophagosomes are accumulated in zebrafish cone photoreceptors with genetic ablation of Synaptojanin-1 (George, Hayden, Stanton & Brockerhoff, 2016), suggesting that Synaptojanin-1 is indispensable to maintain normal autophagy at synapses. Likewise, the Synaptojanin-1 interacting protein, Endophilin (also known as Bif-1) is demonstrated to associate with Beclin-1 to modulate autophagy initiation through regulating the VPS34 complex activity (Takahashi et al., 2007). Additionally, autophagy has been proven to be involved in presynaptic release of dopamine. In ATG7 conditional KO dopaminergic neurons, elevated dopamine release was observed, and the treatment of mTOR inhibitor rapamycin rescued the phenomenon (Hernandez et al., 2012). Subsequent studies revealed that the association of Rab26 and ATG16 (Binotti et al., 2015), Bassoon and ATG5 (Nikoletopoulou & Tavernarakis, 2018), are also important players for synaptic vesicle release. The investigation of autophagy in postsynaptic locus is relatively limited. It seems that the role of autophagy here is just degrading the neurotransmitter receptors including $GABA_A$ receptors and AMPA receptor (Lieberman & Sulzer, 2020).

Autophagy is also a necessity for synaptic plasticity. Synaptic plasticity means the features of synapses that change on structure, number and function to strengthen or weaken the contacts between each other. It is believed that synaptic plasticity is essential for cognitive functions such as learning and memory. At cellular level, there are two forms of synaptic plasticity related with learning and memory, long-term potentiation (LTP) and long-term depression (LTD). Initially, it is reported that BDNF (brain-derived neurotrophic factor) deficiency up-regulated LC3II and accumulation of autophagosomes, while LTP was compromised. Additionally, impaired LTP due to ablation of BDNF was rescued after autophagy was suppressed (Nikoletopoulou, Sidiropoulou, Kallergi, Dalezios & Tavernarakis, 2017), suggesting that autophagy induction impairs LTP. However, a recent study

demonstrated that autophagy stimulation in mouse hippocampus was required for the new memory formation and LTP was blocked after autophagy was pharmacologically inhibited (Glatigny et al., 2019). It seems there is some inconsistency between the two studies. However, it should be noticed that the former study focuses on the function of BDNF on synaptic plasticity, and it is widely accepted that BDNF is a multifunctional secretory protein. Thus, unpredictable side effects might be caused due to the ablation of BDNF.

3.4 Autophagy in mitochondrial dysfunction

Mitochondria are viewed as the organelles created for energy production. Actually, mitochondria participate in multiple metabolic pathways as well as apoptosis (Pickles, Vigie & Youle, 2018). In the process of energy production, mitochondria are impaired by of reactive oxygen species (ROS). Consequently, mitochondria maintain the homeostasis through elimination of damaged ones and synthesis of new mitochondria. In AD pathogenesis, accumulated Aβ potently generates excessive ROS and causes abundant damage to mitochondria (Querfurth & LaFerla, 2010). Mitophagy is the dominant approach to ensure mitochondrial homeostasis (Gatica, Lahiri & Klionsky, 2018). In mammals, mitophagy is commonly induced by the collapse of mitochondrial membrane potential (MMP) caused by ROS overload, and the process is mainly mediated by the PINK1-Parkin pathway (Chu, 2019). PINK1 is a serine/threonine protein kinase in cytoplasm, and it is translocated into mitochondrial matrix in normal functional mitochondria. In damaged mitochondria, however, the compromised MMP blocks PINK1 import and keep it on the outer mitochondrial membrane (OMM) where PINK1 is activated through autophosphorylation (Aerts, Craessaerts, De Strooper & Morais, 2015; Kondapalli et al., 2012; Narendra et al., 2010). Activated PINK1 phosphorylates and activates Parkin, and simultaneously PINK1 phosphorylates ubiquitin on OMM as well to generate phospho-ubiquitin (Kane et al., 2014; Koyano et al., 2014). As an E3 ligase, Parkin recognizes the phosphor-ubiquitin on OMM, thus more Parkin is recruited (Trempe et al., 2013). Typically, ubiquitinated mitochondria are recognized by autophagy receptors p62/SQSTM1 and OPTN, and then undergo autophagic degradation (Chu, 2019; Geisler et al., 2010). Alternatively, Parkin is demonstrated to be dispensable and phosphorylated ubiquitin by PINK1 itself is potent enough for autophagy

receptor NDP52 and OPTN recognition (Lazarou et al., 2015). In addition to PINK1-Parkin-mediated mitophagy, multiple other forms of mitophagy have been reported (Chu, 2019; Gatica, Lahiri & Klionsky, 2018; Pickles, Vigie & Youle, 2018), indicating the complexity of mitochondrial homeostasis maintenance.

Mitochondrial dysfunction is a common character of AD. Postmortem studies have demonstrated that hippocampal mitophagy was markedly reduced in AD patients. Similar phenotype was observed in AD mouse models and neurons derived from induced pluripotent stem cell (iPSC) of AD affected individuals (Lou, Palikaras, Lautrup, Scheibye-Knudsen, Tavernarakis & Fang, 2020), suggesting that mitophagy induction might ameliorate AD pathogenesis. Indeed, a recent study applied pharmacological agonists of mitophagy to treat AD model cells and organisms. The results demonstrated that enhanced mitophagy alleviated both Aβ and tau pathologies, and improved the cognitive functions of AD *C. elegans* and mouse models (Fang et al., 2019). Although the relations between mitophagy and AD pathogenesis still need further investigation, mitophagy mediated clearance of dysfunctional mitochondria displays therapeutic potential for AD interventions.

4. Current therapeutics for AD

AD is an extremely complex disease, and there are still no effective medications to slow or prevent AD progression. At present, only four drugs (rivastigmine, galantamine, donepezil and memantine) have been approved by U.S. FDA for AD treatment, in which three of them (rivastigmine, galantamine and donepezil) are cholinesterase inhibitors and memantine targets NMDA receptor (Long & Holtzman, 2019). The efficacy of these drugs is very limited and varies with different individuals (Knight, Khondoker, Magill, Stewart & Landau, 2018).

Up to date, over one hundred double-blind clinical trials have failed, in which more than twenty compounds were demonstrated to be of no effect after completion of phase 3 trials (Cummings, Lee, Ritter, Sabbagh & Zhong, 2020; Long & Holtzman, 2019). Currently, there are 121 agents are undergoing clinical trials, most of which are targeting Aβ metabolism, tau, inflammation, neurotransmitter receptors, synaptic plasticity and so on. Notably, among these interventions, more agents are designed from other perspectives except amyloid and tau pathologies, compared to the situation of 2019 (Cummings, Lee, Ritter, Sabbagh & Zhong,

2019). For instance, the National Medical Product Administration (NMPA) of China approved GV-971 in 2019 for the treatment of AD at mild-to-moderate stage (Wang et al., 2019). GV-971 is an oligomannate extracted from brown algae, and it is believed to be a multitargeted compound crucial for the balance of gut microbiota. The authors demonstrated that GV-971 administration significantly prevented AD progression through modulating dysbiosis of the gut microbiome induced excessive inflammation in brain (Wang et al., 2019). As GV-971 exhibits high safety in clinical trials and its mechanisms of action are distinctive, cautious optimism might be the right attitude for its commercial application. Besides, another drug in clinical trial, rifaxamin, is also targeting harmful gut bacteria activated neuroinflammation (Cummings, Lee, Ritter, Sabbagh & Zhong, 2020). Additionally, some other agents attempt to suppress AD pathogenesis indirectly via regulating signaling pathways involved in metabolism, epigenetic changes, vascular system, neurogenesis as well as protein homeostasis (Cummings, Lee, Ritter, Sabbagh & Zhong, 2020; Hara, McKeehan & Fillit, 2019). The novel perspectives represent potential approaches for future AD treatment.

5. Autophagy-stimulating strategies for AD therapeutics

Due to the massive failure of compounds targeting amyloid and tau, researcher are considering other therapeutic strategies for AD (Long & Holtzman, 2019). A consensus is being reached in favor of autophagy related interventions. Accumulating evidence indicates that enhanced degradation of misfolded proteins and impaired organelles through inducing autophagy might be an ideal answer to AD therapy. Since autophagy is a conserved, highly dynamic and sophisticatedly-regulated cellular event, so theoretically it can be stimulated at multiple levels, which provides various pharmacological targets to develop agonists or antagonists accordingly.

Although immunotherapy and other therapies are also intriguing options for AD intervention, small molecules are still highly preferable, as they can easily cross the blood-brain barrier (BBB). Hundreds of compounds against AD have been tested clinically in recent years (Cummings, Lee, Ritter, Sabbagh & Zhong, 2019; Cummings, Lee, Ritter, Sabbagh & Zhong, 2020). Here, we select a list of autophagy-stimulating agents that have been investigated in AD animal models and/or proven to be safe in various phases of clinical trials,

even if some of which are not originally designed for AD treatment (Table 1). Next, we discuss the revealed mechanisms of these compounds in autophagy and the performance in animal models and clinical trials.

5.1 Compounds targeting bulk autophagy

Most of the selected agents induce autophagy through inhibiting mTOR and/or activating AMPK (Table 1). Rapamycin and its derivatives are macrolide compounds and they are well-characterized mTOR inhibitors. The administration of rapamycin in AD model mice has been demonstrated to alleviate Aβ aggregation, tauopathies and improve cognitive functions (Lin et al., 2013; Ozcelik et al., 2013; Spilman et al., 2010). The clinical data of rapamycin on AD patients are not available, but it has been shown that low-dose rapamycin improves some aging related markers, suggesting its potential function in slow aging (Singh et al., 2016). Curcumin is a natural product from *Curcuma longa* plants, which potently suppresses PI3K-Akt-mTOR signaling pathway. In AD model mice, the treatment of curcumin has significantly reduced amyloid aggregation and inhibited memory decline (Wang, Zhang, Teng, Zhang & Li, 2014). Clinical results indicate that curcumin functions in suppressing inflammation (Salehi et al., 2019). Considering the damage of neuroinflammation in AD scenario, curcumin is indeed an attractive agent targeting AD treatment.

The anti-psychiatric drug lithium is demonstrated to promote autophagy through activating AMPK (Bao et al., 2019). Animal studies indicate that lithium markedly ameliorates tauopathies in 3xTg AD model mice (Caccamo, Oddo, Tran & LaFerla, 2007). Patient data also showed that lithium administration considerably improved cognition of AD patients and individuals with MCI (mild cognitive impairment) (Matsunaga, Kishi, Annas, Basun, Hampel & Iwata, 2015). Resveratrol is also reported to stimulate autophagy in an AMPK-dependent manner, and the induced autophagy was indicated to attenuate AD pathology in animal model (Vingtdeux et al., 2010). However, resveratrol is a multitargeted compound, and it is also found to enhance autophagy via sirtuin1-mediated signaling pathway (Uddin, Mamun, Labu, Hidalgo-Lanussa, Barreto & Ashraf, 2019). As resveratrol is a grape-derived polyphenol, it is very safe in clinical trial. And it shows some protective effect in AD patients, but inconsistent results were also observed (Kou & Chen, 2017; Turner et al., 2015).

Some other agents are reported to elevate autophagy through both AMPK activation and mTOR inhibition. Glucosamine is an essential component in cartilage, and it is usually used as a supplement to improve the pain caused by loss of cartilage (osteoarthritis). Recent literature indicates that glucosamine is an autophagy agonist via suppressing mTOR and activating AMPK both *in vitro* and *in vivo* (Carames et al., 2013; Chen, Chen, Liang, Tai, Lu & Chen, 2018). There is no report about the effect of glucosamine administration on AD animal models yet. However, glucosamine has been shown to enhance longevity in worms and old mice (Weimer et al., 2014). As age is a major risk factor for AD, glucosamine may have protective effects against AD through facilitating healthy aging. Metformin is a biguanide compound that is widely used for the patients with type 2 diabetes. It activates AMPK and/or inhibits mTORC1 to induce autophagy (Kalender et al., 2010; Onken & Driscoll, 2010). Metformin potently reduces AD-like pathologies and improves cognition in AD mouse models (Table 1). Moreover, metformin displays promising results on improving some cognitive functions in clinical trials (Koenig et al., 2017). Oleuropein is a bitter compound extracted from green olive and it stimulates autophagy by inhibiting mTOR and/or activating AMPK as well (Rigacci et al., 2015). Oleuropein has been demonstrated to markedly reduce Aβ plaques and ameliorate synaptic plasticity in a well-established AD mouse model TgCRND8 (Grossi et al., 2013; Luccarini et al., 2015). Clinical studies have demonstrated that oleuropein has some beneficial effects on several human chronic non-communicable diseases including cardiovascular diseases and diabetes (Nediani, Ruzzolini, Romani & Calorini, 2019). As these chronic disorders are closely correlated with the occurrence of AD, it is worth further investigating the efficacy of oleuropein on AD prevention.

It is not necessary that the autophagy inducers targeting mTOR and/or AMPK. The FDA approved agent for AD treatment, memantine, is shown to enhance autophagy either depending on mTOR or not (Song, Li, Lin & Cao, 2015). Similarly, the FDA approved antiepileptic drug carbamazepine can also induce autophagy in mTOR-dependent or -independent way. Carbamazepine shows intriguing therapeutic potential for AD because of its beneficial property of amyloid aggregates reduction and cognition improvement in 3xTg AD

model mice (Li et al., 2013; Zhang et al., 2017). Nilotinib is a tyrosine kinase inhibitor that is usually applied for the treatment of chronic myeloid leukaemia (CML). A recent study showed that nilotinib stimulates autophagy through c-ABL-mediated mTOR inhibition (Hussain et al., 2019). Although nilotinib is likely to target multiple factors involved in autophagic process (VPS34 complex, for instance) (Yu et al., 2020), its efficacy on autophagy induction is consistent. Besides, *in vivo* studies and clinical trial results demonstrated that nilotinib decreases amyloid deposition (Table 1), making nilotinib an appealing candidate for AD therapeutics. Spermidine is a natural polyamine existing in all eukaryotic cells and it is required for cell proliferation, differentiation and apoptosis (Pegg, 2016). Recent studies revealed that spermidine elevates autophagy *in vitro* through up-regulating the acetyltransferase EP300, which is a Beclin-1 and LC3 binding protein (Sacitharan, Lwin, Gharios & Edwards, 2018). Spermidine has not been applied to AD animal models, however, it displays consistent effect against aging in multiple model organisms, including yeast, *C. elegans*, *Drosophila* and mice (Eisenberg et al., 2016; Eisenberg et al., 2009; Yue et al., 2017). It is presumable that spermidine has the potential to treat AD, the age-related neurodegenerative disorder. Trehalose is a natural disaccharide. In contrast to other autophagy inducers mostly targeting mTOR, AMPK or VPS34 complex, trehalose activates autophagy via the transcription factor TFEB. Studies demonstrated that the administration of trehalose promotes TFEB dephosphorylation, resulting in TFEB nuclear translocation. This leads to the upregulation of multiple TFEB downstream effectors required for autophagy, in an mTOR independent manner (Rusmini et al., 2019). Amyloid and tau pathologies in AD mouse models treated by trehalose are significantly improved (Table 1), indicating that trehalose is a small molecule worthy of consideration as an intervention for AD.

5.2 Compounds targeting mitophagy

In addition to bulk autophagy, researchers have shed more light on selective autophagy to identify potential drug targets for neurodegenerative diseases in recent years. A lot of efforts have been put on screening appealing compounds enhancing aggrephagy and mitophagy. Small molecules targeting aggrephagy have recently been reviewed by Malampati et al. (Malampati et al., 2020). Here, we discuss the anti-aging compound, nicotinamide,

which has been proven to stimulate mitophagy. Nicotinamide is an active form of vitamin B3. It serves as the precursor of oxidized nicotinamide adenine dinucleotide (NAD^+) which is a critical coenzyme in catalyzing a broad range of intracellular metabolic events. Extensive studies have demonstrated that nicotinamide and its derivatives, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), induce mitophagy and are protective against aging (de Picciotto et al., 2016; Mills et al., 2016; Yoshino, Baur & Imai, 2018). Recent evidence indicates that nicotinamide replenishment remarkably ameliorates amyloidosis, tauopathies and cognition impairment in AD mouse models (Table 1). Even though the clinical trial results of nicotinamide on AD patients are not inspiring, nicotinamide exhibits excellent safety even if some subjects were treated with high dose (Di Meco, Curtis, Lauretti & Pratico, 2020). These mitophagy enhancers, including nicotinamide, NR and NMN, are safe and natural molecules that have already been commercially produced. Therefore, it is worth deeper exploration of their effects on AD therapeutics.

6. Concluding remarks and future perspectives

AD and other neurodegenerative disorders are proteinopathies characterized with formation of abnormal and insoluble protein aggregates. Tremendous efforts have been made to develop interventions targeting amyloidosis and tauopathies. However, effective disease-modifying medications are still vacant. Numerous amyloid- and tau-directed compounds failed at clinical trials. Most recently, US FDA rejected the application of aducanumab, a monoclonal antibody targeting Aβ, as a new agent for AD therapy.

In recent years, more and more evidence has demonstrated that dysfunctional autophagy is not just correlated with AD pathologies, but it is likely to be a causative factor for AD development. Accordingly, stimulating autophagy to enhance the elimination of misfolded proteins is proposed to be a potential option for AD therapeutics. Indeed, a variety of autophagy enhancers have been identified to slow down AD progression and improve cognition at least in AD animal models. The beneficial effects of autophagy stimulators on AD patients are either not observed or very limited heretofore. However, clinical trials have identified some candidates that are highly safe and/or approved already by FDA to treat

diseases other than AD. Hence, it is possible to perform clinical trials with larger sample size, and the efficacy of candidate interventions should be examined in distinct subgroups of enrolled subjects with regard to different stages of AD and MCI (mild cognitive impairment). In addition, considering the complexity of AD, single agent may not be effective to alleviate AD symptoms. It is recommended to combine more than one autophagy activators in future clinical trials. Furthermore, as the pathologic changes have been accumulating in the brain years or even decades before the clinical diagnosis of AD, administration of potential interventions (not just autophagy-stimulating compounds but also Aβ-, tau-, APOE-directed agents and so on) at preclinical stage should be employed to evaluate the efficacy.

Autophagy is particularly conserved in eukaryotic organisms. Despite the huge advances in elucidating autophagic process, more efforts are required to understand the mechanisms of the sophisticatedly regulated, highly dynamic cellular event. Recent studies showed that some forms of autophagy selectively degrades specific substrates through autophagy receptors, termed as selective autophagy. Unraveling the molecular basis of cargo selection and recognition is helpful to precisely activate the autophagy pathway. AD is a complex multifactorial disease and researchers have encountered a lot of frustrating drawbacks in AD therapeutics. Autophagy directed strategy provides a new and promising alternative to developing anti-AD medications.

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