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Modulating macroautophagy: a neuronal perspective

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Abstract

Over this past decade, macroautophagy has gained prominence in the field of adult-onset neurodegeneration: from sporadic disorders such as Alzheimer's and Parkinson's disease, to genetic disorders such as Huntington's disease and frontotemporal dementia, the influence of this fundamental pathway has become an important topic of discussion. While there has been particular emphasis on the potential benefits of macroautophagy, there is growing literature that also suggests that macroautophagy contributes towards neurotoxicity. In this review, we discuss the molecular mechanism of macroautophagy and the currently available pharmacological tools, with special emphasis on mammalian macroautophagy in adult brain. Studies indicate that neuronal context strongly influences the role macroautophagy plays in maintaining cellular health, reflecting an ongoing need for better understanding of how macroautophagic regulation is achieved in the heavily differentiated and polarized neurons if we are to effectively manipulate it to treat neurodegenerative disease.

The cell relies upon two routes to degrade cytosolic substrates: the proteasome, which is responsible for the rapid turnover of ubiquitinated proteins [1,2], and the lysosome, which degrades a wide array of cargo from ubiquitinated proteins to organelles [3]. The import of cytosolic cargo into the lysosome is accomplished by autophagy, which is a general term that encompasses microautophagy, chaperone-mediated autophagy (CMA) and **macroautophagy**. In microautophagy, cargo is taken up directly by the lysosome membrane in a manner reminiscent of pinocytosis. Although largely described to occur in the yeast vacuole [4], microautophagy was named for the similar pinocytosis-like events observed in the early ultrastructural studies of lysosomes in starved rat liver [5], and more recent data suggest that cargo is directly taken up in the larger late endosomes [6]. The molecular mechanism of microautophagy is still poorly understood [7,8], limiting our understanding of what role microautophagy may play in neurodegeneration and disease. CMA is another means by which the lysosome directly takes up degradative cargo, and in contrast to microautophagy, CMA has largely been described in mammalian systems in liver [9], but also in other organs including the brain [10–12]. CMA-mediated degradation is achieved by the recognition of substrates that carry a penta peptide motif similar to KFERQ. These proteins are first recognized then trafficked to the lysosome by hsc73, and directed into the

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lysosome through LAMP2A [13]. Although characterized largely as a late-stage response to nutrient deprivation [14], several disease-related proteins have been shown to be CMA substrates, including α-synuclein [15]. A more thorough review of CMA in relation to disease has been presented elsewhere [12,16].

The third and most conserved autophagic pathway is macroautophagy. Macroautophagy delivers cargo to the lysosome through sequestration into a double membrane vesicle known as an autophagosome. The autophagosome further matures, ultimately fusing to the lysosome to permit degradation. Here, we will begin with a brief overview of the molecular mechanism of macroautophagy followed by what is known about the pharmacological interventions that are available. Finally, in light of the growing importance of macroautophagy in neurological disease, we will discuss the outstanding questions of macroautophagic regulation in the mammalian brain. Many of the studies dissecting the molecular underpinnings of macroautophagy have been performed in yeast and invertebrate systems, and we encourage the reader to also consult the following references to read about the differences and similarities observed across phyla [17–21].

Macroautophagy

Macroautophagy is driven by the sequestration of cytoplasmic cargo into a *de novo* synthesized double membrane structure called the isolation membrane or phagophore, which closes to become an autophagosome [22]. The autophagosome ultimately fuses to the lysosome at which point acidification promotes degradation. Much of the mechanistic understanding of macro autophagy centers upon the core proteins involved in formation of the autophagosome, and is driven by a series of autophagy-related (Atg) proteins [23]. Our current understanding divides macroautophagy into three main steps: nucleation, expansion and maturation **(Figure 1A)**. The molecular machineries required for these steps have been extensively reviewed elsewhere [24–27].

Nucleation & expansion

Nucleation and expansion of the growing autophagosome membrane is driven by five core complexes: the ULK 1/2 complex (which is comprised of ULK1/2–mAtg13–FIP200–Atg101) [28]; the Vps34 class III PI3K (which is comprised of Vps34–Vps15–Beclin1–Atg14L/Barkor) [29–31]; mAtg9 and its associated cycling machinery [32]; and the products of two ubiquitin-like conjugation systems, Atg12–Atg5:Atg16L and the mammalian homologs of Atg8 conjugated to phosphatidylethanolamine **(Figure 1B & C)** [33]. ULK1/2 is a serine/threonine protein kinase, and is the mammalian homologue of Atg1. Genetic studies in mice indicate that although only approximately 50% similar, both isoforms can have functional redundancy in its role in macroautophagy [34,35]. The ULK1/2 complex is a stable complex, and the initiation of macro-autophagy is regulated through the phosphorylation status of ULK1/2 and in turn of mAtg13 and FIP200 **(Figure 1B)** [36,37]. The modified phosphorylation status of this complex is what ultimately drives the creation of the isolation membrane. The membrane source of this precursor membrane structure is still an ongoing debate in macroautophagy, but a recent series of live cell and ultrastructural studies from several laboratories have implicated the endoplasmic reticulum (ER) [38–40]. mAtg9, however, the only membrane-spanning Atg protein that is essential for creation of the isolation membrane, is not found in the ER but in the trans-golgi network [41], indicating that mAtg9-containing vesicles are also essential [42]. More recent studies have also implicated the outer mitochondrial membrane [43] and even the plasma membrane [44], suggesting that multiple membrane sources may contribute towards autophagosome biogenesis.

An essential phospholipid in endocytosis and autophagy is phosphatidylinositol-3-monophosphate (PI3P) [31,45]. Creation of PI3P is driven primarily by the class III PI3K, Vps34 [46,47]. To recruit the kinase to the site of autophagosome initiation, the Vps34–Vps15–Beclin1 complex further associates with Atg14L–Barkor [48], and together is known as the autophagy-specific Vps34 complex termed Vps34–class III PI3K complex I. The generation of PI3P recruits two key effector proteins WIPI [49] and DFCP1 [39,50,51]. WIPI 1 and 2 are mammalian homologs of Atg18, and their recruitment is required to initiate the expansion of the isolation membrane **(Figure 1B)** [49]. As such, WIPI and DFCP1 are often used as markers of the autophagosome initiation site in cells [52,53], and protein phosphatases such as MTMR3 and MTMR14 can act as a negative regulator of macroautophagy by diminishing WIPI and DFCP1 recruitment [54,55].

The elongation step during which the isolation membrane grows around the destined cargoes requires two ubiquitin-like proteins Atg12 and Atg8 and their respective conjugation machineries **(Figure 1C)** [33]. Atg12 becomes conjugated to Atg5, while the multiple Atg8 homologs become conjugated to the lipid phosphatidylethanolamine. There are six mammalian homologs to Atg8, MAP1LC3 A, B and C and three GABA receptor-associated proteins (GABARAP) known as GABARAP, and GABARAP-like proteins 1 (GABARAPL1/GEC1) and 2 (GABARAPL2/GATE-16). MAP1LC3B is the most extensively studied and will be referred to simply as LC3 [56] and the non-lipidated and lipidated forms differentiated as LC3-I and -II, respectively. The Atg8 homologs are initially translated in a preprocessed proform, and cleavage by the Atg4B peptidase reveals the requisite glycine for conjugation [57,58]. The conjugations of Atg12 and LC3 depend upon the shared E1-like enzyme Atg7 and then specific E2-like enzymes, Atg10 and Atg3, respectively. Interestingly, the Atg12–Atg5 conjugate forms a large tetrameric complex with Atg16L, to act as a possible E3-like ligase for the recruitment and lipidation of LC3 [59]. In contrast to the other proteins required for nucleation and expansion of autophagic membranes, LC3-II remains membrane bound throughout the process, and is therefore a widely used marker for autophagosomes. The function(s) of LC3-II in membrane formation and closure remains unclear. LC3 has been shown to self-associate [60,61] suggesting it may help tether the membranes that derive the growing autophagosome [60]. Although a role in the ultimate closure of the autophagosome has been suggested [61], further studies may be required to resolve this issue [60].

Maturation

Upon creation and closure, the Atg12–Atg5:Atg16L complex leaves the outer membrane, and the autophagosome then completes maturation by fusing into the endolysosomal pathway and delivering its cargo into the lumen of the lysosome. In mammalian systems, the autophagosome may first fuse with vesicles of the endocytic pathway to become an amphisome [62,63], then fuse with the lysosome to become an autolysosome. Direct fusion of the autophagosome to the lysosome has also been reported, and is the primary mode through which macroautophagic degradation is achieved in yeast [64]. Upon fusion with the lysosome, acidification leads to the degradation of LC3-II on the inner membrane along with the cargo, while the LC3-II pool associated to the cytosolic surface is delipidated by Atg4B and then the LC3 is recycled [65,66].

The maturation of the autophagosome also requires the generation of PI3P. In light of the interaction between macroautophagy and endocytosis in mammalian cells [25,67], this is achieved through the involvement of the endosomal Vps34 class III PI3K complex known as complex II, which instead of Atg14L contains the positive regulator UVRAG or the negative regulator Rubicon [29,30,68,69]. The generation of PI3P is therefore a core regulator of autophagosome formation.

Studies indicate that amphisome formation requires ESCRTs [70–72], Rab11 [73] and the HOPS complex [74], and autolysosome formation requires Rab7 [75–77], presenilin [78], syntaxin 5 [79] and LAMP1 and 2 [80]; however, all of these proteins are also essential for endocytosis and lysosome maturation, thus, whether these proteins directly impact autophagosome fusion into the endolysosomal pathway is unclear. Another vexing series of questions revolves around how autophagosomes achieve fusion at all. Although studies indicate that SNAREs such as VAMP 8 and Vti1b are required for autolysosomes formation [81], how and when Vti1b is incorporated by the outer autophagosome membrane is unclear; early freeze fracture electron miscroscopy studies of autophagosomes suggest that the outer autophagosome membrane is smooth and devoid of transmembrane proteins [82].

Identification of Chmp2b, an ESCRT III protein that modulates **autophagosome maturation**, arose in part from characterization of Chmp2B mutations that give rise to fronto-temporal dementia and amyotrophic lateral sclerosis (reviewed in [83]). Similarly, studies of mutations in VCP/p97, a member of the AAA+ family of ATPase proteins that lead to inclusion body myopathy, Paget's disease of the bone and frontotemporal dementia [84–86], has also been found to be essential for autophagosome maturation [87]. Interestingly, VCP depletion led to a high percentage of ubiquitin-positive contents in the accumulated immature autophagosomes, and was essential to autophagosome maturation upon proteasome inhibition, but not starvation, suggesting that VCP might be required for selective autophagic degradation of ubiquitinated substrates [88].

Selective macroautophagy

In addition to how the autophagosome forms, there have been a growing number of studies examining how the cytosolic cargo is selected for elimination [89]. In light of the diverse array of substrates that can be degraded by macroautophagy, there is growing evidence that substrates are readied for degradation by proteins known as autophagic receptors and selectivity adaptors [83,90]. Several autophagic receptors have been identified, including p62/SQSTM1 [91,92], NBR1 [93], NDP52[94], optineurin [95,96] and NIX [97]. These receptors have been implicated in the selective elimination of ubiquitinated proteins (p62 [98,99], NBR1 [90,94] and NDP52), mitochondria (p62 [100] and Nix [101–103]; reviewed in [104]), peroxisomes (p62 [105]) and invading pathogens (optineurin [106,107], p62 [108] and NDP52 [109]; reviewed in [110,111]). The elimination of ubiquitinated protein aggregates have also been linked to the selectivity adaptor Alfy/WDFY3 [70,112]. A common attribute of the autophagic receptors and selectivity adaptors is that they scaffold substrates with the core autophagic machinery, such as LC3 and Atg12–Atg5. Moreover, their depletion impedes the elimination of their cargo, while general macroautophagy, such as the response elicited by starvation, can remain largely unaffected [70]. This suggests that the macroautophagic elimination of specific cargoes can be manipulated pharmacologically while maintaining basal or housekeeping functions of macroautophagy intact. This selectivity would significantly lessen the pleiotrophic effects of inhibiting or activating the core components of macroautophagy.

Although the mechanistic underpinnings of these selective pathways are still under study [83], there are a growing number of entry points being uncovered. For instance an increased degradation of misfolded proteins by macroautophagy can be evoked by molecular chaperones and co-chaperones such as HSPB8 and BAG3 [113,114] by increasing Alfy expression [70], as well as in a p62-dependent manner by acetylation [115]. Beyond the autophagic receptors, selective degradation of mitochondria requires interaction between PINK1 and Parkin [100,116,117]. Finally, the ability of NBR1 to interact with cargo can be modulated by phosphorylation through TBK1 [106].

Pharmacological manipulation of macroautophagy

Even before a molecular understanding of macroautophagy was revealed by yeast genetic studies [5], biochemical and ultrastructural studies performed in rat liver [5] had already established that starvation and glucagon could promote macroautophagy [118] while insulin and free amino acids could inhibit it [119–121]. This regulation was achieved largely at the level of autophagosome formation [122]. These pioneering studies ultimately led to the identification of two classes of macroautophagy modulators still used today: inhibitors of Vps34 **(Table 1)** [123,124], and inhibitors of the large serine/threonine kinase mTOR **(Table 2)**. These two families of compounds, along with a wide variety of lysosomal inhibitors **(Tables 1 & 2)** allow for very effective global regulation of macroautophagy at early (mTor), middle (Vps34) and late (lysosome) steps in autophagic progression.

Regulation of macroautophagy by mTOR

The identification of these compounds have been invaluable for helping to define the molecular underpinnings of macroautophagy; however, from a pharmacological standpoint, the utility of these compounds is somewhat limited as both Vps34 and mTOR govern multiple processes in the cell, and furthermore, macroautophagy itself plays a prominent basal housekeeping role in many cells and tissues. For example, mTOR is a core kinase that integrates the energy and nutrient status of a cell with its growth, and thus is central to cell growth, cell proliferation, protein translation and protein synthesis, making the effects of mTOR inhibition widespread. mTOR is found in two different complexes, complex 1 (mTORC1) and complex 2 (mTORC2) (reviewed in [125–127]). Rapamycin can initiate macroautophagy because it specifically targets and inhibits mTORC1, the mTOR complex responsible for maintaining the phosphorylation status of the ULK1/2 complex [28]. Besides ULK1/2 phosphorylation, mTORC1 is also responsible for promoting protein synthesis by phosphorylating p70 S6K and elongation inhibition factor 4E binding proteins. Inhibition of mTORC1 directly by rapamycin, or through inhibition of Akt/PKB [128], can thus also inhibit long-term plasticity [129–132] and memory consolidation [133,134]; modulate circadian entrainment [133–136]; diminish myelination [137]; and alter dendritic arborization and local protein synthesis [138].

Regulating macroautophagy: mTOR dependence & independence

Macroautophagic degradation can also be potentiated in an mTOR-independent manner [139,140] **(Table 2)**, although in this case it is unclear how autophagosome building is enhanced in the presence of active mTORC1. One possibility is that mTOR-independent activation of macroautophagy is due to modulation of the ULK1/2 complex through other kinases. Together with mTOR, the evolutionarily conserved kinase AMPK acts as a metabolic regulator of the cell. As ATP becomes hydrolyzed, the ATP:AMP ratio shifts, leading to the activation of this kinase. AMPK has been shown to be an upstream modulator of the ULK1/2 complex [141–144]. The activity of AMPK and mTOR activity can be inter-related [127]; resveratrol can increase phosphorylation of AMPK (through increased Ca^{2+} entry and depletion of extracellular Ca^{2+} stores), leading to an inhibition of mTOR and increased macroautophagy **(Table 2)** [145]. AMPK can also modulate the activity of the ULK1/2 complex directly, independently of mTOR, by phosphorylation of ULK1 [144,146]. Importantly, preventing the ability of AMPK to interact with ULK1 accelerated the ability of cells to mount a starvation response [144]. Thus far, this has only been demonstrated genetically, and a pharmacological decoupling of AMPK and mTOR has not been reported.

Additionally, rather than altering the upstream signaling to increasing the macroautophagic response *per se*, degradation may be enhanced simply by increasing the efficiency of the membrane building that is part of an ongoing basal macroautophagic event. This is

suggested by findings that co-stimulation of mTOR-independent and mTOR-dependent pathways can enhance the degradation of mutant Huntingtin and aggregate-prone α-synuclein [147]. In neurons, there a controversial debate whether rapamycin-mediated mTOR inhibition is the most efficient means by which macroautophagy is activated [148–151]. As mentioned above, Vps34 complex 1 and complex 2 are important for the biogenesis and maturation of autophagosomes, respectively, and can be inhibited by Vps34 kinase inhibitors such as 3-MA or low doses of wortmannin **(Table 1)** [123,124]. Interestingly, direct application of the Vps34 product, PI3P, can potentiate macroautophagy and enhance clearance of selective cargo [140], consistent with the model that membrane-building can be rate limiting.

Regulating macroautophagy: intracellular calcium

A large number of compounds that influence autophagy have implicated intracellular Ca^{2+} levels as an important regulator (reviewed in [152]). Ca^{2+} plays a pivotal role in many cellular processes, and accordingly, its intracellular levels are tightly regulated. Different cells depend upon and respond to changes in Ca^{2+} differently, potentially making it difficult to interpret how Ca^{2+} can impact a cellular pathway. The regulation of macroautophagy is not an exception: elevating intracellular Ca^{2+} has been shown to both inhibit and activate macroautophagy, and modulating Ca^{2+} has been shown to modulate macroautophagy in an mTOR-independent and -dependent manner **(Table 2)**. The precise role of intracellular Ca^{2+} in macroautophagy, therefore, remains unresolved. For example, an mTOR-independent inhibition of macroautophagy by elevated intracellular Ca^{2+} levels may be elicited through the activation of nonlysosomal proteases such as calpains: calpains can cleave several Atg proteins including Atg5 [153,154], diminishing the formation of Atg 12–Atg5 [155]. Another example is the proposed mechanism of action of lithium: lithium has been proposed to activate macroautophagy by diminishing intracellular Ca^{2+} levels. Lithium acts by inactivating inositol monophosphatases, which diminishes IP3 levels and inhibits the IP3 receptor (IP3R), a channel through which Ca^{2+} is released from intracellular stores [156,157]. Although how inhibiting IP3R, and hence diminishing intracellular Ca^{2+} activates macroautophagy, is still uncertain, although an mTOR-independent mechanism has been proposed [156,158]. In a separate study, lithium-induced macroautophagy was shown to require FIP200 and the ULK1/2 complex, indicating that an mTOR-dependent mechanism may also be possible [159]. In hippo campal neurons, increased intracellular Ca^{2+} levels through intracellular stores increased mTOR-dependent phosphorylation of p70 S6K, linking intracellular Ca^{2+} levels with mTOR activation [160,161], and suggests that in these neurons, lithium will lead to an mTOR-dependent activation of macroautophagy. Interestingly, the influx of extracellular Ca^{2+} to raise Ca^{2+} levels did not modulate p70 S6K [160], suggesting that increasing intracellular Ca^{2+} using different methods evoke different signaling cascades.

Consistent with an mTOR-dependent effect, Kroemer and colleagues found that the direct application of a cell-permeable IP3 precursor inhibited starvation-mediated macroautophagy, and proposed that IP3R inhibition increases macroautophagy by upregulating ER stress [157]. ER stress may also lead to the mTOR-dependent upregulation of macroautophagy by thapsigargin **(Table 2)**, however thapsigargin leads to an increase in intracellular Ca^{2+} levels, not a decrease. Strong chronic ER stress might not be desirable to neurons, but mild ER stress has been shown to be protective by priming the autophagic response [162]. Nonetheless, the use of Ca^{2+} to modulate macroautophagy in neurons overall might be difficult, in light of the large transient fluxes of Ca^{2+} that is common to these cells. Moreover, different regions of the neurons may respond different to Ca^{2+} [163,164], and thus compartmentalization may become problematic.

Regulating macroautophagy: lysosomal function

The ultimate goal of macroautophagy is the lysosome-mediated degradation of its cargo, and thus the status of the lysosome is critical for macro autophagy to proceed. Gross inhibition of lysosome function can be accomplished with compounds such as bafilomycin A1, which inhibits the acidification of the lysosome by blocking the vacuolar [H^+] ATPase, or by lysosomotropic agents such as hydrochloroquine **(Table 1)**. More selective inhibition is possible by blocking the activity of a subset of the intralysosomal proteases known as cathepsins. Cathepsins are divided into three subgroups: cysteine, aspartyl and serine cathepsins, of which the cysteine capthepsins B and L and the aspartyl cathepsin D are the most abundant and participate in macroautophagy [165,166]. Cathepsin B and L double knockouts (KOs) lead to juvenile death associated with cerebral and cerebellar neurodegeneration [167], while the cathepsin D KO mice also exhibit juvenile lethality, although toxicity was across multiple organs [168]. Interestingly, the phenotype of cathepsin KO mice differs from those reported with core macroautophagy protein KO mice [34,169–172], suggesting that other cathepsins can compensate for their loss, and also reflects the wider impact the loss of lysosome function could have, since the lysosome would be the point of convergence for all forms of autophagy as well as the degradation of transmembrane proteins.

Regulating macroautophagy: unknown mechanisms

A large number of the compounds that have been identified to activate or inhibit macro autophagic function do so through mechanisms that have not yet been fully defined **(Tables 1 & 2)** and, thus, may ultimately offer greater specificity/selectivity. Furthermore, there are many other possible entry points at which **macroautophagic flux** might be pharmacologically controlled that remain unexplored. For example, Vps34 complexes are under the control of Beclin 1. Beclin 1 was originally identified as an interactor of the anti-apoptotic protein Bcl-2 [173] and its related homologs such as Bcl-xl [174]. These inter actions prevent the ability of Beclin 1 to interact with Vps34, thereby inhibiting autophagosome initiation. The ability of Beclin 1 to switch between the two complexes can be regulated by the phosphorylation of Bcl-2 by JNK1 [175] or phosphorylation of Beclin 1 by DAPK [176]. Thus, specific targeting of one or both of these enzymes may yield a measure of control over autophagic progression. Likewise, Beclin 1-independent forms of macroautophagy have also been reported in cortical neurons, induced by pro-apoptotic compounds such as staurosporine and etoposide [177], but the molecular mechanism of this activation remains unknown. Regardless of how macroautophagy is activated, manipulating general macroautophagic degradation may not be desired. As we learn more about the regulation of selective cargo capture, compounds affecting discrete forms of macroautophagy will be desirable, since the specificity that is gained will likely mitigate many of the problems that may be encountered when modulating energy sensing in the metabolically sensitive neuron.

Macroautophagy in neurons

Neurons are large, postmitotic cells that can undergo extensive remodeling. Furthermore, they are metabolically very active cells with a high energy demand. Neurons are also heavily dependent on protein degradation systems to recycle not only unwanted proteins, but all aspects of the cell, since cell division is not an option for self-renewal. Moreover, due to activities such as synaptic transmission, neurons require a means by which organelles such as mitochondria could be eliminated, as well as an effective bulk degradation pathway to help remodel spines. Macroautophagy will impinge upon each of these aspects of neuronal function and thus, the context in which we study macroautophagy in neurons and the brain will necessarily play an important role in how results should be interpreted.

Different neuronal subtypes express, usually in high levels, the core autophagic machinery [178]. Indeed the Atg8 homologues LC3 and GABARAP were first identified in the brain: LC3 in a complex with MAP 1A and 1B [179], and GABARAP as a protein that helped to traffic the GABA A receptors [180,181]. Conditional genetic deletion of core, nonredundant macroautophagic proteins has firmly established the necessity for macroautophagy in the brain [182–184] – different neuronal subtypes demonstrate an accumulation of ubiquitinated proteins, neuronal dysfunction and degeneration, and early death. However, to permit spatially restricted expression in the brain, these studies relied on the expression of Cre recombinase using the nestin promoter, leading to genetic excision early in development [185,186].

Age & intercellular interactions

The importance of macroautophagy in the adult brain remains uncertain. The currently available data suggest that postnatal deletion of macroautophagy may be less toxic. For example, the conditional deletion of Atg5 during embryonic development (Nestin-cre) leads to a substantial loss of Purkinje cells in the cerebellar cortex at 12 weeks and ataxic gait by 3 weeks [182]. When conditional deletion is instead delayed within the first 2 postnatal weeks (Pcp2-cre), a similar loss of cells is not seen until 48 weeks of age, and ataxic gait at 40 weeks [187]. Similar differences were observed with Atg7 deletion [183,188]. These data suggest that even a modest delay of deletion from embryonic to early postnatal ages (during which time the cerebellum is still undergoing development), can confer significant protection from cell death.

Unfortunately, age is not the only critical variable in these studies, and thus it is not yet straightforward to conclude neurons exhibit an age-dependent reliance upon macroautophagy. In particular, one should note the differing inter-cellular contexts of the two studies. Purkinje cells and cerebellar development require an interplay between the neuronal subpopulations (Purkinje, granular) and a specialized class of glia known as Bergmann glia. All neurons and most glia, including Bergmann glia, express nestin [189], and thus it is likely that in the Nestincre conditional KO mice Atg5 or Atg7 has been deleted in all of the relevant cell types. Pcp2-cre on the other hand has been reported to be exclusively in Purkinje cells [190]. It is therefore possible that the earlier toxicity in the Nestincre conditional mice is due to a contribution of non-cell autonomous toxicity.

An influence of neuronal subtype

Detection of autophagosomes in neurons dates back to the earliest studies on macroautophagy, including ultrastructural studies starting from the late 1960s [191–193]. Acute stressors such as axotomy or nerve crush leads to a rapid accumulation of autophagosome-like vesicles in axon terminals as well as the soma [194,195]. These data indicated that autophagosome-like structures could be formed at the cell body, but importantly, may also be created distally in the axon. A membrane source for creating autophagosomes distally is readily available: the neuronal ER is not limited to the soma, but also found in the axons and terminals [196]. In light of the length of neurons, the ability to locally create autophagosomes would increase the efficiency of macroautophagic clearance by sequestering the cargo. Early studies by Hollenbeck suggest that not only could cargo be sequestered distally, but autophagosomes could mature and form amphisomes while being transported retrograde back to the soma [197]. The presence of autophagosomes in the ER [198–201], and their maturation during regulated transport along the axon [200,202] has been re-explored, confirmed and expanded upon recently using molecular tools such as GFP-LC3. These findings suggest that neuronal macro autophagy of both peripheral and central neurons may be subject to compartmental regulation.

But what is the significance of proliferation of autophagosomes upon acute stress? Historically, both a proregenerative and prodestructive role for macroautophagy had been proposed: in the first instance, autophagosomes clear and recycle the damaged material to promote an environment that permitted axonal regeneration. In the latter instance, the induction of macroautophagy would promote cell death to eliminate the damaged cell [193]. Recently, new genetic and molecular tools have begun to establish that both models are likely correct. For example, two studies each utilized axotomy to produce an acute stress in different neuronal subtypes and then followed the cell survival in the presence and absence of an active macroautophagic response. Axotomy of the medium forebrain bundle leads to the appearance and accumulation of autophagosomes in nigrostriatal dopaminergic axons [203]. To inhibit the macroautophagic response, Burke and colleagues used intranigral injection of adeno-associated virus Cre to selectively eliminate Atg7 in adult dopaminergic neurons. The loss of Atg7 not only inhibited the appearance of autophagosomes, but significantly protected the neurons, leading to not only a preservation of more axons, but also diminished axonal fragmentation and swelling [203]. In contrast, Boya and colleagues found that axotomy of the optic nerve in adult mice was protected upon the induction of macroautophagy, while its inhibition reduced neuronal survival [204].

The neuronal subtypes being examined in the two studies, the midbrain dopaminergic neurons, and the retinal ganglia cells are quite different. For example, dopaminergic neurons project long-range fibers that are unmyelinated, while the retinal ganglia cells of optic nerve have both nonmyelinated and myelinated regions. As mentioned above, the interplay between neurons and glia may underlie how macroautophagy can be toxic in one cell type and protective in another. Nonetheless, there are far more differences to consider: they are morphologically and biochemically distinct, and have different firing activities. While it is unclear how the myriad of differences across neurons contribute towards macroautophagic toxicity, these two studies clearly indicate that augmenting general macro autophagic degradation in the brain may be problematic. It is also possible that the basal dependence on macroautophagy by a neuron may indicate how it responds to macroautophagic induction. For example, the Purkinje cells of the cerebellum have been shown to be especially sensitive to overactivation or inhibition of macroautophagy [182,183,199], indicating that they rely heavily on macroautophagy to survive. Thus, under conditions of excitoxic stress, the **autophagosome accumulation** observed in axonal swellings is thought largely to be protective [188,198,199]. In contrast, studies globally inhibiting macroautophagy in the brain do not report the loss of dopaminergic neurons [182–184]. This suggests that dopaminergic neurons depend less on macroautophagy for everyday survival. Indeed, recent studies indicate that the loss of macroautophagy throughout development leads to a late-onset loss of dopaminergic neurons [205], possibly through the ability of macroautophagy to modulate synaptic transmission [201]. A strong induction of macroautophagy in these neurons due to stress may, therefore, lead to an end stage response that is destructive.

A differential dependence of macroautophagy may pose a significant challenge when trying to modulate macroautophagy in neurodegeneration. Similar to the differential response mounted to acute stress, the impact of macroautophagy to regulate protein degradation in proteinopathies (reviewed in [83]), or to regulate mitochondrial turnover (reviewed in [104]) may differ based on which neurons are particularly affected. For example, in a mouse model of Huntington's disease, the activation of macroautophagy using rapamycin led to a beneficial outcome, partially ameliorating symptoms [148]. In contrast in a SOD1(G93A) model for amyotrophic lateral sclerosis, rapamycin treatment augmented toxicity [206], while lithium administration gave mixed results [207,208]. Many things could account for these differences starting with the affected neuronal subtype, or the interaction between the affected protein and basal macroautophagic processes. Moreover, there may also be a temporal component that contributes: studies of the SOD1(G93A) mice show a continuous

increased accumulation of GFP-LC3 in spinal cord [209]. This could indicate that there is a sustained dysregulation in macroautophagy, leading to what is known as **autophagic stress** [210]. Autophagic stress is defined as a prolonged imbalance in autophagosome formation and degradation. Under such conditions, augmenting macroautophagy with rapamycin would increase the amount of stress, ultimately leading to dysfunction and death. This is especially the case when the disease-causing mutation leads to an unexpected impact on the macroautophagic pathway itself. Similarly, since lysosomal function is essential for macroautophagy to proceed, loss of lysosome trafficking, acidification or maturation will likely eliminate the beneficial impact of macroautophagy and may instead lead to the accumulation of nonmaturing, possibly toxic, autophagic intermediates [78].

Conclusion & future perspective

Like all cells, neurons are dependent upon protein degradation to maintain cellular homeostasis, and both proteasome- and lysosome-mediated degradation are essential. The increased number of genetic and molecular tools continues to shed new insight into how macroautophagy is regulated in neurons. However, as we learn more it becomes clear that we must focus on the structural and biochemical complexities of neurons, as well as the profound variance across the different neuronal subtypes if we are to understand how macroautophagy influences neuronal function and the processes that lead to neurodegeneration. How we examine neuronal macroautophagy and the conclusions we draw may depend upon not only the age of the neuron, but the kind of neuron that is being interrogated as well as where in the neuron macroautophagy is required.

Although the use of genetic tools has increased our knowledge of macroautophagy in the brain, unlike pharmacological tools, they are limited temporally. The ability to interrogate neurons acutely *in vivo* and *in vitro* is a much needed and valued tool. Specific targets within the macroautophagic pathway can be difficult to distinguish and the effects of pharmacologically similar compounds, such as those that alter levels of free cytosolic Ca^{2+}, can be contradictory. Large screens, while useful in the identification of relevant classes of compounds, tell us little about how each compound will act in the spatiotemporal contexts relevant to neurodegenerative disease. Macroautophagy in neurons is a dynamic and multifaceted process; modulating it for disease treatment will require a more thorough understanding of how the compounds suggested by preliminary *in vitro* studies affect not only the canonical degradative pathways, but also the specific degradation of disease-relevant cargo in affected neurons.

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Key Term

Macroautophagy: Drives the clearance of cytoplasmic challenges including protein aggregates and dysfunctional mitochondria that otherwise underlie the development of neurodegenerative disease.

Key Term

Autophagosome maturation: Membrane trafficking events needed to shepherd the encapsulated autophagic cargo to the lysosome, including the formation of amphisomes and autolysosomes. It remains unclear how these membrane trafficking events are regulated and ultimately achieved.

Key Term

Macroautophagic flux: Rate at which autophagosomes are formed and ultimately turned over by fusion with degradative lysosomes. During nutrient deprivation, autophagosome lifetime can be measured in tens of minutes; however, during some disease states the flux is reduced as components needed for the formation of autophagosomes becomes limiting or as steps in autophagosome maturation are impaired.

Key Terms

Autophagosome accumulation:

Autophagosomes accumulate when autophagosome production is upregulated due to a robust cellular response or when autophagic flux is impaired. Accumulation is a hallmark of many diseases and may even constitute the direct cause of some pathologic phenotypes (e.g., Pompe disease). Whether autophagosome accumulation in neurodegenerative disease indicates enhanced autophagic response or a failure to mature is likely both disease and neuronal subtype-specific.

Autophagic stress:

Prolonged imbalance in autophagosome formation and consumption.

Executive summary

Background

- There are three forms of autophagy: microautophagy, chaperone-mediated macroautophagy and macroautophagy, of which macroautophagy is the most conserved.
- Macroautophagy delivers cargo to the lysosome by a double membrane structure known as an autophagosome.

Macroautophagy

- Autophagosome formation is driven by a series of Autophagy-related (Atg) genes.
- The nucleation, expansion and maturation of the autophagosome is driven by a series of essential core protein complexes. The lipidated form of Atg8 is the only protein associated with the autophagosome membrane throughout the process and, thus, is a widely used marker for autophagosomes. There are six mammalian homologs to Atg8, all of which can be lipidated, but MAP1LC3 is the best studied and known is as LC3.
- Macroautophagic degradation can be both nonselective and selective for cargo. Selectivity is achieved by autophagic receptors such as p62/SQSTM1, NBR1, NDP52, Optineurin and NIX, and selectivity adaptors such as Alfy.

Pharmacological manipulations of macroautophagy

- The earliest classes of macroautophagy modulators identified in the pioneering studies are inhibitors of Vps34 and inhibitors of mTOR.
- Both mTOR-dependent and independent forms of autophagy have been reported.

Macroautophagy in neurons

- Neurons express, usually in high levels, the core autophagic machinery, and can form autophagosomes at the soma or distally in axons.
- The importance and role of macroautophagy in neurons can be influenced by a variety of factors including developmental stage, the intercellular interactions between different neuronal subtypes or between neurons and glia, as well as neuronal subtype.
- The differential dependence of macroautophagy by different neuronal subtypes may pose a significant challenge when trying to modulate macroautophagy in neurodegeneration.

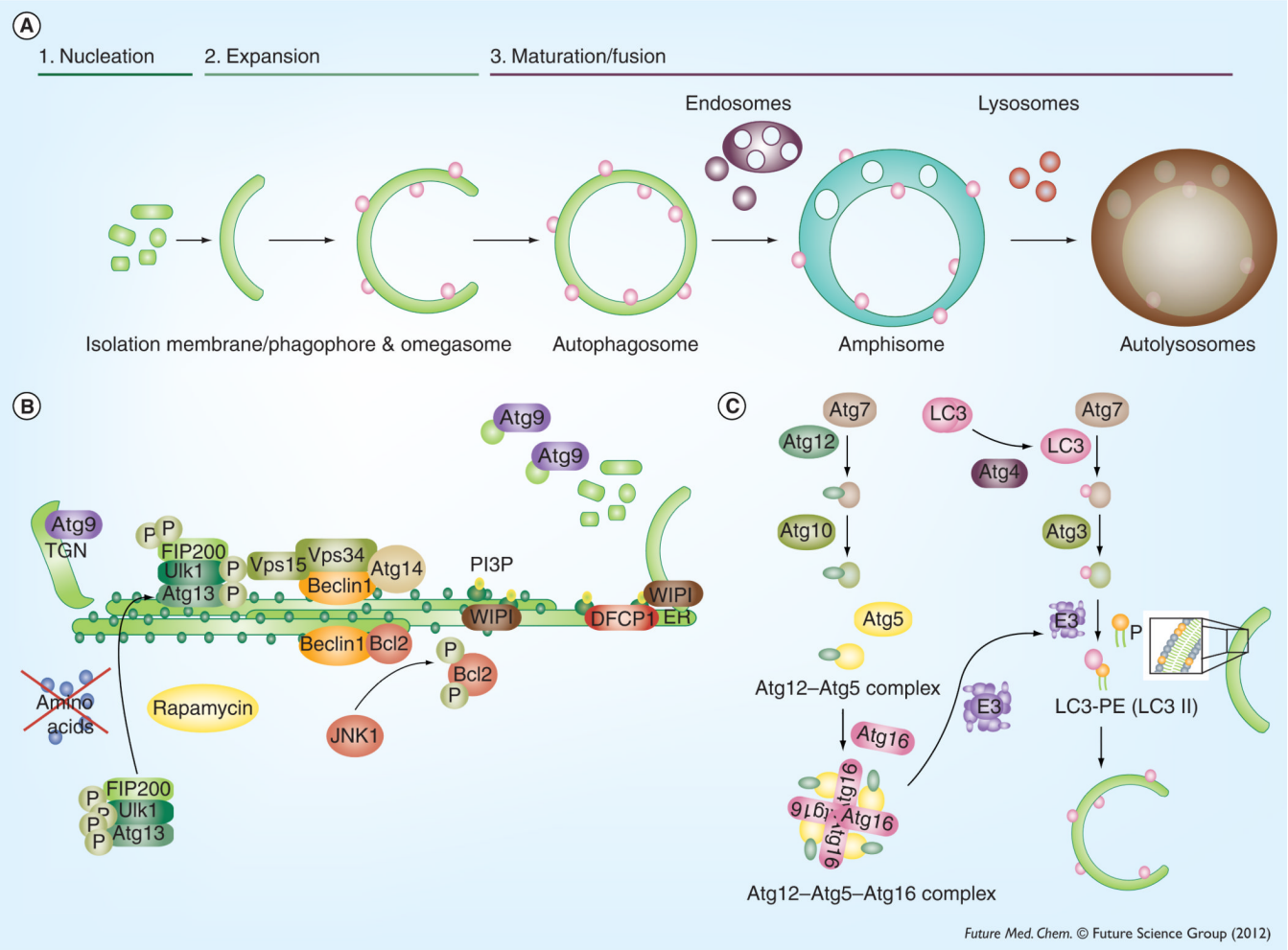

Figure 1. Formation of the autophagosome

(A) Much of the mechanistic understanding of macroautophagy centers upon the core proteins involved in formation of the autophagosome, and is driven by a series of autophagy-related (Atg) proteins. Our current understanding divides macroautophagy into three main steps: nucleation, expansion and maturation. **(B & C)** The initiation and expansion of the autophagosome membrane is dependent upon several core protein complexes. **(B)** The ULK1/2 complex and Vps34 Class III PI3K complex I: in response to mTORC1 inhibition via rapamycin or amino acid withdrawal, the phosphorylation status of the ULK1/2 complex changes and it can localize to the endoplasmic reticulum. The Vps34/PI3K complex 1, which is formed through association with Beclin 1 and Atg14, drives the creation of PI3P. PI3P attracts two effector proteins WIPI and DFCP1, the former of which has been shown to be required for the initiation of the autophagosome. The image depicts the initiation of the autophagosome to occur at the endoplasmic reticulum, but there is a clear importance for a contribution from Atg9, which is found on vesicles originating at the TGN. Other sources of membrane that may contribute to autophagosome biogenesis include the outer mitochondrial membrane and the plasma membrane. **(C)** The two ubiquitin-like conjugation reactions of macroautophagy. The first conjugation reaction leads to the conjugation of Atg12-Atg5, which forms a tetrameric complex with Atg16L. Atg12–Atg5–Atg16 acts as a putative E3-like ligase for the conjugation of LC3 to phosphatidylethanolamine.

TGN: Trans-golgi network.

Table 1

Inhibitors of macroautophagy.

Compound	Mechanism	mTOR	Method of analysis	Ref.
3-methyladenine	Inhibition of macroautophagy through action on class III phosphotidylinositol-3-kinase	Yes	LLPD, ATP levels (luciferase assay), EM analysis of autophagosomes and amino acid levels in rat hepatocytes	[123]
Wortmannin and LY294002	Inhibition of macroautophagy through action on class III phosphotidylinositol-3-kinase	Yes	LLPD, sequestration of electroinjected sucrose, and PI3K levels in rat hepatocytes	[124]
Nitric oxide	Inhibits autophagosome formation in part through interaction with Beclin-1 complex formation	Yes	LC3-II levels in rat primary cortical neurons	[211]
Bafilomycin A_1, hydroxychloroquine and monensin	Inhibition of autophagy through prevention of the fusion between autophagosomes and lysosomes	No		
Haloperidol and clozapin	Inhibition of macroautophagy, mechanism of action unclear but possibly prevents the fusion between autophagosomes and lysosomes	Unknown	Western blot and immunofluorescence of LC3-II in rat primary cortical neuron cultures	[212]
N-acetyl cysteine, cystamine and glutathione	Inhibition of macroautophagy through decreased phosphorylation of JNK and Bcl-2. Counterintuitively, *N*-acetyl cysteine seems to inhibit mTOR activity	Unknown	LC3-II levels in treated COS-7 cells; for *N*-acetyl cysteine only: LC3-I and LC3-II levels in human primary cortical neurons and HeLa cells, confocal microscopy of GFP-LC3 localization in HeLa cells, increased protein aggregation in a zebrafish model of HD, and inhibition of starvation-induced macroautophagy (LC3-II levels) in mice	[213]

EM: Electron microscopy; HD: Huntington's disease; LLPD: Long-lived protein degradation.

Table 2

Activators of macroautophagy.

Compound	Mechanism	mTOR	Method of analysis	Ref.
Rapamycin and rapalogs	Inhibition of TOR signaling	Yes	LLPD and sequestration of electroinjected sucrose in rat hepatocytes; LC3 conversion	[214]
Torinl	Nonspecific inhibition of mTORC1 complex	Yes	Re-localization of GFP-LC3 to autophagosomes and western blots of LC3 conversion in mouse embryonic fibroblasts	[215]
Perhexiline, niclosamide, rottlerin and amiodarone	Inhibition of mTORC1 signaling	Yes	Confocal microscopy of EGFP-LC3 and EGFP-LC3 processing and degradation in MCF-7 cells	[216]
Tamoxifen, C2-ceramide and C6-ceramide	Interaction with mTORC1 and Beclinl complexes	Yes	Localization of GFP-LC3, LLPD, and p62 levels in HeLa cells; EM analysis and measurement of autophagic flux in MCF-7 and HT-29 cells	[217]
Clonidine and rilmenidine	Modulation of cAMP levels	No	LC3 conversion in treated PC12 cells; decreased cell death and decreased aggregation of transfected EGFP-HDQ74 construct in SK-N-SH cells	[158]
Resveratrol	Activation of AMPK	Yes	Clearance of Aβ in HEK293, follow-up in primary hippocampal neurons and APP/PS1 transgenic mice; AMPK LC3 conversion and immunocytochemical analysis	[145,218]
Dexamethasone	Akt and PML dependent induction	Yes	Localization of transiently transfected GFP-LC3 and LC3 conversion in SUP-B15 and RS4;11 cells. EM analysis of autophagic vacuoles	[219]
Phenethyl isothiocyanate	Partly through suppression of the Akt-mTOR signaling axis	Yes	LC3 conversion in treated LNCaP, PC-3, and PrEC cells; Localization of *lgg-1*:GFP in *Caenorhabditis elegans*	[220]
Verapimil	Reduction of intracytosolic Ca^{2+}	No	LC3 conversion in treated PC12 cells; decreased cell death and aggregation of transfected EGFP-HDQ74 construct in SK-N-SH cells	[158]
Niguldipine, nicardipine and amiodaron	Reduction of intracytosolic Ca^{2+}	No	Microscopic analysis of GFP-LC3, LC3 conversion, and reduced accumulation of transiently transfected polyglutamine constructs in H4 cells	[221]
Lithium, L-690,330, carbamazepine and valproic acid	Inhibition of inositol monophosphatase	No	Clearance of tagged mutant huntingtin in SK-N-SH and stable inducible PC12 cells, and western blots of EGFP-HDQ74 in stable inducible PC12 cells	[156]
Calcium phosphate precipitates	Increase of free cytosolic Ca^{2+} in a Beclin 1 and Atg5 dependent manner	Unknown	Localization of GFP-LC3, western blots of LC3-I and -II, and EM analysis of autophagosomes in HEK293 cells	[222]
Vitamin D3 compounds, Ionomycin and thapsigargin	Increase of free cytosolic calcium Ca^{2+}	Yes	Valine levels in MCF-7 cells and GFP-LC3 puncta in stable MCF-7-EGFP-LC3 and transiently transfected HeLa cells	[223,224]
Igoxin, strophanthidin and digoxigenin	Increase of free cytosolic calcium Ca^{2+}	Unknown	Flow cytometric analysis of GFP-LC3 and GFP-Rab7 fluorescence and western blots of LC3-I and -II in stable GFP-LC3 MCF-7 cells	[225]
Alisol B	Increase intracellular Ca^{2+} from internal stores, leading to activation of the CaMKK-AMPK-mTOR pathway	Yes	Localization of GFP-LC3, EM analysis of autophagosomes, and western blots of LC3-I and -II in MCF-7 cells	[226]
Xestospongin B	Inhibition of IP_3R	No	Localization of GFP-LC3, co-localization of GFP-LC3 and Lamp 2A, percentage of	[227]

Compound	Mechanism	mTOR	Method of analysis	Ref.
			vacuolized cells, and levels of LC3-I and -II in transfected HeLa cells	
Cadmium	Increase of free cytosolic calcium (Ca^{2+}) in a Ca^{2+} signaling pathway-dependent manner	Yes	EM analysis of autophagosomes and LC3 conversion in MES-13 cells	[228,229]
Minoxidil	Interaction with K^{+}_{ATP} channels	No	LC3 conversion in treated PC12 cells; decreased cell death and decreased aggregation of transfected EGFP-HDQ74 construct in SK-N-SH cells	[158]
Trehalose	Mechanism of action unclear but reported to be mTOR-independent [139]	Unknown	Macroautophagy-dependent reduction in cell death aggregation of EGFP-HDQ74 in COS-7 cells; LC3 conversion in treated COS-7, PC12, HeLa and QA1/12/9A cells; clearance of EGFP-LC3 aggregates and α-synuclein mutants in dox-inducible PC12 cells	[139]
Fluspirilene, trifluoperazine and pimozide	Mechanisms of action unclear (all three are antipsychotics)	No	Microscopic analysis of GFP-LC3, ratios of LC3-I to -II, and accumulation of transiently transfected polyglutamine constructs in stable H4 cells	[221]
10-NCP and structurally related compounds	Mechanism of action unclear but reported to be Akt and mTOR-independent	No	LC3 conversion in rat primary striatal neurons	[149]
Small molecule enhancers 10, 18 and 28	Mechanisms of action unclear but reported to be mTOR-independent	No	Clearance of aggregates and western blots of LC3-I and LC3-II in HeLa cells transfected with EGFP-LC3; Rhabdomere counts in fly model of Huntington's disease	[139]
Amitriptyline and citalopram	Mechanisms of action unclear (currently used as antidepressants)	Unknown	Quantification of acidic vacuoles, levels of LC3-II and Beclin-1, and localization of GFP-LC3 in rat primary astrocytes and neurons	[230]
Loperamide	Mechanism of action unclear (currently used as an antidiarrheal)	No	Microscopic analysis of GFP-LC3 spots, LC3 conversion, and accumulation of transiently transfected polyglutamine constructs in stable H4 cells	[221]
Staurosporine, MK801 and etoposide	Beclin-1-independent but 3-MA-dependent manner leading to neuronal apoptosis	Unknown	LC3 conversion, and LC3 flux. RFP-GFP-LC3 puncta formation. SDS-soluble p62 levels	[177]

EM: Electron microscopic; HD: Huntington's disease; LLPD: Long-lived protein degradation.