CHAPTER 1

THE CELLULAR PROCESS OF AUTOPHAGY AND CONTROL OF AUTOPHAGY IN NEURONS

Nicole C. McKnight, Noboru Mizushima and Zhenyu Yue

ABSTRACT

Autophagy is a cell self-digestive, lysosomal degradation pathway. The three subtypes of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy, differ in the way that materials are delivered to the lysosomes for degradation. While recent studies reveal that the cellular process of macroautophagy (involving critical steps such as formation, trafficking, fusion and degradation of autophagosomes) is evolutionarily conserved from yeast to mammals, the regulation and functional adaptation of autophagy in various cells and tissues is poorly understood. This chapter provides an overview of what is known about autophagy machinery and highlights the recent findings of autophagy regulation in neurons. Elucidation of neuronal autophagy function will ultimately aid in drug target identification and perhaps lead to a rational therapeutic strategy to combat neurodegenerative diseases.

INTRODUCTION

Neurons are highly differentiated and their complexity arises from their specialized, polarized domains that rely on active membrane transport to connect the cell body to axons and dendrites, which are often long and far away. The neuron has long served as a model for studying autophagy[1] and the presence of double-membrane autophagosomes was described as early as the mid-1960s.[2–6] Upon physical or chemical injury (e.g. axotomy or excitotoxic insult), neurons developed a large number of autophagosomes that sequestered a portion of cytoplasm and were seen at cell bodies (soma) as well as axons. The phenomenon is associated with neuronal subcompartments devoid of organelles and filled with various

types of vesicles,[1] contributing to the histologic appearance of "chromatolysis." Subsequent ultrastructural analysis assisted with electron microscopy (EM) showed accumulation of autophagosomes in neurons of postmortem brains with neurodegenerative diseases, leading to the early hypothesis that autophagy is acutely turned on under pathological conditions perhaps as an attempt to repair/restructure via degradation, but sometimes with destructive or suicidal outcomes. The historical view of autophagy, however, has evolved due to recent genetic and molecular evidence that neurons rely on basal autophagy to ward off intracellular aggregate accumulation and resulting neurotoxicity. In addition, functional autophagy is required under neurodegenerative disease conditions triggered by the expression of disease-associated mutant proteins as these are eliminated by the autophagy. The neuroprotective function of autophagy is conceptually in line with the conservation of autophagy as a survival mechanism. But the idea that autophagy only functions to rescue may not fit with the previous observations in neurons that suppression of autophagy (genetically or pharmacologically) sometimes alleviates the neurotoxicity[7] (for review see Yue *et al.*[7]). Therefore, these parallel hypotheses co-exist, though seemingly conflicting, and may reflect the multi-faceted characteristics of autophagy under different physiological functions.

Our ultimate goal is to resolve these important issues and understand autophagy in detail by thorough investigation of the autophagic process, its regulation and its relevance to human diseases. Several outstanding questions pertinent to this goal remain to be answered: what is the exact cellular process of basal autophagy and how is it controlled in neurons that are highly differentiated, polarized, postmitotic cell types? How does the basal autophagy machinery adapt to the neural pathways that are responsible for rapidly processing and transmitting information by electrical and chemical signals across different subcompartments? How do neurons recognize disease protein aggregates to signal and mobilize the autophagic machinery to selectively degrade the aggregates? Moreover, how does each human disease that exhibits autophagy-associated neuropathology cause altered autophagy? Do these alterations contribute to the disease process and is there any neuronal cell type-specific autophagy regulation? In turn, can targeting autophagy reverse or modify a disease process and as a therapeutic strategy? In fact, emerging evidence suggests that autophagy is a potential drug target. To this end, the research effort has now been increasingly devoted to the search for drugs that enhance autophagy in the brain in order to increase the elimination of disease proteins that are prone to aggregation, though we do not know all the ramifications of such an increase and whether this is even possible in human brains. A recent surge of research into the mechanics of autophagy has, however, greatly increased our knowledge of the process in non-electrical cells. The knowledge

will facilitate our understanding of the conservation and distinct features of neuronal autophagy. Indeed, autophagy occurs in almost every cell of our body and its molecular machinery is highly conserved between organisms and tissues.

AUTOPHAGY PATHWAYS

Autophagy and the ubiquitin-proteasome system (UPS) are the two major degradative pathways in mammalian cells. The latter is responsible for degrading damaged proteins in the proteasome and is specific in its selection of proteins and protein aggregates that are tagged with ubiquitin. Autophagy uses the lysosome for degradation and has three subtypes: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA is induced after prolonged nutrient deprivation or oxidative stress[8] and is selective in its recycling of a subset of cytosolic proteins (see Chapter 4 for details). Microautophagy involves inward invagination of the lysosomal membrane to take up small pieces of cytoplasmic material. Macroautophagy is the main form and is a catabolic, mostly unselective pathway that channels bulk portions of the cytoplasm to the lysosome to generate amino acids and macromolecules; it is generally responsible for degrading long-lived proteins and large cellular structures. By balancing these degradative pathways with new protein synthesis, cells are able to maintain equilibrium of protein pools, which are essential for the survival, development, and proper function of the cells.

Macroautophagy, hereafter referred to as autophagy, is a term originating from the Greek words, "phagy," meaning to eat and "auto," for self. It is a highly conserved intracellular membrane trafficking pathway that is exhibited in all eukaryotes from yeast to humans. It responds to nutrient deprivation as its most evolutionarily conserved function, but is also triggered by cellular stress and the accumulation of protein aggregates and damaged organelles, especially in higher organisms. Autophagy generates the means for survival by degrading cytosolic proteins and whole organelles and recycling these back to the cytosol as amino acids and macromolecules. As a result, autophagy maintains cellular homeostasis by clearing the cell of misfolded or long-lived proteins and damaged parts and has also developed to rid the cell of invading microorganisms.

Autophagy is characterized by the formation of double-membrane vesicles, called autophagosomes that travel to the lysosome where they fuse and the contents of which are degraded by hydrolases. The autophagosome was originally observed using EM in the late 1950s and was first characterized by Christian de Duve in 1966 when he described degradative vesicles that were seen to contain both cytoplasmic components and mitochondria.[9] He saw the vesicles merge with lysosomes and called the process of "non-specific bulk segregation and digestion" autophagy.

Since a massive torrent of studies on the process of autophagy beginning in the early 1990s, cell biologists have dissected the progression of the autophagosome from its initiation or nucleation, its expansion, its maturation including fusion with components of the endocytic pathway and its eventual degradation by the lysosome. Also, great strides have been made to determine how the process is triggered and how the autophagy machinery is initiated. Many autophagy-specific proteins, called Atg proteins, have been discovered in yeast through humans. We now know that the process requires these components as well as other cytosolic proteins and intracellular organelles such as the endoplasmic reticulum (ER), Golgi apparatus, and perhaps mitochondria, in addition to the endosome-lysosome system.

During autophagy, cytoplasmic components are sequestered in a growing phagophore, also known as an isolation membrane (IM), which is generated at the preautophagosomal structure (PAS) in yeast (Figure 1). This double-membrane structure expands around portions of the cytoplasm containing proteins, aggregated proteins and organelles and then closes, forming the autophagic vacuole (AV) or autophagosome. Most mature autophagosomes are approximately 0.5 to 1 μm in diameter; they can fuse with endocytic pathway vesicles including early endosomes, multi-vesicular bodies (MVBs), and late endosomes thus forming amphisomes. Eventually, the autophagosome fuses with the lysosome, becoming an autolysosome, and the acidic hydrolases within break down the captured components to macromolecules and amino acids that are released back to the cytosol by transporters and permeases delivered with the lysosomal membrane.[10] After degradation by autophagy, the recycled products can be used in biosynthetic pathways and to generate new proteins.

In yeast, the cytoplasm-to-vacuole (CVT) pathway, which shares many of the core components with autophagy, and both pexophagy and mitophagy (the specific autophagic degradation of peroxisomes and mitochondria, respectively) are considered selective forms of yeast autophagy. Until recently mammalian autophagy was thought to be a non-selective degradation process, capturing random parts of the cell, but now it is believed to also specifically engulf protein aggregates, organelles and bacteria as these cargos have been seen to selectively and exclusively incorporate into autophagosomes. The organelle-specific pathways of pexophagy, mitophagy and even ER-phagy have been observed in human cells though how these organelles are recognized and targeted by the autophagosome is largely unknown. In addition, the source of the membrane(s) is a subject of intense research and great debate. Although autophagy allows for cellular survival in times of stress such as hypoxia, infection, and starvation, much of the prenucleation process and the steps leading to its initiation remain to be elucidated.

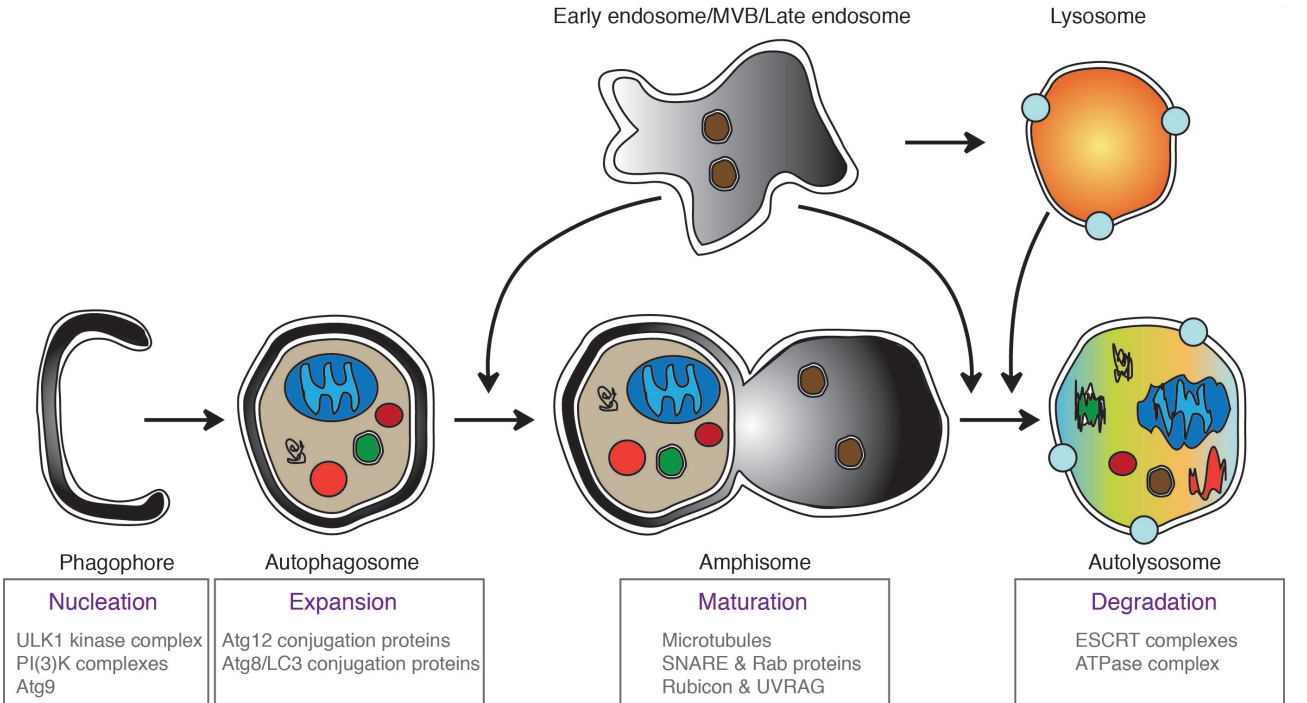

Figure 1. Mammalian autophagy. During mammalian autophagy, first the phagophore (or isolation membrane) is formed or nucleated. The phagophore engulfs portions of the cytoplasm and can take up mitochondria (blue), misfolded proteins and various vesicles in a step called expansion. During maturation, the autophagosome can fuse with endocytic vesicles to become an amphisome. The mature autophagosome or amphisome fuses with the lysosome and becomes degradative.

AUTOPHAGY CORE MACHINERY

The molecular machinery responsible for autophagy initiation and autophagosome nucleation and expansion was discovered first in *Saccharomyces cerevisiae* through genetic screens that identified essential autophagy genes in response to amino acid starvation.[11,12] Currently, there are 35 known Atg proteins involved in both autophagy and the *CVT* pathway in yeast and most, if not all, human counterparts of the autophagy-specific proteins have been discovered.[13] In mammals, there are multiple core and auxiliary proteins that contribute to and control autophagy. The human Atg proteins can be divided into five groups listed in the order that they are thought to act: the ULK1 kinase complex, the Atg9 cycling complex, the Vps34/class III PI3-kinase complexes, the lipid-binding Atg18 homologs, and the ubiquitin-like proteins Atg12 and Atg8/LC3 (and its related proteins) and their conjugation systems. Also, a set of proteins is responsible for transferring degraded material back into the cytoplasm. The five functional groups act at an early stage in the autophagy pathway and are recruited to the phagophore. The recent explosion of autophagy research has led to a somewhat comprehensive view of how they operate to form the autophagosome.

ULK1/ULK2 Kinase Complex (Atg1)

The first yeast autophagy gene identified was *ATG1*.[14] It transcribes a serine/threonine kinase that initiates autophagy downstream of the amino acid sensor and the negative regulator of autophagy, TOR kinase. Atg1 initiates both starvation-induced autophagy and the CVT pathway in yeast and does so through interactions with at least seven other Atg proteins. Autophagy is initiated through the starvation-dependent dephosphorylation of the interacting partner Atg13 that causes it and the third member of the complex, Atg17, to bind Atg1 tightly, activating its kinase activity.[15] This occurs as a result of the direct inhibition of TOR either by amino acid deprivation or treatment with the TOR inhibitor rapamycin. But it is Atg17, not Atg1 or Atg13 that is at the top of the hierarchy of Atg proteins, as determined by systematic analysis of yeast mutants.[16] Atg1 in *Drosophila* and *C. elegans* (in which it is called UNC-51) together with Atg13 and Atg17 are also critical to the control of autophagy in these organisms, but Atg1 is also involved in apoptosis and development through multiple non-Atg binding partners.[17] In addition, Atg1/UNC-51 is required for neuronal vesicular trafficking and development possibly through its indirect interaction with microtubule motors.[18] As in yeast, *Drosophila* Atg13 binds more strongly to Atg1 during autophagy but opposite to yeast, induction of autophagy upon nutrient deprivation in flies is characterized

by increased phosphorylation of Atg13 and a loss of phosphorylation of Atg1 by TOR.[19] Also, TOR remains complexed with Atg1 in both nutrient-rich and starvation conditions.

Two of the mammalian homologues of Atg1, called unc-51-like kinase 1 and 2 (ULK1 and 2) were first discovered to be involved in autophagy in 2007.[20] The discovery of the Atg17 ortholog came shortly after and though it shares little sequence homology to the yeast protein, FIP200, also known as RB1CC1, shares a functional role as an autophagy scaffolding protein.[21] FIP200 has been shown to bind ULK1 in both nutrient-rich and amino acid-free conditions,[22] but ULK1 phosphorylates FIP200 in starvation and they then travel together to the site of autophagosome formation.[23] The Atg13 homologue was discovered through sequence analysis and was shown to not only bind both ULK1 and ULK2 but is also a substrate for both kinases.[24] Atg13 itself binds FIP200[23,25] and it is proposed that Atg13 serves to stabilize the interaction of ULK1 and FIP200. Again, ULK1 kinase activity is highest when it is complexed together with Atg13 (or FIP200) and Atg13 is seen to go to the phagophore upon starvation.[22,24,25]

Exciting new research has shown that the mammalian TOR kinase complex, mTORC1 directly binds to the ULK1-Atg13-FIP200 complex and that Raptor and ULK1 bind in nutrient-rich conditions but dissociate upon starvation.[25] In addition, multiple groups have shown that mTOR directly phosphorylates ULK1/2 and also Atg13.[22,23,25] Atg101, a fourth Atg13-binding protein that is not conserved in yeast was seen to interact with the Atg13-ULK1-FIP200 complex in an Atg13-dependent manner and is required for and possibly promotes autophagy by stabilizing Atg13.[26,27] Corroborating the importance of ULK1 and its interactions with its adaptor proteins and mTOR itself for the initiation of autophagy, a recent study of the hierarchy of mammalian autophagy proteins places ULK1 and FIP200 as the most upstream at the site of autophagosome formation.[28] It has also been recently shown that ULK1 is phosphorylated by AMPK,[29–31] the energy-sensing kinase, adding new information for the mechanism of autophagy initiation after nutrient deprivation.

Atg9 Recycling Complex

The only membrane-spanning autophagy core complex protein is Atg9; it has six transmembrane domains. Although there is no known function designated to mammalian Atg9 (mAtg9), its association with membrane and extrapolation from yeast studies suggests that mAtg9 may deliver membrane to forming autophagosomes. In yeast, Atg9 is seen to cycle between the PAS and a peripheral pool that has recently been shown to comprise of clusters of vesicles and tubules often found

close to mitochondria.[32] The Q/t-SNAREs Sso1/2 and Sec9 are required for formation of the Atg9-positive tubular structures.[33] Atg1 is required for this cycling but Atg9's movement is not dependent on Atg1 kinase activity.[34] The localization of Atg9 to the PAS depends on Atg17 and Atg9 retrieval from the PAS, which occurs prior to autophagosome formation, requires Atg1, Atg2, and Atg18.[16]

Mammalian Atg9 is required for autophagy and is an essential gene for survival; Atg9 knockout mice cannot survive past P0 much like Atg3, Atg5 and Atg7 knockout mice.[35] Similar to yeast Atg9, mAtg9 cycles between two pools, residing in a juxta-nuclear Golgi pool in full medium but dispersing to a peripheral, perhaps endosomal, pool upon amino acid withdrawal.[36] As in yeast, this translocation requires ULK1 and also Atg13.[24] Regulation of autophagy was recently linked to the p38 MAP kinase pathway through Atg9, as it was demonstrated that the cycling of Atg9 requires the Atg9- and p38-interacting protein p38IP and through these interactions, p38α acts as a negative regulator of autophagy.[37]

PI3-Kinase Complexes (Vps34-Beclin 1)

The action of the autophagy-regulating class III PI3-kinase (PIK3C3), also known as Vps34, and its complex occurs early in the process and is required for nucleation of the phagophore. It was discovered previously that treatment with 3-methyladenine (3-MA), an inhibitor of the PI3-kinases, inhibits autophagy.[38] Since then, it has emerged that the production of Phosphatidylinositol(3)P, also known as PtdIns(3)P or PI(3)P, is essential for nucleation and formation of the autophagosome. Vps34 catalyzes the phosphorylation of phosphoinositides (PIs) at position 3 on their inositol ring to produce PI(3)P.[39]

The lipid kinase vacuolar protein sorting (Vps) 34 and its complex, including the Vps15 regulatory subunit and the accessory autophagy proteins Atg14 and Atg6 were first described as requirements for autophagy in yeast.[40] The mammalian homolog of Vps15 is p150 and is also called PIK3R4 for phosphoinositied-3-kinase, regulatory subunit 4; this protein anchors the Vps34 complex to membranes. The mammalian homologue of Atg6 is Beclin 1 or BECN, a coiled-coil protein, and together with the newly identified human ortholog Atg14L (Atg14-like), they form the mammalian Vps34 Complex I.

Human Atg14L, also called BARKOR,[41,42] was shown to be required for autophagosome formation and more specifically for the recruitment of Atg16L and LC3, essential proteins of the phagophore (discussed below) to the site of autophagosome formation.[41,43,44] Unique as a member of the Vps34 kinase complex, Atg14L interacts with both Beclin 1 and Vps34 and its coiled-coil domain is key for the binding. Atg14L is recruited to phagophore structures, but Atg14L

is found on these structures without Beclin 1 and Vps34 and the kinase activity of Vps34 is not required, suggesting Atg14L dictates when and where autophagosome nucleation occurs, possibly by recruiting Vps34 to this site and/or stimulating its activity.[44] Overexpression of Atg14L increases autophagosome formation and it is required for the autophagy-mediated elimination of *Salmonella.*[42]

Another coiled-coil protein UVRAG (UV radiation resistance associated gene) binds to Beclin 1[45] and UVRAG does not associate directly with Vps34 Complex I containing Atg14L, but forms a distinct complex containing p150, Vps34 and Beclin 1.[41,43,46] UVRAG seems to compete with Atg14 for Beclin 1 binding leading to two mutually exclusive complexes.[42] There are conflicting views as to the requirement of UVRAG for autophagy[41,47] and the role of the UVRAG complex in autophagy is still unknown. However, its function in the endocytic pathway was recently shown.[47] The protein Bif-1 (also called SH3GLB1, SH3-domain GRB2-like endophilin B1, or simply endophilin B1) may provide some insight; Bif-1 is required for autophagy.[48] Like many others, it has a coiled-coil domain and interacts with Beclin 1 through direct binding to UVRAG. Bif-1 has other roles in membrane traffic and is required for fission of Golgi carriers. It has been suggested that Bif-1 may be required for autophagosome membrane expansion and curvature through its N-BAR membrane curvature domain and its binding to the tethering factor ARF-GAP.[39] Recently Bif-1 was shown not to act at the level of autophagy initiation, but to regulate degradative endocytic traffic and in turn, autophagosome maturation.[49]

Among the recent eruption of research surrounding the Vps34 kinase complex is the data describing yet-another Beclin 1-binding protein, RUBICON (an acronym for run domain Beclin-1 interacting and cysteine-rich containing protein, still annotated as KIAA0226).[43,44] Rubicon resides with partners Beclin 1, UVRAG, and Vps34 to form the Rubicon complex. Opposite to Atg14L, RUBICON knockdown increases autophagosomes by inhibiting fusion of the autophagosome with the lysosome and also the maturation of endosomal vesicles to lysosomes.[44] Tagged RUBICON is found on endosomes and lysosomes and overexpression of RUBICON inhibits autophagosome turnover confirming its role as a negative regulator of fusion; overexpression of the protein also causes aberrant expansion of late endosomes and lysosomes.[43]

A sixth Beclin 1-binding protein, AMBRA1 may also contribute to the regulation of Vps34 activity.[50] The WD-repeat-containing protein is not only a positive regulator of Beclin 1, but is also required for starvation-induced autophagy. AMBRA1 appears to be an ULK1 substate and mediates autophagy regulation by the dynein motor complex,[51] though the exact molecular function of AMBRA1 in autophagy initiation is not known.

Though p150 is a shared core component of the Vps34 complex, Beclin 1 is the protein that mediates the interactions of the auxiliary proteins that distinguish the multiple known complexes. Through its differential binding, Beclin 1 may act as a switch between the complexes in order to coordinate the regulation of autophagy in highly evolved species. As a master regulator, Beclin 1 may balance autophagosome nucleation and maturation by toggling between Complex1 and the UVRAG complex and also the RUBICON complex.[46] Beclin 1 also plays a role in apoptosis through its binding to the anti-apoptotic protein Bcl-2.[52] Bcl-2 negatively regulates Beclin 1 by keeping it away from its Vps34 kinase complex partners and thereby preventing autophagy. Though it should be said that under certain pathological conditions, autophagy can be induced independently of Beclin 1 to promote cell death.[53,54] Beclin 1 may not only be important for the balance of autophagosome formation and maturation, but is positioned between autophagy and apoptosis, making it an important modulator of cell death versus survival.

Atg18/WIPI PI(3)P-Binding Protein and Atg2

In yeast, retrieval of Atg9 from the PAS, which occurs prior to autophagosome formation requires Atg1, Atg2, and Atg18, which recruits Atg2 and Vps34.[34] In fact, the Atg18-Atg2 complex is essential for nucleation.[16]

Atg18 interacts with Atg9 in yeast and recently the mammalian homologues of Atg18, WIPI (WD-repeat protein Interacting with PhosphoInosides) family proteins, have been characterized.[55,56] Atg18 and the WIPI family proteins contain a non-canonical PI(3)P binding region. Among the WIPI family proteins, WIPI2 is the major ubiquitous isoform, is required for autophagy, and forms puncta upon starvation that partially localize with Atg16L1, GFP-LC3 and ULK1, placing it very early in the hierarchy of autophagy proteins.[56] As it does not bind to endosomes, WIPI2 is thought to be a specific effector of the autophagy-specific cellular pool of PI(3)P. Yeast Atg18, like Atg9, complexes with Atg2 but neither of these interactions has been shown with their mammalian counterparts. The mammalian homologue of Atg2 has been annotated but to this date, has not been characterized.

Ubiquitin-like Conjugation Systems

Two ubiquitin-like conjugation reactions produce the Atg5-Atg12-Atg16 complex and lipidated Atg8 and are essential for the formation and elongation of the autophagosome. The proteins required for these two conjugation reactions are critical for life; Atg3, Atg5, Atg7 and Atg16L1 are essential genes and are

important for maintenance of the amino acid pool during the postnatal starvation period.[57] Many detailed studies have resulted in a thorough understanding of how these critical autophagy proteins contribute to the expansion of the double-membrane vesicle.[58]

Atg12 conjugation reaction

In mammalian cells, Atg12, an ubiquitin-like (UBL) protein is conjugated to Atg5 through a multi-step conjugation reaction that requires the enzymatic activities of E1-like and E2-like proteins. First Atg12 is activated by the E1-like enzyme Atg7 and then transferred to the E2-like enzyme Atg10 which then catalyzes the covalent conjugation of Atg5 to Atg12.[59] Subsequently, the Atg5-Atg12 complex is bound to Atg16L1 and this trimeric complex homodimerizes to become a large complex.[60,61] Mammalian Atg5 is an important marker for early autophagosome formation and it localizes to the phagophore.[62] Mammalian Atg16 (Atg16L1) differs from yeast in that it contains a C-terminal WD40 repeat domain for multi-protein complex assembly and, as mentioned above, is recruited to the phagophore.

Atg8/LC3 conjugation reaction

Atg8, the yeast UBL protein that is lipidated and integrated into the autophagosomal membrane, has multiple homologues in mammals grouped into LC3-like and GABARAP-like subgroups. LC3 (microtubule-associated protein 1 light chain 3) is the best characterized Atg8 homologue and was the first to be shown to localize to the autophagosome using EM.[63] To begin the LC3 lipidation reaction, LC3 is primed by the cysteine endopeptidase Atg4 family proteins, exposing a C-terminal glycine,[64] referred to as the LC3-I form. Next, Atg7, which serves as the E1-like enzyme for both conjugation reactions, activates LC3 and then the E2-like enzyme Atg3 facilitates the conjugation of phosphatidylethanolamine (PE) to LC3 to become LC3-II (or LC3-PE).[65] In the same way, GABARAP and GABARAPL2 (also known as GATE16) can be post-translationally processed and bound to PE.[66] Once LC3 is lipidated and becomes part of the autophagosome, it remains membrane-bound and can be followed from phagophore expansion until fusion with and degradation by the lysosome. As a result, LC3 is a bone fide marker of the autophagosome[67] and we can measure LC3's flux through the pathway visually or by Western blot analysis in order to monitor autophagy.[68]

After closure of the autophagosome, Atg4 cleaves LC3 from PE thereby removing LC3 from the mature autophagosome,[69] a step that is critical for its

fusion with endosomes and lysosomes. Experiments with an Atg4 mutant have shown that priming LC3 is critical for the early Atg7-mediated step of the conjugation pathway but the Atg4 mutant also led to defects in the closure of the expanded autophagosome, suggesting that the lipidated, or LC3-II form of the protein is needed for the membrane curvature required to close the vesicle.[61]

Current evidence points to an interplay between the ubiquitin-like conjugation pathways, beyond sharing their E1-like enzyme Atg7. It is generally believed that the Atg5-Atg12 reaction occurs upstream of the LC3 lipidation pathway and Atg16L1 is required for the recruitment of LC3II to the site of autophagosome formation.[61] In addition, some evidence shows that the Atg5-Atg12 complex acts as an E3-like enzyme, conferring specificity to the conjugation of LC3 to PE[70] and conversely, overexpressed Atg3 enhances Atg5-Atg12 conjugation.[65]

SOURCE OF THE AUTOPHAGOSOME MEMBRANE

Although many of the essential proteins and machinery required for autophagosome nucleation and expansion are known (as discussed above), the source of the membrane and the mechanism for phagophore formation are mostly unknown. The origin of the membrane has been in question since the discovery of the double membraned vacuoles and is recently the subject of intense debate and concentrated research. Autophagy scientists strive to determine whether the membrane forms *de novo* from localized lipid synthesis or is derived from a pre-existing organelle or organelles. In yeast autophagosomes always form at the PAS and next to the vacuole. But in mammalian cells, autophagosomes seem to be able to form anywhere in the cytoplasm including the axo-dendritic extensions, and a distinct place of origin, like the PAS, does not exist. Phagophores may form from a PAS-like structure, or the phagophore itself could be the mammalian equivalent of the PAS, but this is not known. Perhaps the difficulty arises from a lack of protein markers, both for the autophagosome and of other membrane sources found in the autophagosomal membrane.[71] Using current methods, it is difficult to differentiate between the autophagosome membrane and the membrane of the organelles it engulfs and the two primary marker proteins, Atg5 and LC3 associate with the phagophore only after it begins to form so earlier, non-transient markers are needed.[72] Multiple recent studies have increased our knowledge of the origin of the autophagosome and point to the ER, mitochondria and even the plasma membrane as possible sources of the membrane.[73–76]

AUTOPHAGY SIGNALING/REGULATION OF AUTOPHAGY

In yeast, autophagy is directly activated by the loss of negative regulation by TOR through amino acid starvation or TOR inhibition with rapamycin.[77] In mammalian cells, the mTOR kinase complex mTORC1 also acts as the key negative regulator of autophagy as well a positive regulator of cell growth. Amino acids stimulate mTOR and promote the phosphorylation of ribosomal S6 protein (S6) by p70S6 kinase and a decrease in phosphorylated S6 reflects an induction of autophagy by starvation or rapamycin.[78] Recently, Rag GTPases and three proteins in a complex called the "Ragulator" have been shown to signal mTOR in response to amino acid starvation and also translocate mTOR to the lysosome membrane.[79] mTOR loss stimulates autophagy by directly affecting ULK1, but adding complexity, ULK1 and ULK2 have been shown to feedback to the mTOR-p70S6 kinase pathway (for review, see Ref. 17). In fact, in *Drosophila* autophagy inhibits cell growth and Atg1 itself can directly inhibit Tor signaling suggesting positive feedback.[80]

Nutrients like amino acids can directly signal mTOR through the Rag-Ragulator complex but a lack of amino acids can also activate autophagy independently from mTOR. For instance, amino acids inhibit the Raf-1-MEK1/2-ERK1/2 signaling cascade that in turn inhibits autophagy.[81] Other mTOR-independent autophagy activation pathways have been reported including that induced by lithium chloride, which appears to act through modulation of myo-inositol-1,4,5 triphosphate (IP_3) levels.[82] Although conflicting data does exist, one group reports that resveratrol also drives autophagy independent of mTOR by activating sirtuins that deacetylate Atg's, which activates autophagy.[83] PKA was also shown to directly modulate autophagy proteins; it suppresses autophagy by directly phosphorylating both LC3 and ULK1 kinase complex proteins.[84]

As previously discussed, 3-MA and also wortmannin inhibit autophagy through the inhibition of the class III PI3-K product PI(3)P; but 3-MA also inhibits class I PI3-K which is a negative regulator of autophagy through the Akt-TSC1/2-Rheb-mTOR signaling pathway.[85] Growth factor (insulin) signaling inhibits autophagy through stimulation of IRS1 and IRS2, which activate the class I PI3-kinase. Alternately, overexpression of PTEN stimulates autophagy by inhibiting the inhibitory Akt pathway.[86] Interestingly, a recent study has shown an unexpected role of class IA PI3K catalytic p110-β in positively regulating autophagy, adding to the complexity of autophagy control by the PI3K family.[87]

The anti-apoptotic protein Bcl-2 inhibits autophagy by sequestering Beclin 1 away from Vps34 and it was recently demonstrated that starvation removes Bcl-2, through direct phosphorylation by JNK1, from the class III PI3-kinase complex 1.[88]

In addition, energy inhibits LKB1, a positive regulator of autophagy that activates AMPK, and this pathway is activated in stress conditions;[89] AMPK itself senses a reduced ATP:AMP ratio and is reported to directly phosphorylate ULK1.[29–31] Autophagy is also influenced by p53, a master regulator of cell cycle progression and apoptosis, in opposing ways. Basal levels of the cytoplasmic form of the protein inhibit autophagy and its degradation leads to the induction of autophagy;[90] but in times of cellular stress, p53 can activate both autophagy and apoptosis.[91]

THE ROLE OF LC3-BINDING PROTEINS IN AUTOPHAGY

p62, also known as sequestosome 1 (SQSTM1) is a multi-functional adaptor protein that binds polyubiquitinated proteins and aggregates by oligomerization.[92] Though p62 is not required for starvation-induced autophagy, a body of evidence shows that it is a specific substrate for the process and its autophagy-induced degradation can be used as a read-out for autophagy function.[93–95] In addition to binding ubiquitin (Ub), it also binds to LC3, perhaps recruiting the ubiquitinated proteins to the autophagosome.[96,97] Loss of autophagy resulted in accumulation of ubiquitinated proteins and the formation of ubiquitinated inclusion relies on the presence of p62, suggesting a role for p62 in assembly of ubiquitinated proteins into aggregates.[98,99] p62 has recently been demonstrated to translocate into the nucleus where it co-localizes with promyelocytic leukemia (PML) bodies. The nucleoplasm to cytoplasm shuffling of p62, which is possibly controlled by phosphorylation events, may serve as a regulatory mechanism for its role in autophagy.[100] Though the previous view was that the UPS and autophagy act in a mutually exclusive way, recent work has shown that the two pathways are linked through their common modulator, p62/SQSTM1.[101]

Another LC3- and Ub-binding protein, neighbor of BRCA1 gene 1 (NBR1), also serves as autophagic receptor for ubiquitinated targets.[102] Like p62, NBR1 contains an LC3-interacting region (LIR) and this motif is required for NBR1's degradation by autophagy. It seems that NBR1 cooperates with p62 to direct autophagic degradation of ubiquitinated targets and both can serve as markers of pathological inclusions. The clearance of these inclusions is an important homeostatic function that autophagy has gained in higher organisms, although the exact mechanism of how autophagy selectively degrades these substrates is not known.

AUTOPHAGY IN NEURONS

Autophagy is critical for the maintenance of homeostasis for intracellular proteins and cellular organelles to ensure their proper functions as demonstrated in recent

studies with autophagy-deficient mouse models. Suppression of basal autophagy through the genetic deletion of *ATG5* or *ATG7* specifically in neural tissue causes neurodegeneration in mice.[103,104] The mice experience motor and reflex deficits but perhaps most striking pathology is the ubiquitin-positive inclusion bodies that accumulate in the neurons. Atg7 itself is essential for the maintenance of axonal homeostasis; without it, intracellular proteins and aberrant membrane structures accumulate to toxic levels and axons degenerate.[105] Neurons are especially sensitive to the accumulation of damaged or aggregated cytosolic proteins or membranes because they are extremely polarized, large cells; constitutive basal autophagy thus serves an important protective role in neurons.[7]

Multiple studies have suggested that the levels of lipidated LC3 and the numbers of autophagosomes detected in neurons are relatively low in normal as well as nutrient withdrawal conditions.[106,107] One hypothesis is that autophagosomes are formed normally, but there is an extremely high rate of turnover of autophagosomes due to efficient autophagy-lysosomal degradation in neurons.[107,108] An alternate hypothesis, however, is that autophagosome synthesis is tightly controlled in neurons; the constantly low rate of formation of typical autophagosomes may be sufficient to maintain the homeostatic balance. Perhaps neurons are prevented from undergoing extensive autophagy induction in response to starvation because this could lead to loss of mass and cells, as neurons cannot be regenerated. When an organism encounters a period of nutrient deprivation the brain is typically shielded by peripheral production of nutrients through autophagy, insuring that they can maintain essential normal function regardless of such conditions. Future studies of the unique regulation of autophagy in neurons should examine these possibilities.

Compartmentalized Autophagy in Neurons

The degree of functional subcompartmentalization (soma, axons and dendrites) of the neuron distinguishes it from other cell types (Figure 2). Accordingly, autophagy may be adapted to different neuronal functions in different subcompartments. It is known that an intact microtubule network is required for the formation, maturation and degradation of autophagosomes in mitotic cells[109] and that autophagosomes traffic towards the centrosome, where most lysosomes reside.[110] Similarly in neurons, the retrograde trafficking of autophagosomes from axons or neurites towards the cell body has been observed.[111] Recently it was shown that though autophagosomes and autolysosomes travel in both anterograde and retrograde directions (using kinesin and dynein, respectively), there is bias of traffic towards the nucleus of PC12 neurites during starvation.[112] In Alzheimer's

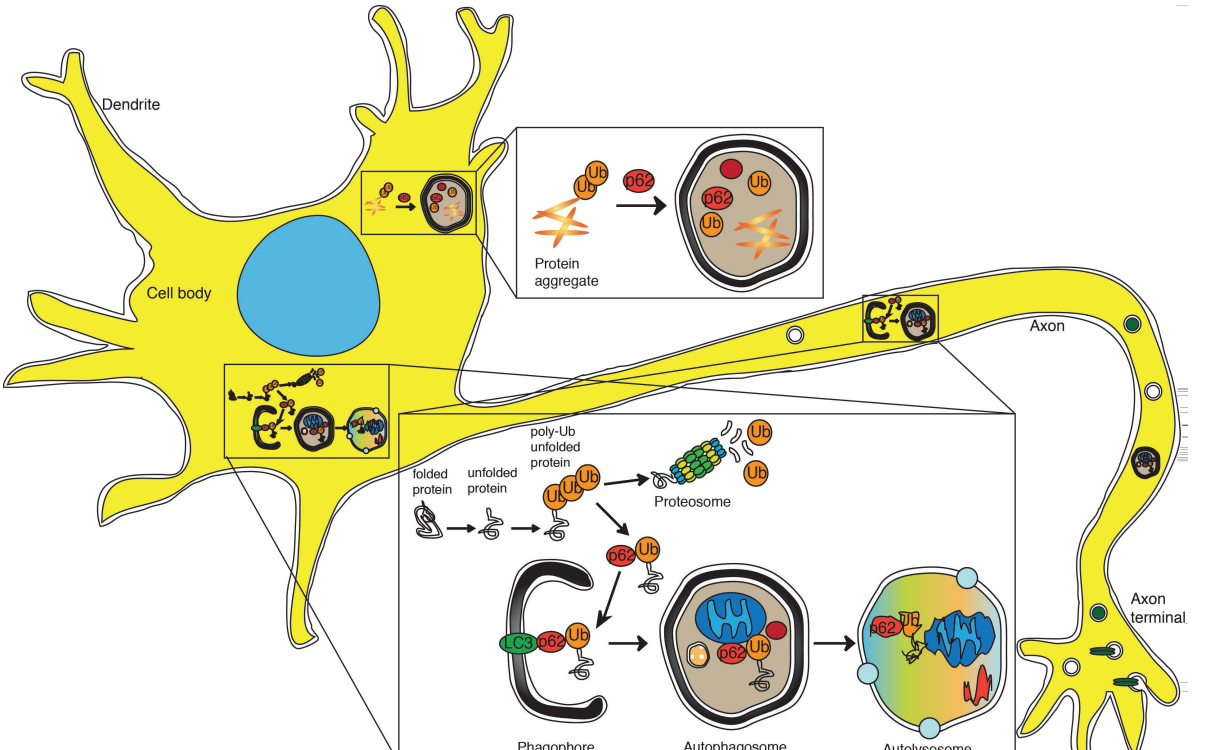

Figure 2. Autophagy in neurons. Membrane trafficking directs vesicles including endosomes and autophagosomes from the axons or the dendrites to the cell body. Ubiquitinated (Ub) proteins are either degraded by the proteasome or carried by the LC3- and Ub-binding protein p62 to the phagophore. Growth factors and neurotrophins bind to receptors at the axon terminal and are endocytosed. Adapted from Tooze and Schiavo.[116]

brains, undigested autophagosomes in dystrophic neurites are unable to travel to the lysosomes for degradation and accumulate, a disease hallmark further exacerbated by impaired lysosomal proteolysis.[113] Motor proteins such as dynein were shown to participate in autophagosome trafficking in axons[111,113] and another protein that may be related to autophagosome trafficking in the axon is MAP1B, a microtubule- and LC3-binding protein.[93] There is increased autophagosome production in the axon after injury,[114] suggesting that autophagosome can be synthesized locally in axon terminals. Perhaps autophagy is also important during axonal outgrowth and development. MAP1B is implicated in the autophagy that participates in the axon remodeling that takes place after excitotoxic stress or neurodegeneration.[93] Axonal or neuritic autophagy is further discussed in Chapters 16 and 17.

Autophagy Signaling in Neurons

Autophagy is a constitutive, basal process in the central nervous system (CNS). Autophagy is activated without starvation signals in the brain,[115] suggesting that it is not amino acid sensitive (and also suggesting tissue-specific functions for autophagy in organisms). Several lines of evidence suggest that neurotrophin receptor signaling and responses are connected to the regulation of autophagy.[116] Reduced levels of ULK1/2 causes impaired endocytosis of nerve growth factor (NGF), excessive axon arborization, and retarded axon elongation, suggesting that the autophagy machinery plays a role in controlling NGF/TrkA levels and signaling.[117] The investigations as to whether autophagy signaling in neurons requires mTOR have yielded somewhat mixed results. Some evidence points to an mTOR-independent mechanism[118–120] and in fact, a small molecule screen for inducers of autophagy in neuroblastoma cells found compounds that induced autophagy through distinct mechanism from rapamycin.[121] Recently, an siRNA screen searching for inducers of basal autophagy in neuroblastoma cells suggested that the Vps34 kinase complex but not mTORC1 regulates autophagy.[122] However, other evidence has been provided that mTOR inhibition by rapamycin does indeed induce autophagic flux in neurons.[123–125] Furthermore, an interesting study searching for autophagy regulators in cell culture medium showed that insulin withdrawal triggers the autophagy process[126] (and the authors suggest these effects are mTOR-dependent). Taken together, these data suggest that there are parallel signaling pathways that regulate neuronal autophagy with distinct mechanisms.

Despite the observations noted above regarding autophagy signaling in neurons, it remains unknown whether or not similar results occur in the intact brain *in vivo*.

It has been shown that neuronal autophagy can be induced by hypoxic-ischemia, overexcited glutamate receptors (caused by excitotoxic stimuli such as NMDA and kainic acid), methamphetamine, proteasome inhibition, and secondary to alterations in lysosomal enzyme activity or lipid storage (for review see Yue *et al*.[7]). There is evidence that Akt signaling negatively regulates autophagy in various cell types, but very recently the Akt/Rheb/mTor signaling pathways were shown to regulate acute retrograde axon degeneration which is achieved not by programmed cell death but by autophagy.[127] New research adds other signaling pathways including IGF-1,[128] Cdk5/endophilin B1(Bif-1),[129] JNK/FoxO,[130] and mutant LRRK2[84] to the list of autophagy inducers in pathophysiological conditions.

Autophagic Clearance of Protein Aggregates in Neurons

The studies of *ATG5* and *ATG7* conditional knockout brains reveal a critical role for autophagy in the suppression of spontaneous protein aggregation.[103,104] They also suggest that basal autophagy in neurons is a protein quality control pathway for continuous turnover of misfolded and/or unfolded proteins. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice[99] and proper turnover of p62 by autophagy is critical to prevent spontaneous aggregate formation, which is directly mediated by p62 itself.[99] Furthermore, if p62 accumulates, through inhibition of autophagy, proteasomal function is hindered.[101] Autophagy is likely an alternative degradation pathway for ubiquitinated proteins and supports the UPS to clear potential aggregating proteins when the UPS is overloaded[131] (Figure 2). There are at least two main hypotheses that explain how autophagy protects against neurodegeneration: (1) the autophagy machinery specifically recognizes and eliminates protein aggregates and inclusions, or (2) inclusion bodies that form after impaired protein turnover are degraded by autophagy through an unspecific, secondary mechanism. Supporting the latter, it is suggested that the generation of inclusions and aggresomes of mutant proteins is actually a cytoprotective mechanism.[132,133] Yet most recent studies, especially those involving p62 and other ubiquitin- and LC3-binding proteins suggest that these function as adaptors to specifically identify damaged proteins and direct the degradation of aggregates, almost all of which are tagged with ubiquitin.[98,134]

Aggrephagy, or the specific autophagocytosis of protein aggregates or inclusion, is an example of a selective form of autophagy.[135] Alfy, a FYVE domain-containing protein associated with autophagy, interacts with PI(3)P, p62, and Atg5, and recently has emerged as a conductor of aggrephagy.[134] Therefore, autophagy can be selective, and its selectivity is mediated by adaptor-like proteins

such as p62 and Alfy. Protein inclusions in both liver and brain of Atg7 KO mice disappear in Atg7/p62 double KO mice.[99] Whereas the liver damage was dramatically improved, neurological dysfunction was not improved even though inclusion bodies were not generated. This study suggests that prevention/elimination of inclusions is not a major role of autophagy in the brain, and that autophagy may have more general roles in protein/organelle turnover in the brain. Histone deacetylase 6 (HDAC6), which is associated with microtubules and regulates a variety of cellular functions, may also participate in autophagic degradation of polyubiquitinated proteins[136,137] and promotes the clearance of polyQ containing protein aggregates.[136]

Regulation of Neuronal Autophagy by Endosomal and ESCRT Machinery

ESCRT complexes I–III are required for multi-vesicular body (MVB) formation and growth factor signaling in neurons.[116] The fusion of autophagosomes with MVBs creates amphisomes, which likely represent an important route for the degradation of the contents of both original organelles. It was demonstrated that subunits of these complexes, including CHMP2B, plus some regulatory proteins are required for autophagy, and specifically the maturation and delivery of autophagosomes to the lysosome.[138,139] Interestingly, mutations in CHMP2B are implicated in both frontotemporal dementia (FTD)[140] and amyotropic lateral sclerosis (ALS).[141] Though intact ESCRT complexes are required for dendritic development and maintenance, the pathology of these mutations is due to dysfunctional autophagosome degradation.[142]

It seems that neurons are especially vulnerable to aberrant ESCRT function. The emerging evidence strongly suggests the convergence of the endocytic/MVB and autophagic pathways in neurons.[143] The merge may provide an additional layer of control or an alternative pathway for autophagosome turnover in higher eukaryotes; thus, the ESCRT-mediated route may be a critical one in neurons. ESCRT-deficient neurons phenocopy the *ATG5* and *ATG7* deleted neurons, suggesting that ESCRTs/MVBs may be indispensible for basal autophagy. Moreover, functional MVBs are required for the clearance of the disease-related protein TDP-43 and polyQ-containing proteins.[139] In fact, the accumulation of ubiquitin- and p62-labeled inclusions and non-digested autophagosomes in ESCRT-deficient neurons suggests that ESCRT is not only important for homeostasis, but also for selective autophagy in the brain. Finally, membrane dynamics of ESCRT-mediated multi-vesicular body formation is similar to microautophagy, and in fact, a recent paper showed that endosomal microautophagy degrades cytosolic proteins in a somewhat selective manner.[144]

Neuroprotective versus Neurotoxic Role of Autophagy

While most evidence points to autophagy as a protective process in neurons, there is some intriguing evidence to suggest that autophagy is responsible for cell death in the brain. Increased autophagosome number has been observed during hippocampal neuron death and in fact, Atg7 is critical for neuronal cell death after hypoxic/ischemic brain injury.[145] In another model of neuronal cell loss, FTD, inhibition of autophagy actually delays the neurodegeneration progression.[146] Also, loss of the critical autophagy protein Atg7 increases resistance to retrograde degeneration in axons after toxin injury.[127] A consensus in the literature is emerging that the axon, which in part incorporates autophagic cell death, dies differently than the soma, which uses apoptotic cell death.[147] The paradox of autophagy also extends to cancer: (1) autophagy has tumor suppressor properties and helps to keep the cell alive, (2) yet, it is a form of apoptosis-independent cell death. Also, growing tumors can hijack the process and live off of autophagic recycling until angiogenesis occurs. Perhaps basal autophagy in the brain is essential for neuronal homeostasis, but in the case of acute neural injuries that induce massive expansion of autophagy-lysosomal degradation, it becomes harmful and responsible for degeneration, especially in axodendritic projections. The context is therefore very important when considering the application of drugs to increase autophagy in the brain.

AUTOPHAGY IN NEURODEGENERATIVE DISEASES

Basal autophagy serves a neuroprotective function by maintaining neural homeostasis under healthy conditions; but perhaps the more impressive role of autophagy is its ability to degrade and clear disease-related mutant proteins. A body of evidence has increasingly shown that autophagy is primarily responsible for the turnover of aggregate-prone proteins or macromolecular complexes. The examples include polyQ-containing proteins that cause neurodegenerative diseases such as Huntington's disease (huntingtin),[148] spinocerebellar ataxia (SCA1),[149] and spinobulbar muscular atrophy (androgen receptor).[137] In addition, disease-linked mutants of alpha-synuclein in Parkinson's disease, mutant SOD1 in ALS, and tau in Alzheimer's disease and other tauopathies were also shown to be autophagy substrates.[106,150–152] An intact, efficient autophagy system is undoubtedly important and beneficial for the neurons to prevent the build-up of these disease proteins.

Autophagy in mammals is highly evolved and has become a tightly regulated process; but when autophagy is disrupted or misregulated multiple diseases can

occur.[153] The evidence for the disturbance of various autophagy pathways by disease proteins has been presented. For example, alpha-synuclein (mutant or wildtype) is linked to impaired autophagy[150] and CMA[154] separately; Parkin and PINK1 mutants were shown to inhibit autophagy of mitochondria (mitophagy);[155] PolyQ-Htt may take part in compromising the efficiency of autophagy.[156] The most remarkable evidence was provided by Nixon's group in their study of Alzheimer's disease (AD)-associated mutations and their effects on multiple stages of autophagy. In particular, the impairment of acidification and substrate proteolysis within autolysosomes is a potential mechanism underlying autophagy failure in AD.[157] In the most common form of familial onset AD, mutant presenilin 1 blocks autophagy by impeding lysosomal proteolysis.

CONCLUSION

Despite the recent surge of publications delving into the molecular mechanisms of autophagic function, there are many remaining questions. Of critical importance is to determine how and where the phagophore (isolation membrane) and thus the autophagosome forms in mammalian cells. What other regulatory proteins are required to act at this site and how might this differ in various tissues and cell types, particularly neurons? In addition, it is imperative to understand how endocytic, MVB/ESCRT, and lysosomal acidification pathways participate in and are required for autophagy completion in neurons. Autophagy is critical for maintaining proper cellular function, especially in differentiated, specialized neurons that are especially vulnerable to aggregated protein accumulation. In turn, disrupted autophagy is implicated in many neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's (discussed in detail in chapters to follow). Striving to uncover the mysteries of the process, autophagy scientists will find new drug targets that may provide the framework for therapies for multiple conditions including aging, cancer and neurodegeneration.

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