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The Journey of the Synaptic Autophagosome: A Cell Biological Perspective

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Autophagy is a key cellular degradative pathway, important for neuronal homeostasis and function. Disruption of autophagy is associated with neuronal dysfunction and neurodegeneration. Autophagy is compartmentalized in neurons, with specific stages of the pathway occurring in distinct subcellular compartments. Coordination of these stages drives progression of autophagy and enables clearance of substrates. Yet, we are only now learning how these distributed processes are integrated across the neuron. In this review, we focus on the cell biological course of autophagy in neurons, from biogenesis at the synapse to degradation in the soma. We describe how the steps of autophagy are distributed across neuronal subcellular compartments, how local machinery regulates autophagy, and the impact of coordinated regulation on neuronal physiology and disease. We also discuss how recent advances in our understanding of neuronal autophagic mechanisms have reframed how we think about the role of local regulation of autophagy in all tissues.

A Brief Overview of Autophagy in Neurons

Macroautophagy, hereafter autophagy, is a cellular degradative pathway, important for development and for maintenance of cellular homeostasis. In neurons, autophagy has been implicated in development, physiology, and aging (Azarnia Tehran et al., 2018; Kulkarni et al., 2018; Liang and Sigrist, 2018; Lüningschrör and Sendtner, 2018; Menzies et al., 2017; Stavoe and Holzbaur, 2019; Vijayan and Verstreken, 2017). While most of the studies examining synaptic autophagy have focused on autophagosomes in axons, autophagy has also been observed to occur in the neuronal soma and the dendrites. In this review, we discuss how the cell biology of the neuron impacts the journey of the synaptic autophagosome, from biogenesis to breakdown. We begin with a brief synopsis of the autophagy pathway, focused on its importance in neuronal health and disease and how its regulation is uniquely adapted in neurons to meet their needs.

Autophagy is essential for neuronal physiology and survival. Neurons rely on autophagy to efficiently remove cellular debris and toxic materials, with imbalances leading to neuron death. Neuron-specific depletion of autophagy in mice results in axon degeneration, accumulation of ubiquitin-containing protein aggregates, and neuronal cell death (Hara et al., 2006; Komatsu et al., 2006, 2007). These findings underscore the importance of autophagy for neuronal physiology and function.

Autophagy has also been implicated in human neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and amyotrophic lateral sclerosis. For example, neurons from Alzheimer's and Parkinson's disease patients show an abnormal accumulation of autophagosomes in distal neuronal processes and at synaptic terminals (Gowrishankar et al., 2015; Nixon et al., 2005; Tammineni et al., 2017; Yue et al., 2009). Emerging evidence supports that loss of lysosomal function may act as a primary disease mechanism contributing to neuronal death (Wallings et al., 2019). Autophagic and lysosomal markers have been proposed as biomarkers for disease detection (Mputhia et al., 2019), and a growing number of pharmacological agents seek to modulate the autophagy pathway as a therapeutic intervention for neurodegenerative diseases associated with autophagy (Malik et al., 2019). But while much evidence supports the idea that defects in autophagy contribute to neurodegenerative diseases, the pathogenic mechanisms that directly link the steps of autophagy to disease outcomes are not fully understood. For instance, excessive autophagy can contribute to neuronal stress, but loss of degradative activity can also prevent the removal of toxic substrates, affect neuronal physiology, and contribute to disease (Malik et al., 2019). Moreover, in neurons, autophagic organelle biogenesis, transport, and degradation occur in varied subcellular compartments, and these local environments impact neuronal autophagy. In this review, we describe our current understanding of the cell biology of autophagy in axons, focusing on biogenesis events at presynaptic sites, trafficking along axons, fusion with late endosomes/lysosomes for degradation, and the orchestrated regulation of these processes across subcellular compartments during neuronal autophagy. Understanding how autophagic cargo engulfment coordinates with lysosomal degradation across the structure of the neuron will be important to link the mechanisms of autophagy with neurodegenerative diseases during autophagy dysfunction.

Most of our understanding of autophagy has come from studies conducted in non-neuronal cells. Autophagy was first discovered in yeast as a mechanism to support biosynthesis under nutrient deprivation by degrading and reusing cellular

materials (Mizushima and Komatsu, 2011; Tsukada and Ohsumi, 1993; Wen and Klionsky, 2016). First, double-membrane structures, called autophagosomes, form around cellular cargoes such as aged organelles or proteins and then fuse with proteolytic late endosomes or lysosomes to mediate degradation. Autophagy is regulated by a series of protein complexes, which include 30+ proteins involved in processes from biogenesis of autophagosomes and cargo recognition to transport and degradation.

The core enzymatic processes of autophagy are evolutionarily conserved and are necessary for autophagy in neurons. However, the signals inducing autophagy, the physiological roles for autophagy, and autophagy's subcellular distribution in neurons are distinct. Processes associated with neuronal function, such as synaptic transmission, are linked to the regulation of autophagy in neurons (Hernandez et al., 2012; Shehata et al., 2012; Soukup et al., 2016; Wang et al., 2015). Neuronal autophagy has been linked to and shown to influence processes like neurotransmitter receptor turnover (Rowland et al., 2006), synaptic development (Shen and Ganetzky, 2009; Stavoe et al., 2016), synaptic pruning (Tang et al., 2014), and synaptic plasticity (Glatigny et al., 2019; Nikoletopoulou et al., 2017), among other processes essential for neuronal physiology.

The steps of autophagy are modified to fit the context of the polarized neuron and the substrates within the neuron being targeted for degradation. For example, one of the most striking aspects of neuronal autophagy is its spatial organization. Autophagosomes form in distal axonal compartments near synapses and undergo retrograde transport. During transport, they fuse with late endosomes and lysosomes before their cargo is degraded in the cell body (Bunge, 1973; Katsumata et al., 2010; Lee et al., 2011; Maday et al., 2012; Ravikumar et al., 2005; Soukup et al., 2016; Stavoe et al., 2016). This spatial specificity prompts a number of questions about the regulation of autophagy in neurons. What signals instruct autophagosome biogenesis at the synapse? How are different autophagic steps distributed and coordinated within distinct subcellular compartments of the neuron? How do the specialized environments of those compartments contribute to the regulation of autophagy?

In addition to autophagy's key roles in neuronal physiology, the spatial separation of the steps of autophagy in neurons affords the autophagy field an opportunity to rigorously examine the compartmentalized events of autophagosome biogenesis and degradation with a greater resolution than in non-neuronal cells. Concepts emerging from neurons regarding how cells compartmentalize and coordinate the different steps of autophagy across time and space will likely illuminate our understanding of the regulation of autophagy in other cell types.

Autophagosome Biogenesis at Presynaptic Sites

The first evidence for compartmentalized activity of autophagosomes came from electron micrographs of neurons which revealed the presence of double-membrane structures (which were later termed autophagosomes) in growing axon terminals (Bunge, 1973). This study described cup-like isolation membranes in axons, consistent with biogenesis of autophagosomes. It also described fully formed, closed, double-membrane autophagosomes and electron-dense cargo-containing multilamellar structures that reflect autolysosomes arising from autophagosome and lysosome fusion in axons. While the electron microscopy data could not reveal the progression of individual structures over time, these studies did provide evidence that distinct steps of autophagy, ranging from biogenesis to autolysosome formation, occur in axon terminals.

More recent studies have made use of translational fusions with fluorescent proteins to examine the dynamic progression of autophagy in neurons. The preferred markers for autophagosomes include yeast Atg8 and its orthologs, such as the LC3 and GABARAP families in mammals and zebrafish, Atg8 in *Drosophila melanogaster*, and LGG-1 and LGG-2 in *C. elegans* (Klionsky et al., 2012; Meléndez et al., 2003; Zhang et al., 2015). Atg8 orthologs are ubiquitin-like proteins that are anchored to autophagic membranes via a covalent bond between the last glycine in Atg8 and a phosphatidylethanolamine (PE) phospholipid in the autophagosome membrane. Since Atg8 orthologs localize to immature and mature autophagic structures, tracking Atg8 family proteins enables *in vivo* tracking of autophagosome biogenesis, transport, and maturation. Soukup et al. (2016) bridged these strategies by examining presynaptic *Drosophila* neuromuscular junctions using correlative light and electron microscopy (CLEM). They also demonstrated that Atg8-containing structures correspond to autophagosomes forming near presynaptic sites (Soukup et al., 2016). While autophagosomes can form in neuronal cell bodies (Lee et al., 2011; Maday and Holzbaur, 2016), autophagosome formation in axons is independent from cell body input and occurs even in axons that have been severed from their cell bodies (Hernandez et al., 2012; Soukup et al., 2016). We note that most of the studies on the dynamics of autophagy in neurons have been performed in invertebrate organisms or in cultured neuron systems, in which synapses could arguably be more prone to autophagy-dependent remodeling. It will be important to establish how the observed cell biology of autophagy in these systems compares to that of intact myelinated or aged brains of mammals.

But together, these studies in cultured neurons and intact invertebrate systems demonstrate that axonal autophagosomes do not necessarily arise from autophagosomes formed in the cell body and trafficked into the axon and indicate that autophagosome biogenesis occurs in the axon. These studies demonstrate that autophagosome biogenesis is compartmentalized in neurons, occurring at axonal terminals and near presynaptic compartments (Maday et al., 2012; Soukup et al., 2016; Stavoe et al., 2016).

Signals Inducing Neuronal Autophagy

In non-neuronal cell types, starvation is a major trigger for autophagy, inducing non-specific degradation of cellular materials to provide nutrients for metabolic processes. In neurons, starvation and starvation-related pathways can also induce autophagy. For example, nutrient deprivation in primary cortical neurons leads to increased autophagy (Young et al., 2009). mTOR, a canonical regulator of starvation-induced autophagy in non-neuronal cells, can also induce autophagy in neurons. mTOR is a kinase that is activated during growth and suppressed during starvation to promote autophagy (Yang and Klionsky, 2010). In mouse brains, short-term fasting led to decreased mTOR levels and increased numbers of autophagosomes in neurons (Alirezaei et al., 2010).

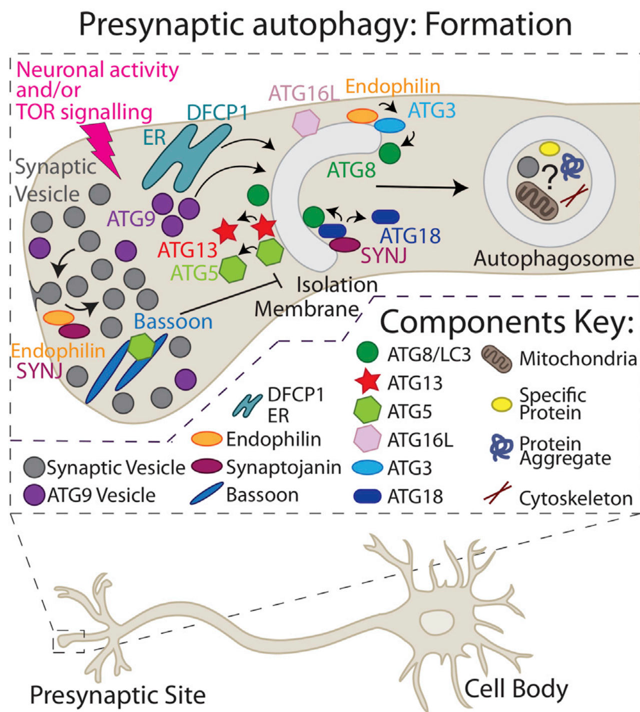

Figure 1. Autophagosome Formation at the Presynaptic Site Uses Repurposed Synaptic Machinery

Synaptic autophagosome biogenesis can be initiated by starvation-induced TOR signaling or by neuronal activity. Synaptic autophagosomes can engulf substrates either non-selectively or selectively including mitochondria, synaptic vesicles, disease-related protein aggregates, cytoskeletal elements, and specific proteins.

Autophagic membranes at the synapse can arise from a DFCP1-enriched portion of the endoplasmic reticulum. Synaptically localized ATG9 vesicles also facilitate autophagic membrane nucleation. ATG13 and ATG5 transiently localize to the autophagosome before dissociating, while ATG8/LC3 persists on the membrane. ATG16L also colocalizes with ATG8/LC3 puncta at the synapse. The active zone protein Bassoon can inhibit synaptic autophagy, potentially by sequestering ATG5. The endocytosis protein Endophilin A localizes to curved membranes and recruits ATG3, which is part of the lipidation machinery that attaches ATG8/LC3 to the autophagic membrane. Another endocytosis protein, Synaptojanin (SYNJ), facilitates the dissociation of ATG18, which can also recruit ATG8/LC3 but must be removed for autophagic progression. The use of synaptic machinery to regulate local autophagy provides an opportunity for crosstalk and may promote co-regulation of neuronal activity and synaptic autophagy.

In dopaminergic axons from striatal brain slices, inhibition of mTOR by the drug Rapamycin resulted in an increased number of autophagosomes near synaptic terminals, as well as a decrease in synaptic vesicle numbers (Hernandez et al., 2012). Rapamycin-induced autophagy also regulates synaptic growth (Shen and Ganetzky, 2009) and axon elongation in cortical neurons (Ban et al., 2013), consistent with a role for mTOR in starvation-mediated induction of autophagy and autophagic regulation of physiological processes in neurons.

It is important to note that while starvation can induce autophagy in neurons, autophagosome biogenesis can also occur in neurons in a constitutive manner. In cultured neurons, autophagosomes are observed to constitutively form in the distal neurite even in the absence of stimuli such as starvation (Maday and Holzbaur, 2016; Maday et al., 2012). In *Drosophila* neurons, basal levels of autophagy also occur regardless of starvation but neuronal autophagy increases upon starvation (Soukup et al., 2016). In *C. elegans* neurons, autophagy occurs at a basal level but increases following stimuli known to trigger autophagy in non-neuronal cells, such as starvation or exposure to noxious temperatures (Hill et al., 2019 and unpublished data). Together, these studies indicate that while stimuli known to trigger autophagy in non-neuronal cells can enhance autophagy in neurons, likely there are other pathways that regulate the observed basal levels of neuronal autophagy.

Neuronal activity impacts the levels of neuronal autophagy. Applying the glutamate analog *N*-methyl-*D*-aspartic acid (NMDA), an excitotoxin, increased autophagosome biogenesis (as revealed by increases in autophagy under Bafilomycin A conditions where degradation is inhibited) and increased the numbers of autophagosomes in axons (Katsumata et al., 2010). Consistent with these findings, exposing tissue culture neurons to media with high levels of potassium chloride (which facilitates neuronal depolarization) caused an increase in autophagosomes at nerve terminals (Shehata et al., 2012; Wang et al., 2015). In *Drosophila*, prolonged neuronal activity induced by activating a temperature-sensitive TrpA1 channel or through bouts of direct electrical nerve stimulation also increased autophagosome biogenesis at the neuromuscular junction (Soukup et al., 2016). *In vivo* studies in *C. elegans* examining autophagosome biogenesis in single neurons in response to physiological stimuli demonstrated that the number of synaptic autophagosomes predictably changes based on the firing state of the neuron. Firing state was manipulated by altering the physiological stimuli that promote neuronal responses, by genetically inhibiting synaptic transmission, or by chemo-genetically altering the response state of the neuron (Hill et al., 2019). We note that while general increases in autophagosome number can result from either enhanced biogenesis or defective degradation, the local increases of autophagosomes seen at synapses, combined with transport studies (see next section), suggest that firing states in neurons promote biogenesis of autophagosomes at synapses.

Interestingly, while single physiological action potentials may induce synaptic processes like synaptic vesicle cycling, they appear to be insufficient to alter levels of autophagy in neurons. Instead, the discussed studies have consistently observed that *prolonged* neuronal stimulation correlates with increased neuronal autophagy, suggesting a link between the firing *state* of neurons and the level of autophagosome biogenesis. While direct mechanistic links between the synaptic vesicle cycle and autophagy are not known, it is possible that second messenger signals, such as calcium, or shared molecular machinery (as discussed below) could help coordinate these two pathways.

Roles for the Synaptic Machinery in Autophagy

Increasing evidence indicates that the canonical machinery involved in synaptic transmission and function, including endocytosis and active zone proteins, are also necessary for instructing autophagy at presynaptic sites. For example, studies in *Drosophila* have demonstrated that components of endocytosis also facilitate synaptic autophagy (Figure 1). Endophilin A, a protein mainly known for its role in endocytosis, directly regulates

autophagosome formation by inducing curved membranes that can recruit autophagic machinery like ATG3. Endophilin A requires LRRK2, a kinase associated with Parkinson's disease, to activate synaptic autophagy (Soukup et al., 2016).

Intriguingly, the phosphatase Synaptojanin, another endocytosis gene associated with Parkinson's disease, plays conserved roles in regulating autophagy in zebrafish and *Drosophila*. Synaptojanin contains two phosphatase domains: a central inositol 5′ phosphatase domain and an N-terminal Sac1 domain that can dephosphorylate other inositol substrates (Guo et al., 1999). In zebrafish, Synaptojanin regulates autophagy in photoreceptor neurons via its 5′ phosphatase domain, but not its Sac1 domain (George et al., 2016). In *Drosophila,* Synaptojanin regulates synaptic autophagy in neuromuscular junctions through its Sac1 domain, which eliminates Phosphatidylinositol 3-phosphate (PI(3)P) from immature autophagosomes, resulting in loss of the PI(3)P-binding-protein Atg18a/WIPI2 (Vanhauwaert et al., 2017). *Drosophila* Synaptojanin Sac1 mutants display a reduction in numbers of Atg8-containing puncta (a marker of autophagosomes) and an accumulation of Atg18a/WIPI2 puncta at synapses. The difference between *Drosophila* and zebrafish studies might be due to specific roles for the different domains of Synaptojanin at different stages of autophagy. For example, studies of the zebrafish Synaptojanin 5′ phosphatase domain mutants demonstrate an increase in non-acidified LC3/ATG8 puncta (consistent with a defect in autophagosome maturation), while the studies in *Drosophila* Synaptojanin Sac1 mutants display a loss of ATG8 puncta (consistent with a defect in autophagosome biogenesis) (George et al., 2016; Vanhauwaert et al., 2017). Importantly, both studies indicate Synaptojanin contributes to autophagy regulation near neuronal synapses. Of note, the roles of Endophilin A and Synaptojanin in synaptic autophagy are genetically separable from their roles in endocytosis (Soukup et al., 2016; Vanhauwaert et al., 2017). The use of common molecular machinery between endocytosis and synaptic autophagy suggests a mechanism used by the synapse to both monitor and then respond to changes in synaptic activity by repurposing of these canonical endocytic molecules.

The active zone protein Bassoon can also negatively regulate synaptic autophagy (Okerlund et al., 2017). It has been proposed that Bassoon inhibits synaptic autophagy by binding to autophagy protein ATG5 and sequestering it, thereby preventing autophagosome formation. Bassoon mutants show an increase in the constitutive formation of autophagosomes in axon tips (Okerlund et al., 2017). The dual roles of Endophilin A, Synaptojanin, and Bassoon in synaptic activity and autophagy place them in an ideal position to provide regulation for activity-dependent synaptic autophagy.

From these studies, we learn that synaptic machinery can genetically interact with the autophagy machinery at the synapse. While proximity might be one reason for synaptic proteins to perform specialized regulation of synaptic autophagy, there may be another purpose. Autophagosomes may act as signaling endosomes between the axon terminal and cell body. This has been observed in studies where autophagosomes transport brain-derived neurotrophic factor (BDNF)-activated TrkB receptors to the soma to prevent neurodegeneration (Kononenko et al., 2017).

These studies also suggest a link between the synaptic vesicle cycle (which is tied to synaptic activity) and autophagy. Neurons fire at varied rates, and the firing rates exert different amounts of stress on the synaptic machinery and demand for protein turnover. It is interesting to speculate that synaptic machinery may relay the extent of neuronal activity to the local autophagy machinery in order to fine-tune the amount of synaptic autophagy needed at any given time to degrade the worn-out synaptic machinery in neurons.

Cargoes of Axonal Autophagy

Local formation of axonal autophagosomes results in the degradation of axonal cargoes. Early studies examining retrograde moving organelles in axons during neuronal outgrowth detected that autophagosomes form near growth cones and contain cytoskeletal elements, including tubulin and neurofilaments (Hollenbeck, 1993; Hollenbeck and Bray, 1987). Consistent with a role for autophagy in degrading cytoskeletal elements present in growth cones, studies in cortical neurons demonstrate that autophagy negatively regulates levels of hnRNP-Q1, a RhoA regulator, to control early axon elongation (Ban et al., 2013). These studies reveal that autophagy can degrade local components of the cytoskeleton to regulate growth cone dynamics.

In addition to cytoskeletal cargoes, autophagy can also degrade synaptic material. Electron microscopy studies of neuronal autophagosomes show synaptic-vesicle-like cargo inside autophagosomes (Hernandez et al., 2012). Autophagosomes can also enclose synaptic vesicles, as shown in vertebrate hippocampal neurons (Binotti et al., 2015), and incorporate the synaptic vesicle protein VAMP2 into the phagophore membrane (Okerlund et al., 2017). A study in hippocampal neurons, which used a light-activated reactive oxygen species generator to specifically damage the synaptic protein Synaptophysin, determined that autophagy can degrade damaged synaptic proteins without engulfing the whole synaptic vesicle (Hoffmann et al., 2019). They hypothesize that this might occur via an intermediate endosomal sorting step, where damaged synaptic proteins are selectively removed for degradation. *In vivo* studies in *C. elegans* neurons showed that presynaptic proteins, including active zone proteins SYD-1 and SYD-2/Liprin alpha and synaptic vesicle protein SNB-1/synaptobrevin, can be found inside acidified autophagosomes in neurons (Hill et al., 2019). Together, these studies demonstrate that synaptic autophagy can degrade local components of the synapse, including synaptic vesicles, synaptic vesicle proteins, and components of the presynaptic active zone.

While autophagosomes can engulf synaptic components in neurons, the mechanisms by which synaptic cargoes are recognized remain unclear. In non-neuronal cells, autophagy is described as either selective or non-selective, based on two criteria—the type of cargo being degraded, and the adaptor proteins used to confer cargo specificity (Figure 1). In neurons, autophagy can similarly depend on adaptor molecules or not, based on context, and both forms of selective and non-selective autophagy contribute to degrading local organelles and protein aggregates. The best-examined instances of selective autophagy include mitophagy (degradation of mitochondria) or aggrephagy (degradation of protein aggregates), and defects in these processes have been linked to Parkinson's, Alzheimer's, Huntington's, and amyotrophic lateral sclerosis, among other

diseases. Mitophagy and aggrephagy in neurons have been recently reviewed elsewhere (Deng et al., 2017; Evans and Holzbaur, 2020). We will not discuss them here but will highlight that these studies further suggest that local biogenesis of autophagosomes in axons is critical to degrade specific cargoes and that disrupting this process can contribute to neurodegenerative diseases.

Intriguingly, recent studies have also suggested the existence of "vesiculophagy," or the selective degradation of synaptic or secretory vesicles in neurons. In these studies, a specific small GTPase, RAB26, associates with clusters of synaptic vesicles enclosed by autophagosomes in a process dependent on the GTPase state of RAB26 (Binotti et al., 2015). In a subsequent study, a guanine nucleotide exchange factor (GEF) for RAB26, PLEKHG5, was shown to be required for RAB26-dependent autophagy of synaptic vesicles in motor neurons (Lüningschrör et al., 2017). Together, these findings suggest the existence of a selective mechanism for degrading synaptic vesicles in neurons.

Most selective autophagy adaptors were first identified in non-neuronal cells. Despite emerging evidence of specific cargoes in synaptic and axonal autophagosomes, little is known, outside of mitophagy and aggrephagy, regarding the role of selective autophagy adaptors in selecting specific cargoes in neurons. While more work is necessary to identify adaptors that act in neurons, we hypothesize that non-selective autophagy may also play an important role in specifically degrading synaptic material.

How might a non-selective process like bulk autophagy specifically degrade synaptic material? We speculate that the unique cell biology of the neuron contributes to the "specificity" of bulk autophagy without requiring adaptor proteins. The concept of "selective autophagy," best understood in non-neuronal cells, is a molecular-centered concept, in which cargo specificity is conferred based on molecular recognition of a target by an adaptor protein. But specificity could also be conferred through a cell biological mechanism by instructing the site of autophagosome biogenesis. Driving bulk autophagy within subcellular compartments of the neuron, such as the synapse, by default engulfs cargoes located in that sub-compartment without requiring specific adaptor proteins. This mechanism may also be a cell biological principle of autophagosome specificity in non-neuronal cells but is arguably harder to track in cells that do not have the pronounced polarity of neurons. This is consistent with the notion that cell biology instructs cargo degradation within specific compartments; in the next section, we discuss how regulating the localization of autophagic components at a cell biological level can mediate local autophagosome biogenesis in neurons.

Biogenesis of Synaptic Autophagosomes

Biogenesis of autophagosomes initiates through signals that recruit membranes. While the mechanisms instructing membrane recruitment are similar between neuronal and non-neuronal cells, mechanisms in neuronal cells can be locally targeted to axons. For example, a DFCP1-positive subdomain of the endoplasmic reticulum provides membranes for autophagosome biogenesis but in neurons does so locally at axon terminals to drive axonal autophagy (Maday and Holzbaur, 2014) (Figure 1).

ATG9, the only known transmembrane protein involved in the core autophagy pathway (Lang et al., 2000; Noda et al., 2000), is also enriched at synaptic sites (Soukup et al., 2016; Stavoe et al., 2016; Tamura et al., 2010; Vanhauwaert et al., 2017). In *C. elegans*, the conserved motor protein kinesin KIF1A/UNC-104 is required to transport ATG-9-containing vesicles from the soma to presynaptic regions to support local autophagosome biogenesis near synapses (Stavoe et al., 2016). While ATG9 is essential for autophagosome biogenesis, its role is not fully understood. ATG9 is present on small vesicles thought to participate in autophagosome biogenesis, either through direct fusion and nucleation of the phagophore as in yeast (Yamamoto et al., 2012) or through dynamic transient interactions with the phagophore membrane as in mammals (Orsi et al., 2012). These studies indicate that transporting key components of the autophagic machinery, like ATG9, to synapses can instruct local autophagosome biogenesis. Importantly, in the case of ATG9, transport occurs via a specific kinesin best known for its role in transporting synaptic material. Using a synaptic kinesin for ATG9 transport may link autophagosome biogenesis to the subcellular synaptic site.

In non-neuronal cells, autophagosome biogenesis involves an ordered and processive recruitment of autophagic machinery to the site of formation (Itakura and Mizushima, 2010; Koyama-Honda et al., 2013; Suzuki et al., 2007). The biogenesis of the synaptic autophagosome is similarly ordered and spatially regulated. In axon terminals, ATG5 and ATG13 are first recruited prior to LC3/ATG8. Then, ATG5 and ATG13 dissociate while LC3/ATG8 signal persists (Maday and Holzbaur, 2014). Additional factors including p62, ATG5, and ATG16L also associate with synaptic autophagosomes in cultured neurons (Okerlund et al., 2017). In the fly, Atg3 (Soukup et al., 2016) and ATG18a can be found on synaptic autophagosomes (Vanhauwaert et al., 2017). In zebrafish photoreceptor neurons, the PI(3)P probe 2xFYVE colocalizes with LC3/ATG8 at synaptic terminals but eventually dissociates (George et al., 2016). Another PI(3)P binding protein, PLEKHG5, a RAB26 GEF, can participate in autophagosome biogenesis to promote autophagy of synaptic vesicles (Lüningschrör et al., 2017). Together, these studies suggest that synaptic autophagy proceeds in a stepwise manner at specific subcellular compartments to facilitate local target engulfment.

Sequential factor recruitment events are contingent upon each other (Itakura and Mizushima, 2010; Koyama-Honda et al., 2013; Suzuki et al., 2007). For example, autophagy rates decrease in aged neurons due to a local loss of WIPI2B, which can stall ATG13-positive, ATG5-positive, and ATG9-positive autophagosomes at axon terminals that fail to recruit LC3B (Stavoe et al., 2019). These findings suggest that proper autophagy progression likely requires removal of ATG13, ATG5, and/or ATG9. When autophagosome formation is genetically interrupted at a late stage, such as in *atg-2* or *epg-6* mutant animals, autophagosomes fail to undergo retrograde transport and accumulate at the synapse (Stavoe et al., 2016).

These studies are consistent with a locally coordinated signaling network that regulates autophagy at the synapse from biogenesis to maturation and transport. Importantly, these studies indicate that regulated signaling governs local recruitment of autophagic factors into distal axonal sites. The hierarchical recruitment of autophagy machinery influences axonal autophagy, from the local cargoes the autophagosome engulfs

to the timing of autophagosome formation and regulated progression toward maturation and degradation. Eventually, the synaptic autophagosome and its cargoes undergo degradation, a step that does not happen at the synapse and requires transport. What signals coordinate the completion of biogenesis at the synapse and the initiation of transport toward the cell body?

Transport and Degradation of Autophagosomes in Neurons

Autophagosomes must fuse with acidic, proteolytic lysosomes for degradation. In non-neuronal cells, the distance between a lysosome and the Golgi correlates with the lysosomes' degradative capacity, as peripheral lysosomes that are far from the Golgi are less acidic and have lower cathepsin enzymatic activity (Johnson et al., 2016). One explanation is that the Golgi-derived production of proteins and efficient delivery is necessary for the maturation of nearby lysosomes. In neurons, the Golgi apparatus predominantly resides in the soma and is absent from axons. Consistent with observations from non-neuronal cells, classic studies examining pH in cultured neurons found that few, if any, endocytic or autophagic organelles in the axons were acidic, while compartments in the cell body ranged from acidic pH <5 to a more neutral pH around 7 (Hollenbeck, 1993). These findings are also consistent with more recent studies demonstrating low lysosomal proteolytic activity in axons (Ferguson, 2018; Gowrishankar et al., 2015; Lee et al., 2011). The immature state of these organelles in the axon indicates that autophagosome acidification and degradation require transport toward the soma.

The importance of the link between retrograde transport and autophagosome degradation is perhaps best exemplified by the consequences of its disruption. When retrograde transport is disrupted, as occurs in some neurodegenerative diseases, it results in autophagosome accumulation at synapses, Alzheimer's disease-like autophagic accumulations, and axonal pathology (Lee et al., 2011; Nixon et al., 2005; Tammineni et al., 2017). We examine this key cell biological relationship between the retrograde transport of autophagosomes and degradation by describing first the relationship between acidification and transport and then how mechanisms that regulate autophagosome transport contribute to clearance of autophagosomes.

Autophagosome Maturation from the Synapse to the Cell Body

Autophagosome transport toward the cell body is linked to autophagosome acidification and maturation. Maturation can be detected in live cells using tandem mCherry::GFP::LC3/ATG8 markers. Since GFP fluorescence is preferentially quenched in an acidic environment as compared to mCherry, these markers reveal acidification through differences in the relative fluorescence of the GFP/mCherry fluorophores in individual autophagosomes (Kimura et al., 2007). Using these assays, it was shown that autophagosome acidity gradually increases during autophagosome retrograde transport (Maday et al., 2012). About 50% of autophagic structures in cultured neurons or fly neuromuscular junctions are already acidic in the distal axon or synaptic regions (Maday et al., 2012; Vanhauwaert et al., 2017). This suggests that the acidification process may begin at the synapse for some neurons (Maday et al., 2012). In different neurons, the degree of acidification in the cell body varies. For example, in fly larval motor neurons, all of the autophagic structures in the cell body are mature (Neisch et al., 2017), while in *C. elegans* interneurons, about half of the structures in the cell body are mature (Hill et al., 2019). Despite these cell-specific differences, it is generally accepted that a gradient of acidification from the synapse toward the cell body exists and that autophagosomes require transport toward the cell body for degradation.

From studies in non-neuronal cells, we learn that only fully formed autophagosomes (not immature phagophores) are competent for retrograde trafficking (Fass et al., 2006). Fully formed autophagosomes associate with dynein motor proteins to initiate retrograde transport. One mechanism to recruit dynein is through autophagosome fusion with late endosomes that contain dynein subunits (such as dynein intermediate chain 2C) at the axon terminal (Cai et al., 2010; Cheng et al., 2015). However, a study in *Drosophila* motor neurons found that blocking fusion with late endosomes did not alter autophagosome mobility (Neisch et al., 2017), which indicates the presence of other redundant pathways to facilitate autophagosome recruitment of dynein and retrograde transport.

Processive acidification of autophagosomes occurs through multiple sequential fusions with late endosomes and lysosomes during transport to the cell body (Eskelinen, 2005; Kimura et al., 2007). Consistent with this, the lysosomal protein LAMP1 and autophagosome protein LC3/ATG8 do not extensively colocalize in the distal axon (Maday et al., 2012; Wang et al., 2015). However, they do increasingly colocalize during transport. Nearly all retrogradely moving autophagosomes also contain LysoTracker red staining (Maday et al., 2012), which reports acidic pH, as well as late endosome-associated protein Rab7 (Cheng et al., 2015; Lee et al., 2011), consistent with retrograde moving autophagic structures being amphisomes or autolysosomes (Figure 2). The importance of multiple fusion events is demonstrated by retrograde transport defects in autophagosomes of Huntingtin-depleted neurons. These autophagosomes initially appear to acidify normally but are then less acidic and degradative toward the cell body (Wong and Holzbaur, 2014). Proper fusion between autophagosomes and lysosomes requires autophagosomal transport. In axons treated with vinblastine, which disrupts the microtubule cytoskeleton, colocalization between LAMP1 and LC3/ATG8 is disrupted (Lee et al., 2011). Similarly, loss of autophagosome retrograde transport, through impairment of the transport adaptor molecule JIP1, causes a secondary defect in autophagosome acidification, as revealed using an LC3/ATG8 tandem construct (Fu et al., 2014). Together, these studies underscore a tight mechanistic connection between autophagosome transport and maturation. Below, we detail the distinct mechanistic steps of transport and fusion and their cell biological regulation during clearance of synaptic autophagosomes.

Neuronal Autophagosomes Require Dynein Motor Activity and Scaffolding Proteins

In both neuronal and non-neuronal cells, autophagosomes rely on microtubules for their transport (Fass et al., 2006; Kimura et al., 2008). In axons, microtubules are uniformly oriented plus-end-out with plus-end-directed kinesin motor proteins primarily regulating movement away from the cell body, while minus-end-directed dynein motor proteins canonically regulate

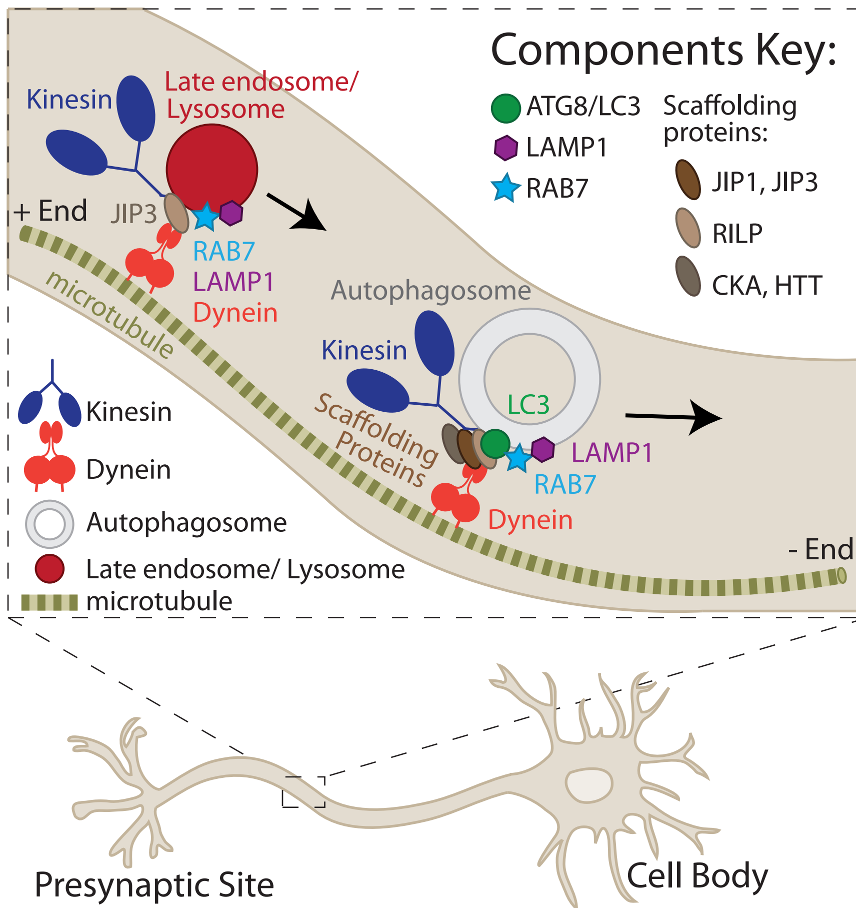

Figure 2. Scaffolding Proteins Regulate the Activity of Dynein and Kinesin Motors to Support Transport of Autophagic Structures from the Axon to the Cell Body

Scaffolding proteins likely interact with the autophagosome via ATG8/LC3 to suppress kinesin activity and facilitate dynein activity. Examples include JIP1, JIP3, RILP, CKA, and HTT, shown here on one autophagosome. However, it is unknown if these scaffolding proteins localize to distinct sub-populations. JIP3 also regulates endosome and lysosome transport. Retrograde-moving autophagosomes also contain the late endosome/lysosome markers LAMP1 and RAB7, suggesting prior fusion with late endosomes/lysosomes in the synaptic region.

movement toward the cell body (Baas and Lin, 2011; Hirokawa et al., 2010). What begins as bidirectional movement of the autophagosome in the distal axon eventually switches to a unidirectional retrograde transport toward the cell body (Maday et al., 2012). For autophagosomes to utilize retrograde transport (toward the cell body), they must associate with minus-end-directed motor proteins like dynein.

The importance of dynein in retrograde transport and maturation of autophagosomes is most clearly seen when dynein motor proteins are inhibited. Studies using a combination of non-neuronal cells, cultured neuronal precursor cells, and mouse models find that pharmacological inhibition of dynein, or expression of a dominant negative version of dynein components, result in phenotypes of stalled autophagy, such as accumulation of lipidated LC3/ATG8, reduced fusion events with lysosomes, and accumulation of protein aggregates (Ravikumar et al., 2005). Similarly, reducing dynein activity using dynactin RNAi in *C. elegans* increases axonal accumulation of autophagosomes, neuronal dysfunction, and neurodegeneration (Ikenaka et al., 2013). In Alzheimer's disease, neurons show an impairment in retrograde transport of autophagosomes, which drives the accumulation of pathological amphisomes in axons (Tammineni et al., 2017). Taken together, these studies reveal a pivotal role for dynein to coordinate the retrograde transport of autophagosomes toward the cell body with autophagosome acidification and clearance.

Regulated transport by motor proteins is modulated by scaffolding molecules, which can bind to transported organelles and selectively activate either kinesin or dynein to ensure processivity in an anterograde or retrograde direction (Fu and Holzbaur, 2014) (Figure 2). Scaffolding proteins interact with autophagosomes via the autophagy protein LC3/ATG8. For example, the RILP-RAB7 complex that recruits dynein to direct lysosome transport (Jordens et al., 2001) also colocalizes with LC3/ATG8 in axons (Bains et al., 2011) potentially regulating autophagosome retrograde transport. In another example, the motor scaffolding protein CKA (a component of the Striatin-interacting phosphatase and kinase [STRIPAK] complex) binds both Atg8a (an LC3/ATG8 ortholog) and dynein to mediate retrograde transport of autophagosomes, with loss of CKA resulting in autophagosomes accumulation in terminal axon boutons (Neisch et al., 2017). Disrupting the interaction between LC3/ATG8 and scaffolding proteins, such as through antibodies against LC3/ATG8, abolishes autophagosome movement, underscoring the importance of this interaction for autophagosome retrograde transport (Kimura et al., 2008).

Other key scaffolding molecules that mediate the interaction between autophagosomes and motor proteins include JIP family proteins JIP1 and JIP3. JIP1 and JIP3 bind both dynein and kinesin motors (Arimoto et al., 2011; Bowman et al., 2000; Fu et al., 2014) and can act together to affect axonal transport (Sun et al., 2017). JIP1 in its dephosphorylated state promotes processive retrograde transport of autophagosomes (Fu et al., 2014). Studies in *C. elegans* neurons demonstrated that JIP3/UNC-16/Sunday Driver is critical for dynein-mediated retrograde transport of lysosomes, early endosomes, and autophagosomes, with defects leading to organelle jams (Edwards et al., 2013, 2015; Hill et al., 2019). Consistent with the important role for JIP3 in autophagosome and lysosome transport, axons

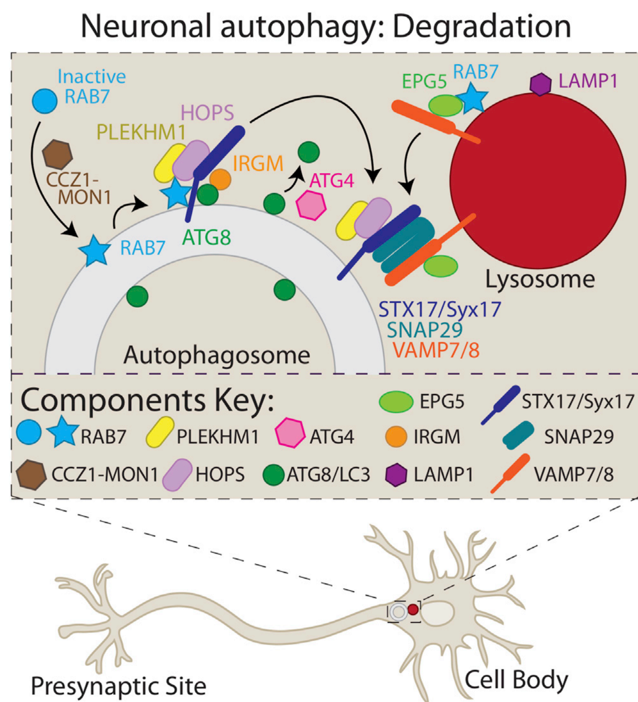

Figure 3. Autophagosomes Fuse with Late Endosomes/Lysosomes via a SNARE Complex
Autophagosome-lysosome fusion requires the small GTPase RAB7, which is activated by a GEF complex, CCZ1-MON1. RAB7 interactor PLEKHM1 forms a complex with the HOPS complex and the autophagosome-specific SNARE, STX17, to support SNARE complex formation and stability. STX17/Syx17 is a cytosolic protein recruited to the autophagosome via IRGM, a GTPase, and LC3. The SNARE complex also contains a lysosomal SNARE VAMP7/8 and a cytosolic SNARE SNAP29 containing two alpha helices. ATG8 is delipidated or removed from the autophagosome by the cysteine protease ATG4, perhaps prior to fusion with lysosomes. A RAB7 effector, EPG5, interacts with VAMP7/8 to promote autophagosome-lysosome fusion. While factors contributing to autophagosome-lysosome fusion are shown here to act in a series of events, their order of recruitment and activity are unknown, with this presenting only one of many possible scenarios.

from JIP3 knockout mouse neurons swell and accumulate Alzheimer's disease-like plaques, Aβ peptides, and immature lysosomes (Gowrishankar et al., 2017). Disruption of autophagosomal transport has also been linked to Huntington's disease. For example, Huntingtin (HTT) and its adaptor HAP1 act together as scaffolding proteins to direct autophagosome retrograde transport (Wong and Holzbaur, 2014). A disease allele version of huntingtin with polyQ-HTT caused a specific defect in autophagosome retrograde transport, but not defects in autophagosome formation or cargo loading. This result highlights a possible mechanistic link between specific defects in retrograde transport of autophagosomes and neurodegenerative disease (Wong and Holzbaur, 2014).

Machinery of Fusion with Late Endosomes and Lysosomes

Most of the studies investigating the machinery of autophagic fusion have been conducted in non-neuronal cells. While the machinery is likely similar between neurons and other cells, the distinctive structure of neurons adds a spatial dimension to fusion, which may have unique consequences for neurons. In this section, we describe mechanistic insights from non-neuronal cells and discuss how they relate to neurons.

Fusion between autophagosomes and late endosomes or lysosomes occurs as autophagosomes are transported toward the cell body and is mediated by SNARE complexes. The SNARE complex is a bundle of alpha helices located on opposing membranes to be fused (Hong, 2005; Südhof and Rothman, 2009). Autophagy SNARES have three components: SNAP29, VAMP8 (called VAMP7 in *Drosophila*) on the late endosome/lysosome, and STX17/Syx17 on the autophagosome outer membrane (Itakura et al., 2012; Takáts et al., 2013) (Figure 3). STX17 is likely inserted into the autophagosome membrane from a cytosolic pool, as it is relatively hydrophilic for a SNARE (Itakura et al., 2012). Its recruitment also requires a GTPase (IRGM) and a LIR motif on STX17 (Kumar et al., 2018).

In *Drosophila* neurons, the STX17 ortholog, Syx17, is important for autophagy, with mutants accumulating autophagosomes in neuronal cell bodies, and disruption of neuronal function (Takáts et al., 2013). A study in cultured vertebrate neurons also revealed that STX17 knockdown induced axonal accumulation of non-degradative autophagosomes (Cheng et al., 2015). While these studies show different locations for autophagosome accumulation, importantly, they consistently reveal a role for the SNARE complex in autophagosome clearance.

Interestingly, loss of STX17 does not result in the accumulation of autophagosomes docked on late endosomes/lysosomes but rather in the accumulation of immature autophagosomes. This suggests that STX17 may also participate in upstream events like docking or tethering of the lysosome prior to fusion. The tethering complex, HOPS, is also required for efficient autophagosome maturation and fusion with lysosomes (Jiang et al., 2014; Manil-Ségalen et al., 2014; Takáts et al., 2014) (Figure 3). HOPS forms a complex with the autophagosomal SNARE and the endocytic adaptor PLEKHM1, a RAB7 interactor that is required for efficient autophagic flux (McEwan et al., 2015). RAB7, a small GTPase and a member of the RAB family of tethering factors, is recruited to autophagosomes to facilitate their maturation into late autophagic vacuoles (Gutierrez et al., 2004; Jäger et al., 2004). In *Drosophila*, recruitment of Rab7 depends upon the Ccz1-Mon1 guanosine exchange complex, but not the HOPS complex or Syx17, and is important for autophagosome-lysosome fusion (Hegedűs et al., 2016) (Figure 3). Downstream of RAB7, the effector protein EPG5 helps mediate fusion of autophagosomes with late endosomes by directly interacting with the endosomal component of the SNARE complex, VAMP7/8 (Wang et al., 2016). Consistent with these conserved roles, both SAND-1/MON1 and EPG-5 are critical in *C. elegans* neurons to clear autophagosomes in the neuronal cell bodies (Hill et al., 2019).

A recent study using cancer cell lines and fly neurons identified a Parkinson's disease-associated ATPase, ATP13A2, as critical for regulating autophagosome-lysosome fusion (Wang et al., 2019). ATP13A2 is not required for autophagosome tethering to lysosomes or for recruitment of the autophagosomal SNARE STX17. Rather it acts on lysosomes to recruit a deacetylase, HDAC6. HDAC6 then deacetylates a cytoskeletal regulator, cortactin, to promote autophagosome-lysosome fusion, likely by

modulating the local cytoskeleton (Wang et al., 2019). Together, these studies demonstrate that regulated mechanisms of lysosomal fusion are linked to the retrograde transport of the autophagosome, its acidification and clearance.

Activity of ATG8 Family Proteins Is Associated with Autophagosome Biogenesis at the Synapse and Clearance in the Soma

ATG8/GARABRAP/LC3/LGG-1/LGG-2 (hereafter ATG8s) are a family of proteins that have recently emerged as key components for autophagosome biogenesis and clearance. The ATG8s are ubiquitin-like proteins that associate with the autophagosomal membrane. Because of their stable association with autophagosomes, they are frequently used as markers for autophagy (Klionsky et al., 2012). The stable association of ATG8s with autophagosomes effectively "bookends" the process of autophagy, providing molecular components that travel the length of the neuron and autophagy process, from biogenesis to fusion with degradative lysosomes.

The ATG8s may coordinate biogenesis and clearance of synaptic autophagosomes. ATG8s associates with autophagosome membranes in a process that depends on cleavage of a pro-form by a cysteine protease, ATG4 (Kirisako et al., 1999, 2000). This process is important for ATG8 activity and thus autophagosome biogenesis. ATG8s are also cleaved for removal from the autophagosomal membrane. This second cleavage, also mediated by the ATG4 proteases, is necessary for the autophagosome to ultimately fuse with the vacuole (Yu et al., 2012) (Figure 3). Therefore, the ATG4 proteases perform two cleavages that bookend the association of ATG8/LC3 with the autophagosome.

In metazoans, specialized isoforms of ATG4 proteases preferentially perform distinct cleavage roles during autophagosome biogenesis and clearance. For example, *C. elegans* has two ATG4 protease isoforms encoded by two distinct genes, ATG-4.1 and ATG-4.2. While these two genes are partially redundant, biochemical assays and *in vivo* studies revealed distinct phenotypes, indicating specialized functions in the cleavage of ATG8 during autophagosome biogenesis and clearance. Indeed, genetic lesions in *atg-4.2*, but not *atg-4.1*, resulted in a dramatic accumulation of autophagosomes in the cell body of neurons since they were unable to mature and be cleared (Hill et al., 2019). In mammals, the ATG4 family of proteases comprises four isoforms, and consistent with findings in *C. elegans*, the distinct isoforms display different biochemical activities to promote delipidation of the ATG8s (Kauffman et al., 2018).

In metazoans, there is an expansion of the genes coding for ATG8s into two major subgroups, the GABARAPs and the LC3s. Recent findings indicate that different family members may specifically modulate distinct steps in the progression of the autophagy pathway. For example, the GABARAPs in mammals play a more significant role than the LC3s do in promoting the recruitment of PLEKHM1, a RAB7 interactor required for facilitating autophagosome-lysosome fusion (McEwan et al., 2015; Nguyen et al., 2016). However, the *C. elegans* LC3 ortholog, LGG-2, preferentially promotes tethering of autophagosomes and lysosomes via direct interaction with the HOPS complex protein VPS-39 (Alberti et al., 2010; Manil-Ségalen et al., 2014). Despite species differences for ortholog preference, these findings suggest that specific ATG8s may also specialize in distinct steps of the autophagy pathway.

Together, these studies indicate the existence of specialized functions for the ATG4 protease isoforms and the ATG8s substrates in metazoans. Importantly, disrupting these distinct isoforms results in different cell biological phenotypes regarding autophagosome biogenesis and clearance, indicating that a series of regulated processes coordinate autophagy progression in neurons. Precise knowledge of the specific roles of ATG4 protease isoforms and ATG8 substrates in metazoans could provide therapeutic targets to alter specific steps of the autophagy pathway.

The Flux of Autophagy in Neurons

Our description of the cell biology of neuronal autophagy might give the reader the impression of a linear pathway starting with autophagosome biogenesis at the synapse and ending with its degradation in the cell body. While that description is accurate for an individual autophagosome, from a cellular perspective the autophagy process is a flux—a continuous and integrated cycle with regulated feedback loops. As such, blocking downstream processes, such as autophagosome maturation, can affect upstream processes, such as autophagosome transport and biogenesis, in distinct subcellular compartments. Knowledge of flux is important for targeted therapeutic interventions seeking to alter a specific step of the autophagy pathway in neurons.

For example, blocking lysosomal proteolysis activity with the protease inhibitor Leupeptin causes autophagosomes to lose dynein and stall in the axon (Lee et al., 2011). Similar results occur after short-term (2–4 h) exposure to Baflomycin A (Lee et al., 2011; Wang et al., 2015), a drug that blocks lysosomal acidification (Fass et al., 2006; Klionsky et al., 2008) or Chloroquine exposure, which increases lysosomal pH, as seen in studies of the fly neuromuscular junction where autophagosomes increase in number in the synapses (Soukup et al., 2016). Loss of acidity via Baflomycin A can also cause transport reduction. In this case, autophagosomes accumulate in the cell soma (Maday and Holzbaur, 2016). Further support for regulatory feedback loops comes from *in vivo* studies in *C. elegans* where genetic mutants that block downstream steps of the autophagosome pathway, such as *atg-2*, *epg-6*, and *epg-5*, inhibit the formation of new autophagosomes in *C. elegans* neurons (Stavoe et al., 2016). While it is unknown how such feedback systems might operate, it is possible that mechanisms occurring over longer timescales may be influenced by changes in transcriptional networks. Such transcriptional networks are already known to influence autophagy levels in non-neuronal cells by regulating autophagosome and lysosome biogenesis based on nutrient state (Di Malta et al., 2019), and we speculate that similar mechanisms might occur in neurons.

Together, these studies suggest that defects in downstream events like maturation and clearance, which occur near the cell body, impact upstream events like autophagosome biogenesis and transport, which occur in the synapse and the axon. These findings underscore the main message of this review: that the distinct steps of the autophagy pathway are distributed

throughout the neuron and coordinated to regulate the progression of the autophagy pathway. We speculate that the discussed mechanisms which ensure effective coordination of autophagy in neurons will be important to prevent neurodegenerative disease, both by ensuring efficient degradation of neuronal substrates and by preventing the accumulation of potentially toxic autophagic intermediates. Furthermore, by gazing through the lens of the cell biologist, where location in the cell dictates the substrates available for biochemistry, we can better focus our understanding of neuronal autophagy in physiology and disease.

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