Autophagy in the presynaptic compartment in health and disease

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Synapses are functionally distinct neuronal compartments that are critical for brain function, with synaptic dysfunction being an early pathological feature in aging and disease. Given the large number of proteins needed for synaptic function, the proliferation of defective proteins and the subsequent loss of protein homeostasis may be a leading cause of synaptic dysfunction. Autophagic mechanisms are cellular digestion processes that recycle cellular components and contribute to protein homeostasis. Autophagy is important within the nervous system, but its function in specific compartments such as the synapse has been unclear. Evidence from research on both autophagy and synaptic function suggests that there are links between the two and that synaptic homeostasis during aging requires autophagy to regulate protein homeostasis. Exciting new work on autophagy-modulating proteins that are enriched at the synapse has begun to link autophagy to synapses and synaptic dysfunction in disease. A better understanding of these links will help us harness the potential therapeutic benefits of autophagy in combating age-related disorders of the nervous system.

Introduction

Neurons in the brain form synaptic connections to generate neuronal circuits whose emergent properties include phenomena such as sensory perception, behavior, memory, and emotion. Information is encoded as electrical impulses in neurons and can be relayed from the presynaptic compartment of one neuron to a postsynaptic compartment of another neuron by the release of neurotransmitters. Neurons are postmitotic, with new neurons being formed very infrequently; therefore, most neurons and a majority of their synapses have to be maintained for the lifetime of an organism (Bishop et al., 2010). Synapses are also dynamic, as their properties can be changed by experiences such as stress or learning, a phenomenon known as synaptic plasticity (Glanzman, 2010; Christoffel et al., 2011). Therefore, if synapses become dysfunctional, the circuits they form are fundamentally altered and brain function is severely affected. This leads to the important question of how synaptic dysfunction arises within the presynaptic compartment.

Synapses are particularly susceptible to accumulating damaged proteins

The presynapse may contain a mean of ∼300,000 proteins within a volume of 0.37 ± 0.04 μm^3 (Wilhelm et al., 2014). After a neuron fires, an action potential arrives at the presynapse, causing the release of neurotransmitters that trigger neuronal activity in the postsynaptic neuron. The release of neurotransmitters involves the fusion of synaptic vesicles with the plasma membrane, followed by the retrieval of fused vesicle membranes by endocytosis. These processes require the coordinated efforts of a large group of proteins (Südhof, 2004). The synaptic vesicle cycle is initiated very rapidly and can occur repeatedly, with a frequency exceeding 100 Hz in some neurons (de Kock and Sakmann, 2008). These conditions place considerable stress on presynaptic proteins, leading these proteins to accumulate damage and becoming functionally impaired. Further complications arise from the unique morphological aspects of neurons and synapses. Within postmitotic neurons, defective proteins cannot be diluted away by repeated cell cycle divisions. Furthermore, synapses are often located very far from the soma of a neuron, the main location of protein synthesis. For example, human neurons have synapses separated from the soma by axons of more than a meter in length (Standring, 2008). Considering these distances, the slow cytoplasmic transport of protein (∼10 mm/d) means that new protein replacements will not arrive quickly (Roy, 2014). Collectively, these unfavorable circumstances increase the possibility of a buildup of defective proteins that could then lead to synaptic dysfunction, as when synaptic proteins are knocked out or mutated (Verstreken et al., 2002; Kasprowicz et al., 2008; Uytterhoeven et al., 2011).

Mechanisms for removing damaged proteins

Neurons have evolved several strategies for removing damaged proteins and maintaining the integrity of the proteome, a process termed protein homeostasis (Kaushik and Cuervo, 2015; Labbadia and Morimoto, 2015). These strategies include molecular chaperone activity and protein degradative pathways consisting of the ubiquitin-proteasome system and lysosomal degradation

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Abbreviations used: AD, Alzheimer's disease; CMA, chaperone-mediated autophagy; CR, caloric restriction; PD, Parkinson's disease.

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via autophagy, a process in which parts of the cytoplasm are engulfed by membrane and sent for degradation. As noted, neurons have unique morphologies in which specific compartments such as synapses are distant and functionally distinct from the soma, with their own unique complement of proteins (Wilhelm et al., 2014). Therefore, protein quality control mechanisms may be spatially regulated to meet the specific requirements of protein homeostasis at the synapse. In this review, we focus on autophagy, but we refer the reader to several excellent reviews on the ubiquitin-proteasome system and chaperones (Bingol and Sheng, 2011; Alvarez-Castelao and Schuman, 2015; Labbadia and Morimoto, 2015).

Types of autophagy

Pioneering work in the 1960s by Nobel laureate Christian de Duve and others led to the detection of double-membrane vesicles that seemed to be engulfing proteins and organelles (Yang and Klionsky, 2010). These observations led de Duve to coin the term autophagy, or "self-eating." Nobel prize–winning genetic studies on autophagy in yeast by Ohsumi and colleagues ushered in a molecular era in which many of the constituent parts of the autophagic machinery have been elucidated and shown to be conserved from yeast to mammals (Ohsumi, 2014). Distinct forms of autophagy occur in the cell: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA; Mijaljica et al., 2011; Mizushima and Komatsu, 2011; Kaushik and Cuervo, 2012). These types of autophagy have been studied mostly in the context of nonneuronal cells or neuronal cell bodies, yet there is emerging evidence that functionally distinct compartments such as synapses may have specialized forms of autophagy. Macroautophagy is a bulk degradative process whereby cytoplasmic entities such as proteins, sugars, lipids, RNA, and occasionally entire organelles are engulfed within a double-membrane vesicle termed the autophagosome. The autophagosome will then fuse with the lysosome, where the engulfed products can be degraded by hydrolases. The resulting end products are amino acids and lipids, which can be exported from the lysosome and used by the cell in various capacities. Therefore, macroautophagy is a catabolic pathway that assists in protein homeostasis by removing dysfunctional cellular components and contributing toward the creation of new components.

Microautophagy is a specialized form of autophagy in which cytoplasmic proteins are degraded by direct transport into lysosomes through invaginations of the lysosomal membrane. Microautophagy may also take place via the delivery of proteins into late endosomes, a process that is dependent on the chaperone Hsc70 (Sahu et al., 2011; Uytterhoeven et al., 2015). The Hsc70 chaperone is also involved in CMA, a selective degradative process. During CMA, Hsc70 recognizes proteins with a specific amino acid motif and associates with the lysosomal membrane protein LAMP2A, resulting in client protein translocation into the lysosome (Kaushik and Cuervo, 2012). Increasing evidence suggests that there is cellular cross-talk between all three types of autophagy. In this review, we focus on macroautophagy and its potential roles at the presynapse (referred to as synaptic autophagy). We refer the reader to other reviews discussing microautophagy and CMA in greater detail (Mijaljica et al., 2011; Kaushik and Cuervo, 2012).

The process of autophagy

The progression of autophagy occurs through clearly defined steps regulated by distinct sets of autophagy-related gene (Atg) proteins (Fig. 1). A brief overview is provided herein to introduce autophagy proteins involved in the studies discussed here. A more detailed description of the general mechanisms of autophagy is covered by several comprehensive reviews (Mizushima and Komatsu, 2011; Bento et al., 2016; Maday, 2016).

Autophagy can be broadly divided into three phases: (1) initiation of autophagy, leading to the formation of an expanding, double-membrane preautophagosomal structure; (2) formation

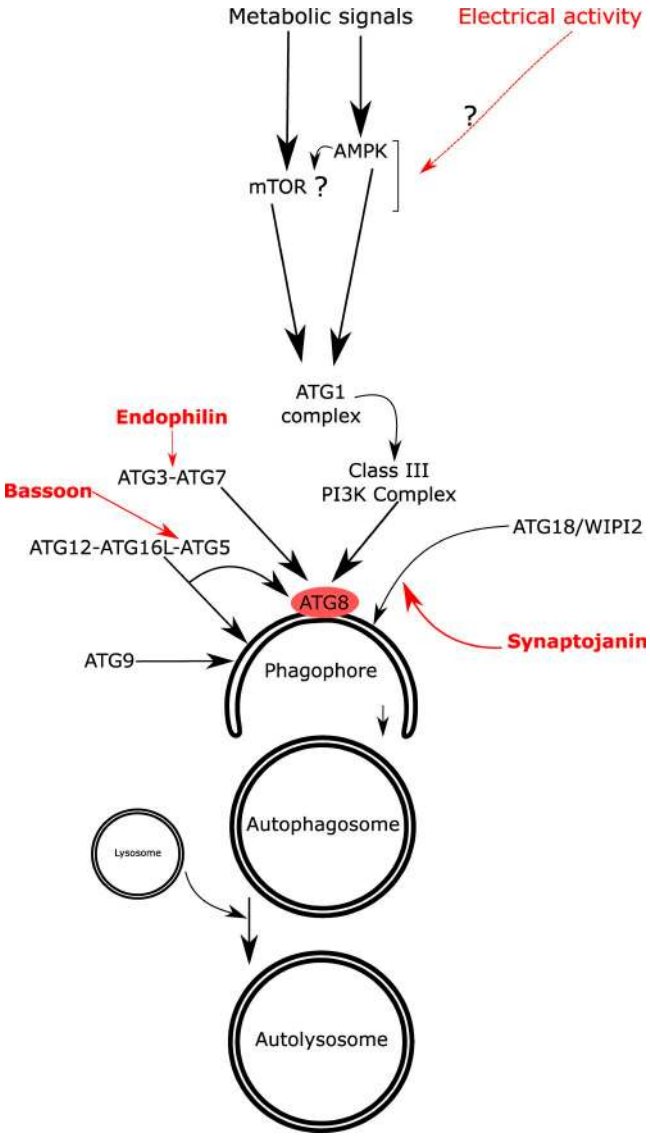

Figure 1. **Overview of autophagy and potential synaptic modifications.** Autophagy is triggered by metabolic signals elicited by nutrient starvation or electrical activity of the neuron. Starvation states signal through mTOR or AMPK to activate the ATG1 complex, the most upstream kinase complex in autophagy. The ATG1 complex and subsequently the phosphatidylinositide 3-kinase complex act on the isolation membrane. Important ATG protein complexes required for autophagy are highlighted. These include the ubiquitin-like conjugation systems: the ATG12-ATG16L-ATG5 and the ATG3-ATG7 complexes that conjugate lipidated ATG8 proteins to the growing phagophore. ATG9 and ATG18/WIP2 complex also associate with the phagophore. Recent studies have demonstrated that the synapse-enriched proteins Endophilin, Synaptojanin, and Bassoon interact with Atg proteins (see text for details). The phagophore will mature into an autophagosome, a completely closed-off vesicle structure that has engulfed cytoplasmic elements and organelles. Finally, the autophagosome will fuse with the lysosome to form the autolysosome.

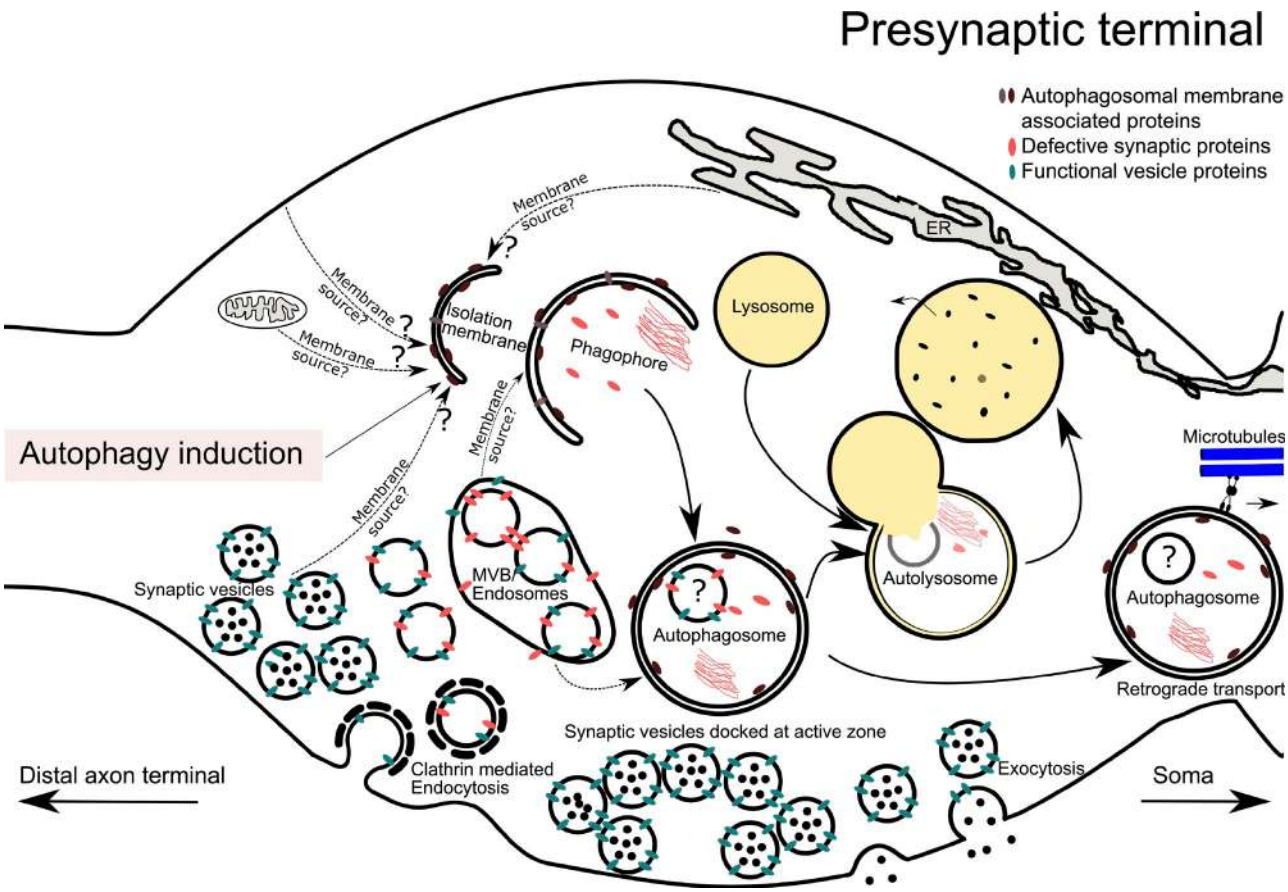

Figure 2. **A hypothetical model for autophagy at the presynaptic terminal; schematic of how autophagy may potentially proceed at the presynaptic terminal.** At the presynaptic terminal, defective synaptic proteins can be recycled by autophagy. Synaptic vesicles are retrieved after neurotransmitter exocytosis by clathrin-mediated endocytosis and can be restored to the vesicle pool or recycled. The exact mechanisms by which synaptic vesicles could end up in autophagosomes remain elusive. The membrane source for the isolation membrane could be the ER, the plasma membrane, mitochondria, or potentially even the synaptic vesicles. Fully formed autophagosomes could have two fates: either to be retrogradely transported to the cell soma and fuse with lysosomes or to locally fuse with the lysosome. Lysosomal fusion will result in a breakdown of material that has been autophagosed, and the resultant amino acids and lipids can be released back in to the cytoplasm.

of the autophagosome, a completely closed-off vesicle structure that has engulfed cytoplasmic elements and organelles; and (3) fusion of the autophagosome with the lysosome.

The origin of the membrane that contributes to the formation of the preautophagosome has been a matter of intense study and debate. Studies have shown that diverse organelles including the ER, Golgi, and plasma membrane can be the source of autophagic membranes (Bento et al., 2016). Ultimately, it may simply be a case of the most accessible or abundant source of membrane near where autophagy is taking place. At synapses, the main source of autophagic membrane is not known, although the ER has been suggested for autophagosomes formed at distal axons (Maday and Holzbaur, 2014). The large synaptic vesicle pool may also be good candidate, but this has not been investigated (Fig. 2).

The ULK1/Atg1 complex, comprising the kinase ULK1/2, Atg13, and FIP200, is the first autophagy complex formed upon the induction of autophagy (Fig. 1). The ULK1/Atg1 complex recruits a phosphatidylinositide 3-kinase class III kinase complex comprised of Beclin-1, Vps15, Vps34, and Atg14L to the growing isolation membrane or phagophore. This complex leads to an enrichment of PtdIns3P within the growing membrane and the recruitment of proteins such as Atg18/WIP2, which then brings the Atg12-Atg5-Atg16L complex to the membranes. The Atg12-5-16L complex functions as a ubiquitin ligase–like enzyme that conjugates proteins of the Atg8/LC3 family to the lipid phosphatidylethanolamine. Atg8 is initially processed by Atg4 and Atg7 and finally by an E2-like enzyme, ATG3, which coordinates with the Atg12-5-16L complex to conjugate Atg8 to phosphatidylethanolamine on the growing autophagosomal membrane. Once Atg8/LC3 is lipidated and incorporated into the phagophore membrane, the growing membrane engulfs cytoplasmic elements as it enlarges and closes in on itself. Although autophagic engulfment is generally a nonselective process, certain adaptor proteins such as p62 and optineurin can direct specific cargoes to the preautophagosome (Johnson et al., 2012). The autophagosome will fuse with the lysosome, where its contents can be degraded and the constituent building blocks, for example amino acids and lipids, can be released back into the cytoplasm. It is important to realize that many other proteins are involved in the process of autophagy, and the interactions between Atg proteins are inevitably more complex than what is presented as a simplified, canonical pathway in Fig. 1.

Neuronal and synaptic autophagy

In the 1960s, nerve crush studies on the rat sciatic nerve established the presence of autophagic vesicles within the nervous

Assays for the study of autophagy

Transmission electron microscopy offers the best resolution to view various stages of autophagy. Transmission electron microscopy studies have been able to visualize within neurons growing phagophores to fully formed double-membrane autophagosomes with cargo inside (Nixon et al., 2005; Petralia et al., 2013). Work from our laboratory has used correlative light and electron microscopy to colocalize fluorescently tagged Atg proteins with autophagic vesicles at synapses (Soukup et al., 2016; Vanhauwaert et al., 2017).

Lipidated Atg8/LC3 is commonly used as a marker of autophagosome formation and autophagy in general. An Atg8 with a fluorescent tag (for example, RFP-Atg8) changes from a diffuse distribution to be localized in punctae that mark autophagosomes. A tandem-tagged Atg8 (mCherry-GFP-Atg) is also commonly used (Kimura et al., 2007). In this case, the autophagosome has both GFP and mCherry fluorescence, but upon fusion with the lysosome, the GFP fluorescence is quenched within the acidified environment of the autolysosome, whereas the mCherry fluorescence remains. Therefore, the change in red signal over yellow can be used to monitor the rate of autophagy or autophagic flux. The nonprocessed Atg8 (Atg8-I) and the lipidated form (Atg8-II) can also be tracked by immunoblotting, because Atg8-II migrates faster in polyacrylamide gels than Atg8-I, and therefore, two distinct bands are detected. When autophagy is upregulated, higher levels of Atg8-II can be detected. Other proteins can also be used to track different stages of autophagy. Atg5 and WIPI2 localize to early growing autophagosomes (Dooley et al., 2014), whereas STX17 is detected on mature, closed autophagosomes (Takáts et al., 2013). Finally, during autophagy, lysosomal markers such as LAMP1 also have increased punctate staining that marks autolysosomes (Juhász et al., 2008; Soukup et al., 2016). Given the variations in autophagy from cell to cell, it is critical that multiple markers be used.

It is important to assess whether the measured levels of autophagy (autophagic flux) are different because of a change in autophagosome biogenesis or in autophagosome degradation rates. Therefore, Atg8 levels are often assessed in the presence of lysosomal inhibitors such as bafilomycin A1 or chloroquine. If decreased autophagosome biogenesis causes fewer Atg8 punctae, then a block in degradation by inhibitors would have no effect; if it is caused by increased degradation, then the number of punctae will increase. A comprehensive survey of methodologies available to monitor autophagy is regularly published (Klionsky et al., 2016) that helps set community-wide standards for studying autophagy.

system (Holtzman and Novikoff, 1965). Despite the early start, our knowledge of autophagy within the nervous system has lagged, especially with regard to tissues such as the liver, where autophagy has been extensively studied (Boland and Nixon, 2006). Neuronal structure is unique, with a distinct cell soma and dendrites and axons with presynaptic regions; accordingly, neuronal autophagy is highly compartmentalized (Maday and Holzbaur, 2016). Within the soma, there is a population of autophagic vesicles derived from the axon and synapse that are distinct from the autophagic vesicles generated in the cell body (Maday and Holzbaur, 2016). In axons, autophagosomes form continuously at distal tips and contain cargoes derived from synapses (Maday et al., 2012; Maday and Holzbaur, 2014, 2016; Wang et al., 2015). Autophagosomes or autolysosomes are transported to the soma in a dynein-dependent manner (Hollenbeck, 1993; Cheng et al., 2015; Fig. 2). Here we explore how autophagy may be triggered within the nervous system, what physiological functions it performs, and finally, the role of autophagy in the context of aging and neurodegenerative conditions. Among these, the role of autophagy during the physiological or "normal" functioning of the brain remains the most enigmatic.

Autophagy during neuronal development

The severe degenerative phenotypes and neonatal lethality observed in *Atg*-knockout mice points to the importance of autophagy (Kuma et al., 2004; Komatsu et al., 2005; Saitoh et al., 2008; Sou et al., 2008; Malhotra et al., 2015). The loss of *Atg7* or *Atg5* specifically in the brain causes neurodegeneration and the formation of inclusion bodies that have intracellular protein aggregates (Hara et al., 2006; Komatsu et al., 2006). These are likely caused by a developmental reduction of autophagy, since the genes were conditionally deleted in the brain during embryonic development using the Nestin-cre driver. In *Drosophila melanogaster*, mutations in several *Atg* genes (1, 2, 6, and 18) result in significant reductions in the number of neuromuscular junction boutons (Shen and Ganetzky, 2009; Wairkar et al., 2009). Conversely, overexpression of *Atg1*, which can induce ectopic autophagy (Scott et al., 2007) results in a severe overgrowth of synapses (Shen and Ganetzky, 2009). However, it is unclear whether autophagy is needed specifically at synapses for synaptic development. Shedding some light on this issue, recent work demonstrated that during *Caenorhabditis elegans* neurodevelopment, the localization of *Atg9* to specific sites in an axon and the consequent formation of autophagosomes are required for proper synapse formation at those sites (Stavoe et al., 2016). On the postsynaptic side, lack of autophagy because of a loss of *Atg7* leads to a synaptic pruning deficit that results in an overabundance of dendritic spines in forebrain neurons in vivo and causes autism-like phenotypes (Tang et al., 2014). These studies suggest that autophagy has an important role in synaptogenesis and neurodevelopment that needs to be further elucidated.

Signals inducing neuronal autophagy

Metabolic regulation of neuronal autophagy. One key question is how neuronal autophagy may be triggered. Autophagy is a catabolic process that can respond to a lack of amino acids by degrading proteins; therefore, autophagy is strongly regulated by metabolic pathways that sense nutrient levels. Starvation has classically been used as a means to experimentally induce autophagy; for example, starvation causes the liver to rapidly up-regulate autophagy. However, the links between starvation and autophagy in the brain are less clear. In mice, even after 48 h of starvation, there was no detectable up-regulation in autophagy in the brain as measured by the presence of GFP-LC3 dots. In contrast, there was a strong induction of autophagy in the liver, muscle, heart, and other tissues (Mizushima et al., 2004). In contrast, other work has shown that food restriction can up-regulate autophagy in cortical, Purkinje, and hypothalamic neurons (Alirezaei et al., 2010; Kaushik et al., 2011) or *Drosophila* motor neurons (Soukup et al., 2016). In vitro studies of cultured neurons have also yielded disparate results. Recently, it was shown that metabolic starvation of cultured hippocampal neurons does not induce autophagy (Maday and Holzbaur, 2016), but earlier work with cortical neuron cultures indicated otherwise (Boland et al., 2008; Young et al., 2009). Differences in cell types and starvation protocols may contribute to these contradictory results. A key node within the cellular pathway that links metabolism and autophagy is the kinase complex mTORC1 (Shimobayashi and Hall, 2014). mTORC1 inhibits autophagy by the phosphorylation of ULK1/Atg1, and nutrient deprivation relieves the inhibition, thus triggering autophagy. As with starvation, there is conflicting in vivo and in vitro evidence as to whether mTORC1 inhibition can induce autophagy in the nervous system (Ravikumar et al., 2004; Fox et al., 2010; Tsvetkov et al., 2010; Roscic et al., 2011; Maday and Holzbaur, 2016). It is likely that different cell types in the brain may be set up to respond differently to stimuli such as starvation or mTORC1 inhibition. Additionally, mTORC1-independent pathways may exist (Fig. 1; Egan et al., 2011).

Regulation of autophagy by neuronal activity. Acute stimuli such as neuronal activity can also transiently up-regulate autophagy. At the *Drosophila* neuromuscular junction, high-frequency stimulation results in a rapid increase in Atg8 (LC3) puncta formation at presynaptic terminals (Soukup et al., 2016; Vanhauwaert et al., 2017), whereas in rat hippocampal neurons, neuronal stimulation induces autophagosome formation pre- and postsynaptically (Shehata et al., 2012; Wang et al., 2015). Given that neuronal activity is a fundamental property of the brain, the study of neuronal stimulation–induced autophagy may be more physiologically relevant than starvation-induced autophagy. It will be important to uncover how neuronal firing induces synaptic autophagy. One potential mechanism is calcium signaling. At a synapse, an action potential induces a drastic increase in calcium concentration, which drives exocytosis (Rizzoli, 2014). However, autophagy can be either inhibited or activated by calcium, based on the experimental context (Johnson et al., 2012). Therefore, the specific effect of calcium on autophagy at the synapse needs to be investigated. Several calcium-sensing proteins exist at the synapse, for e.g., the synaptotagmins (Südhof, 2013), but none of them have been studied in the context of autophagy.

Autophagy at the synapse and its effect on synaptic function

Within the presynaptic region of dopaminergic neurons, basal and induced autophagy can reduce the kinetics of neurotransmitter release and the density of synaptic vesicles (Hernandez et al., 2012). The modulation of vesicle numbers or evoked neurotransmitter release by presynaptic autophagy could therefore potentially contribute to synaptic plasticity mechanisms such as synaptic potentiation and depression. Autophagy has also been implicated in the degradation of postsynaptic receptors such as inhibitory GABAA receptors and AMPAR receptors, thereby inducing synaptic long-term depression (Rowland et al., 2006; Shehata et al., 2012). mTORC1 has been implicated in synaptic plasticity, but largely in the context of its ability to regulate protein synthesis (Casadio et al., 1999; Tang et al., 2002). However, mTORC1-mediated protein degradation by autophagy could play an equally important role and deserves to be studied in better detail. Along with removing defective proteins, autophagy is also important for transporting extracellular cargo internalized at synapses during endocytosis back to the cell soma (Wang et al., 2015). Although we are just scratching the surface, these early studies show that autophagy can be used for specific roles at the synapse. What has not been clear is whether synaptic autophagy and autophagy in the soma are distinct forms of autophagy.

Recent studies suggest that this may be the case, because presynaptic proteins have now been shown to regulate autophagy, and these proteins are largely excluded from the soma. Presynapses have active sorting mechanisms regulated by Rab proteins that direct endocytosed vesicles back to the synaptic vesicle pool or toward the endosomal–lysosomal pathway (Wucherpfennig et al., 2003; Uytterhoeven et al., 2011; Fernandes et al., 2014). These sorting mechanisms can act to remove older, potentially defective, transmembrane synaptic vesicle proteins and rejuvenate the vesicle protein pool (Fernandes et al., 2014). Furthermore, Rab26 was found to be associated with both synaptic vesicles and autophagosomes (Binotti et al., 2015), hinting at a potential parallel synaptic trafficking pathway that recycles older vesicles and their associated proteins via autophagy as well.

Recent work from our laboratory and the Milosevic laboratory has shown that Endophilin A (EndoA), a protein highly enriched at presynapses, has an unexpected role in autophagy (Murdoch et al., 2016; Soukup et al., 2016). In addition, we also found that the EndoA binding partner Synaptojanin 1 (Synj1), a protein also enriched at synapses, can regulate autophagy (Vanhauwaert et al., 2017), corroborating data from zebrafish (George et al., 2016). Both EndoA and Synj1 have well-established roles in synaptic vesicle endocytosis. During synaptic vesicle endocytosis, EndoA is recruited to clathrin-coated pits, which then recruit Synj1 to uncoat the vesicle after fission from the membrane (Song and Zinsmaier, 2003). However, it is now clear that they have additional, independent roles as well to regulate autophagy at the synapse.

Within the *Drosophila* neuromuscular junction presynaptic endings, EndoA is required for starvation, and neuronal stimulation induced increases in Atg8-mCherry and Lamp-GFP punctae (Soukup et al., 2016). The Atg8-positive punctae at the synapse were shown to be associated with large membranous structures dubbed "synaptic autophagosomes" using correlative light and electron microscopy. Furthermore, the phosphorylation of EndoA at serine 75 by the LRRK2 kinase, shown previously to happen at the synapse (Matta et al., 2012; Arranz et al., 2015), selectively activates synaptic autophagy. A phosphomimetic EndoA ($Endo^{S75D}$) or an active LRRK2 (G2019S) increases Atg8 and Lamp markers even in the fed state. Furthermore, phosphorylated EndoA recruits Atg3 to membrane and causes it to colocalize with Atg8. Because EndoA is capable of sensing and inducing membrane curvature (Farsad et al., 2001; Gallop et al., 2006), the proposed model suggests that EndoA may be creating docking sites for Atg3 on the growing autophagosomes (Fig. 1).

The lipid phosphatase Synj1 promotes the uncoating of endocytosed synaptic vesicles by dephosphorylating PI(4,5)P_2 through the actions of its 5-phosphatase domain (Cremona et al., 1999; Verstreken et al., 2003). Synj1 also has another domain, the SAC1 domain, that dephosphorylates different phosphoinositides, including PI(3)P and PI(3,5)P_2 (Guo et al., 1999), two phosphoinositides essential during the formation of autophagosomes (Noda et al., 2010; Dall'Armi et al., 2013). Recently, two Parkinson's disease (PD) mutations within SAC1 domain were described, and one of them has been studied in detail. The R258Q mutation blocks the ability of Synj1 to dephosphorylate PI(3)P and PI(3,5)P_2 (Krebs et al., 2013). Likely, the more recently identified mutation R459P that also resides in the SAC1 domain (Kirola et al., 2016) also affects the dephosphorylation of these phosphoinositides, but that remains to be tested. In *Drosophila* and patient induced pluripotent stem cell–derived human neurons, this mutation severely reduces autophagic flux at synapses upon starvation or neuronal stimulation (Vanhauwaert et al., 2017). In contrast, it does not have overt effects on synaptic transmission at fly neuromuscular junctions. In mouse, a R258Q knock-in mutation does appear to affect synaptic vesicle endocytosis, but only during mild stimuli, not during regular or intense activity (Mani et al., 2007; Cao et al., 2017). Whether the mouse knock-in neurons display defects in synaptic autophagy, similar to the patient-derived neurons, has not yet been assessed. One of the effects of the lack of dephosphorylation of phosphoinositides in R258Q mutants is the presynaptic accumulation of Atg18a, a member of the PROPPIN domain–containing protein family that binds PI(3)P and PI(3,5)P_2 and has a role in autophagy (Dove et al., 2004; Baskaran

et al., 2012). Therefore, Synj1 dephosphorylation is required for Atg18a to leave nascent autophagosomes. The data suggest a model by which the cycling of Atg18a onto and then off of the autophagosomal membranes, mediated by Synj1, aids in the concentration of Atg8 and the formation of mature autophagosomes (Fig. 1; Vanhauwaert et al., 2017). The loss of EndoA and Synj1 function also results in an activity-dependent degeneration of photoreceptor neurons in *Drosophila* (Soukup et al., 2016; Vanhauwaert et al., 2017). Furthermore, both the EndoA phosphomutants and the Synj1 mutation result in the death of dopaminergic neurons in *Drosophila* brains at 30 days of age. These observations are interesting because the R258Q mutation in Synj1 causes PD (Krebs et al., 2013; Quadri et al., 2013; Olgiati et al., 2014), and the kinase LRRK2 that phosphorylates EndoA to induce autophagy is also mutated in PD (West et al., 2005; Greggio et al., 2006). Dopaminergic neurons also degenerate in PD (Fearnley and Lees, 1991), and the data therefore suggest a role for synaptic autophagy defects in the pathogenic cascade of PD.

In contrast to the autophagy-promoting roles of EndoA and Synj1, another presynapse-specific protein, Bassoon, has a role in actively inhibiting autophagy. Bassoon is known to play a key role in organizing the presynaptic active zone from which docked synaptic vesicles are released (Ackermann et al., 2015). Okerlund et al. (2017) found that the loss of Bassoon in cultured hippocampal neurons results in the up-regulation of ATG8 puncta at synapses, and an increased number of autophagic vesicles can be seen via EM. Furthermore, Bassoon inhibits autophagy by binding to Atg5, an E3-like ligase that is crucial for the attachment of LC3 to autophagosomes (Fig. 1). The loss of Bassoon also results in the reduction of a synaptic vesicle marker, suggesting that Atg5-driven autophagy results in the degradation of synaptic vesicles (Okerlund et al., 2017).

These new results suggest that synapse-enriched proteins have evolved to interact with Atg proteins to regulate a form of autophagy at synapses that uses the same core Atg components but is regulated by these synaptic proteins. Interestingly, these proteins have functional links to both vesicle cycling and autophagy. It is tempting to speculate that they may be part of a system that senses the amount of vesicle recycling taking place and triggers autophagy if there is a buildup of dysfunctional components.

Autophagy and neuronal dysfunction

Loss of protein quality control is highly correlated with aging and age-related neurodegenerative diseases (López-Otín et al., 2013; Labbadia and Morimoto, 2015). In both these pathological contexts, synaptic failure is a common feature that is manifested much earlier than neuronal loss within the brain. Commonly seen phenotypes such as memory deficits and loss of motor control can be traced back to structural and functional changes at synapses (Morrison and Baxter, 2012; Yeoman et al., 2012; Petralia et al., 2014). These changes can severely disrupt neuronal circuits, reduce coordination between brain areas, and cause the brain-wide abnormalities seen in aging or diseased human brains (Andrews-Hanna et al., 2007; Bishop et al., 2010). Because a large number of proteins are required for synaptic function (Rizzoli, 2014), it stands to reason that dysregulated protein homeostasis caused, at least in part, by alterations in autophagy is what leads to the synaptic phenotypes in disease and aging. Regular autophagy seems to decline with age (Rubinsztein et al., 2011). In *Drosophila*, autophagosomes and assorted multivesicular bodies accumulate within presynapses in old flies, indicating a defect in autophagosome clearance via lysosomal fusion (Beramendi et al., 2007), whereas in the human brain, expression of key autophagy genes is reduced during aging (Lipinski et al., 2010). Conversely, a growing list of studies have demonstrated that up-regulation of autophagy increases lifespan and neuronal health in many organisms (Harris and Rubinsztein, 2011).

A role for autophagy in mediating the beneficial effects of caloric restriction? A large body of evidence shows that one of the strongest modulators of aging and longevity in organisms from worms to monkeys is caloric restriction (CR; Blagosklonny, 2012; Fontana and Partridge, 2015). In humans, CR can improve memory (Witte et al., 2009), which may be a result of improved synaptic plasticity. CR affects many pathways in the body, but one of its major effects is the up-regulation of autophagy (Donati, 2006; Bergamini et al., 2007; Hansen et al., 2008). Notably, as discussed earlier, starvation is used widely as a tool to induce autophagy. Potential molecular links between CR and autophagy are the mTOR and AMPK pathways (Kapahi et al., 2010; Cantó and Auwerx, 2011), both of which probably function at the synapse in certain neurons (Yang et al., 2011; Shen et al., 2015). AMPK plays an important role in energy homeostasis by sensing adenosine nucleotide levels (Mihaylova and Shaw, 2011) and can directly phosphorylate and activate the autophagy-initiating kinase, ULK1/Atg1 (Egan et al., 2011; Kim et al., 2011). mTORC1 actively inhibits autophagy, and the inhibition can be relieved by the mTORC1 inhibitor rapamycin, which has been repeatedly shown to increase the lifespan of animal models (Bjedov et al., 2010; Wilkinson et al., 2012; Bitto et al., 2016). Excitingly, even transient rapamycin treatment given late in adulthood is capable of producing beneficial lifespan effects (Bitto et al., 2016). The widespread benefit of CR is one of the strongest indications that autophagy may have incredible therapeutic potential, but the links between CR, autophagy, and improved synaptic and neuronal outcomes are mostly correlational in nature at this point. Therefore, it will be important to understand the exact molecular mechanisms that link these phenomena.

Modulating autophagy to improve synaptic function during aging. In the mouse retina, age-related changes to both the synaptic structure of photoreceptor neurons (Liets et al., 2006; Terzibasi et al., 2009; Samuel et al., 2011) and synaptic function (Kolesnikov et al., 2010; Samuel et al., 2011) have been documented. Recent work has shown that reducing AMPK signaling also results in similar synaptic defects, whereas these defects can be reduced by increasing AMPK activity through genetic means, CR, or administering the AMPK-activating drug metformin (Samuel et al., 2011). Although it was not explicitly tested, it seems possible that AMPK could induce some of these synaptic changes through up-regulation of autophagy. Similarly, age-related changes seen at the neuromuscular junction synapses of mice (Jang and Van Remmen, 2011; Carnio et al., 2014) can also be improved by caloric restriction and exercise (Valdez et al., 2010).

Administering spermidine, a natural polyamine, increases the lifespan in yeast, worms, flies, and human cells in an autophagy-dependent manner (Eisenberg et al., 2009). A pair of studies in *Drosophila* have elegantly shown that spermidine can specifically affect synapses to improve neuronal outcomes. In aged *Drosophila* (30 d), age-induced memory impairment can be ameliorated by spermidine feeding, whereas

this improvement is lost in flies in which key autophagy genes *Atg7* and *Atg8* were deleted (Gupta et al., 2013). Furthermore, the authors followed up by showing that age-induced memory impairment is connected to an age-related increase in the level of synaptic proteins that results in structural changes at the presynaptic active zone, concomitantly leading to enhanced neurotransmitter release (Gupta et al., 2016). These age-related protein changes are reversed when spermidine is added to the diet of flies. These studies seem to suggest that modulations that up-regulate autophagy can remedy the age-related changes occurring at synapses. Once again, a better understanding of molecular mechanisms could potentially mean that we can modulate synaptic autophagy directly without resorting to organism-wide interventions such as caloric restriction.

Autophagy as a means to combat synaptic dysfunction in Alzheimer's and Parkinson's diseases. A common hallmark of Alzheimer's disease (AD), PD, and other neurodegenerative conditions is the presence of protein aggregates inside or outside the cell (Ross and Poirier, 2004). Whether these aggregates represent the loss of protein quality control or are the remnants of the cellular attempt to restore homeostasis remains a matter of debate. Autophagy has also been linked to several neurodegenerative diseases (Nixon, 2013). All the autophagy modulations discussed above—from CR to spermidine—positively impact animal models of AD and PD. Therefore, detailed knowledge about autophagic processes that impact the synapse is critically required for a better understanding of disease etiology and to develop potential therapeutic strategies.

AD is the most common cause of dementia. It is characterized by the buildup of two forms of protein aggregates: amyloid plaques mostly outside neurons and neurofibrillary tangles within neurons. Amyloid plaques are composed primarily of β-amyloid protein, and neurofibrillary tangles contain the microtubule-binding protein tau. AD is a synaptopathy in which failure of the synapse precedes neuronal dysfunction and eventual loss of the neuron (Selkoe, 2002; Oddo et al., 2003; Gouras et al., 2010). Intracellular neurofibrillary tangles are formed when hyperphosphorylated tau comes off microtubules and accumulates within or near synapses and may cause synaptic dysfunction (Pooler et al., 2014). In animal and cell models of tauopathies, increased autophagy can remove toxic tau aggregates (Berger et al., 2006; Rodríguez-Navarro et al., 2010), which may have important therapeutic significance because a reduction in all forms of tau reduces neurodegeneration (Santacruz et al., 2005; Roberson et al., 2007; Lasagna-Reeves et al., 2016). Up-regulating autophagy at the synapse could therefore eliminate early tau isoforms that could be driving the synaptic dysfunction. In line with this, inhibiting mTORC1, by rapamycin or other means, reduces AD-related cognitive and synaptic deficits in several AD animal model studies (Ma et al., 2010; Spilman et al., 2010; Caccamo et al., 2013; Ozcelik et al., 2013). One potential complication is that changes in autophagy itself have been linked to the progression of AD. A build-up of autophagic vacuoles was seen within swollen neurites, including synapses, of AD patient brains and AD mouse models (Boland et al., 2008; Nixon and Yang, 2011). This suggests that there is a defect in autophagosome maturation and fusion with the lysosome. Other data suggest that autophagy induction and autophagosome biogenesis/maturation may be reduced in AD (Pickford et al., 2008; Rohn et al., 2011). Therefore, one can imagine a situation in which autophagy may be reduced in the initial stages of the disease, driving a build-up of harmful proteins. The cell then attempts to clear via increased autophagy, which then fails, further compounding the disease pathology.

PD is characterized by a loss of motor control, dementia, and other pathophysiologies. Like AD, a growing list of studies describing synaptic dysfunction in PD reflects the fact that PD, like AD, can be considered a synaptopathy (Plowey and Chu, 2011). Although a majority of PD cases are idiopathic, PD has been linked specifically with several dysfunctional proteins, among which α-synuclein, LRRK2, PINK1, and PARKIN have attracted the most amount of scientific scrutiny (Bonifati, 2014). Mutations that enhance the kinase activity of LRRK2 are linked to PD (West et al., 2005; Greggio et al., 2006). LRRK2 functions at the synapse (Piccoli et al., 2011; Matta et al., 2012; Arranz et al., 2015) and is also linked to the regulation of autophagy (Plowey and Chu, 2011). We recently demonstrated that LRRK2-dependent phosphorylation of EndoA is required for autophagy at *Drosophila* synapses (as described in the section Autophagy at the synapse and its effect on synaptic function; Soukup et al., 2016). Therefore, LRRK2 dysregulation in PD may drive alterations in synaptic autophagy, starting a cascade leading to synaptic defects in PD. Like LRRK2, two other PD-related genes, Parkin and Synaptojanin, also interact with EndoA (Trempe et al., 2009; Krebs et al., 2013; Quadri et al., 2013; Cao et al., 2014), suggesting that EndoA may be a crucial node in the network linking PD with autophagy regulation at the synapse. Finally, many PD cases are caused by increased α-synuclein expression and aggregation into Lewy bodies. α-Synuclein functions at the synapse, and increased expression results in decreased neurotransmitter release and decreased autophagy (Chandra et al., 2005). Conversely, genetic reduction in autophagy by the deletion of Atg7 increases α-synuclein at the synapse and within Lewy bodies (Friedman et al., 2012).

Overall, three major concepts emerge when considering the connection between autophagy and synaptic dysfunction. In aging and disease, autophagy is reduced, defective proteins accumulate, and synapses become dysfunctional. In some cases, synaptic pathologies are caused by defective proteins directly disturbing normal autophagy. Finally, clearing defective proteins by increasing autophagy could help ameliorate synaptic deficits seen in aging and neurodegenerative diseases. Because synaptic dysfunction is an early phenomenon in pathological brain states, autophagy could be an important strategy for targeting diseases at a nascent stage where neuronal loss has not yet occurred and reversibility of phenotypes is feasible. Any specific therapeutic strategy will be contingent on whether the disease state directly affects autophagy. If not, then autophagy up-regulation may be useful. However, if autophagy is itself inhibited, any up-regulation could exacerbate the disease pathology by causing a buildup of autophagy intermediates (Fig. 3).

Conclusions and perspective

There is growing appreciation that autophagy plays distinct roles in different tissues and that its role in the nervous system is unique. However, very little is known about the role of autophagy during normal neuronal function and behavior. Evidence shows that neuronal firing and synaptic activity trigger autophagy. But what role does autophagy play at the synapse after it is triggered? It is known that specific patterns of synaptic activity trigger changes to the synapse that will modify its future activity. This type of activity-dependent change is crucial for processes such as memory encoding and the learning of

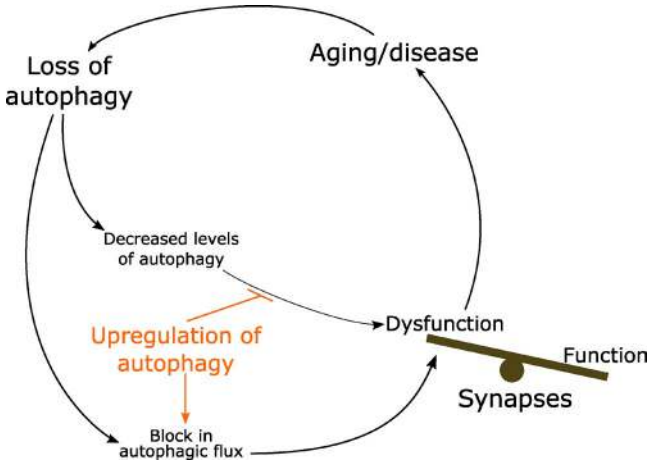

Figure 3. **Potential connections between autophagy and synaptic dysfunction in aging and disease.** Synaptic dysfunction and a reduction of autophagy are both common phenomena in aging and age-related neurodegenerative diseases. Therefore, it is possible that synaptic dysfunction is driven, in part, by a reduction in autophagy at the synapse. The up-regulation of autophagy has been considered a potentially beneficial therapeutic strategy. However, it is crucial to analyze whether the loss of autophagy in a particular context is caused by lower levels of autophagy induction or a block in autophagic flux, i.e., the cycle of autophagosome generation and subsequent fusion with the lysosome. In the first case, up-regulation of autophagy is useful because it can counter the lowered levels of autophagy. However, in the second case, the up-regulation of autophagy could exacerbate the disease state because of a buildup of autophagic intermediates that are not cleared because of the block in autophagic flux.

new behaviors. One possibility that remains to be investigated is that autophagy is one of the mechanisms used to effect activity-dependent synaptic change. Another scenario is that autophagy is useful to ameliorate the toxicity resulting from the intense, repeated process of exo- and endocytosis. If so, it will be important to understand how the autophagy machinery detects the levels of toxicity and how dysfunctional proteins are marked for degradation by autophagy.

As with many other fields, our ability to answer key questions is contingent on the development of better experimental tools (see Assays for the study of autophagy). For example, neuroscience research has been revolutionized by the development of excellent tools in two categories. One set of tools allows for the activation and inactivation of a specific set of neurons (i.e., optogenetics and chemogenetics; Aston-Jones and Deisseroth, 2013). The other set are sensors that let researchers monitor neuronal activity, often in real time and in vivo (Rose et al., 2014). Similarly, autophagy research would benefit from methodologies able to regulate autophagy in a temporally and spatially restricted manner, thereby allowing us to investigate autophagy at the level of specific synapses or neurons. Furthermore, a tool for inducing autophagy would allow us to definitively test whether the induction of autophagy can ameliorate synaptic defects seen in neurodegenerative conditions. Interestingly, a small peptide derived from the domain of Bassoon that inhibits Atg5 was able to inhibit synaptic autophagy (Okerlund et al., 2017) and could be developed into a synapse-specific autophagy inhibitor. This finding also highlights the importance of identifying region- or compartment-specific (for example, presynapse) modulators of autophagy. Finally, the development of sensors that can accurately measure autophagic flux in vivo or in real time is critical. Sensors that are more sensitive will be important for testing how specific interventions or drugs can modulate autophagy. Furthermore, we need sensors or markers of autophagy that can be applied in humans. These tools will accelerate our understanding of autophagy within the nervous system.

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