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REVIEW

The Role of Autophagy in Excitotoxicity, Synaptic Mitochondrial Stress and Neurodegeneration

Charleen T. Chu

Department of Pathology/Division of Neuropathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

ABSTRACT

Brain and nervous system functions depend upon maintaining the integrity of synaptic structures over the lifetime. Autophagy, a key homeostatic quality control system, plays a central role not only in neuronal development and survival/cell death, but also in regulating synaptic activity and plasticity. Glutamate is the major excitatory neurotransmitter that activates downstream targets, with a key role in learning and memory. However, an excess of glutamatergic stimulation is pathological in stroke, epilepsy and neurodegeneration, triggering excitotoxic cell death or a sublethal process of excitatory mitochondrial calcium toxicity (EMT) that triggers dendritic retraction. Markers of autophagy and mitophagy are often elevated following excitatory neuronal injuries, with the potential to influence cell death or neurodegenerative outcomes of these injuries. Interestingly, leucine-rich repeat kinase 2 (LRRK2) and PTEN-induced kinase 1 (PINK1), two kinases linked to autophagy, mitophagy and Parkinson disease, play important roles in regulating mitochondrial calcium handling, synaptic density and function, and maturation of dendritic spines. Mutations in LRRK2, PINK1, or proteins linked to Alzheimer's disease perturb mitochondrial calcium handling to sensitize neurons to excitatory injury. While autophagy and mitophagy can play both protective and harmful roles, studies in various excitotoxicity and stroke models often implicate autophagy in a pathogenic role. Understanding the role of autophagic degradation in regulating synaptic loss and cell death following excitatory neuronal injuries has important therapeutic implications for both acute and chronic neurological disorders.

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I. Excitatory neuronal injuries

It has been recognized for the past 70 years that excess stimulation of the nervous system by the excitatory amino acid glutamate elicits neuronal

CONTACT Charleen T. Chu ctc4@pitt.edu Department of Pathology/Division of Neuropathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

injury. In the 1950s, researchers discovered that administration of glutamate to the brain or retina caused seizures and cell death [1,2]. In subsequent years, it was established that glutamate functions as the major endogenous excitatory neurotransmitter in the brain. Glutamate binds to three classes of receptors that function as ion channels, with differing sensitivities to exogenously applied agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainic acid (KA). Glutamate also interacts with G protein-coupled metabotropic receptors and both plasma membrane and vesicular glutamate transporters. As the molecular nature of ionotropic glutamate receptors was elucidated [3], studies in the 1980's definitively showed that glutamate receptor antagonists could ameliorate ischemic brain injuries [4,5]. More recently, it has become clear that glutamate-dependent postsynaptic neurotoxicity also contributes to chronic neurodegenerative diseases [6].

Excitotoxicity was initially implicated in epilepsy and ischemic brain injuries, commonly referred to as strokes. Excitotoxic cell death is also observed during hypoglycemic brain injury [7]. A growing body of literature implicates excitatory mechanisms in multiple chronic neurological disorders, including Alzheimer disease (AD) [8,9], Parkinson disease (PD) [10,11], Huntington disease (HD) [12], and the amyotrophic lateral sclerosis-frontotemporal dementia ALS-FTD spectrum [13]. Whereas acute neuronal insults typically lead directly to cell death, chronic neurodegenerative diseases are more often characterized pathologically by dendritic degeneration and spine loss, as observed in AD, tauopathies, HD, ALS, prion diseases [14] and PD [15]. Interestingly, sublethal excitatory neuronal injuries often elicit dendritic atrophy in culture [10,16,17], potentially providing a mechanistic linkage between glutamatergic dysregulation and synaptic loss observed in these diseases.

In the following sections, I will review excitatory neuronal injury mechanisms observed in acute and chronic neurodegeneration with an emphasis on the mechanistic interplay with autophagy and mitophagy. Autophagy is the process by which cellular constituents are delivered to the lysosome for degradation; mitophagy refers to the selective targeting of mitochondria for autophagic degradation. After reviewing common methods used to monitor autophagy and mitophagy, I will summarize the literature linking autophagy and mitophagy, or associated proteins, to excitatory injuries. Finally, the impact of autophagy or mitophagy on the outcome of excitatory neuronal injuries will be evaluated and discussed with respect to cell death, dendritic/neuritic retraction and behavioral measures.

IA. Excitotoxic cell death

The intracellular mechanisms that lead to cell death triggered by excess glutamate receptor ligation differ depending upon the dosage of glutamate receptor agonists utilized. Extremely high levels elicit massive influx of Na+ and Cl-, creating an osmotic stress that leads to plasma membrane rupture and necrotic cell death. In contrast, excitotoxicity triggered during epilepsy or hypoxic-ischemic injuries centers around large shifts in intracellular calcium levels [18]. As discussed below, although cell death during excitotoxicity may involve initiation of the intrinsic pathway of apoptosis, in practice, the morphology observed more closely resembles oncosis (the "red dead neuron") or necrotic cell death. Often described as showing biochemical features of apoptosis with ultrastructural features of necrosis, excitotoxic cell death *in vivo* falls along the apoptotic-necrotic continuum [19]. In addition, compartmentalized activation of necroptosis and mitophagy contributes to excitotoxic axonal and dendritic degeneration, respectively [16,17,20,21].

As a key molecule involved in cellular signaling, intracellular calcium is tightly controlled. Following the rapid, post-synaptic entry of extracellular calcium through the NMDA receptor and other calcium channels, calcium signal recovery is mediated by multiple mechanisms. These include ion channel inactivation, exchangers that pump calcium back outside the cell, and the rapid uptake of calcium into mitochondria, endoplasmic reticulum, and other intracellular stores. Physiologically regulated calcium influx is essential for synaptic transmission and activity-dependent transcriptional responses [22], acting to promote mitochondrial function [23] (Figure 1A). In contrast, the sustained high levels of cytosolic calcium observed during excitotoxic neuronal cell death serve to activate CAPN2/m-calpains, death associated protein kinase 1 and neuronal NO synthase, while suppressing trophic signaling through PKA-CREB [24] (Figure 1B).

Elevations in calcium and reactive oxygen species may trigger the mitochondrial permeability transition, leading to loss of mitochondrial antioxidants, release of mitochondrial calcium, and release of cytochrome *c* to trigger apoptotic cell death [25] (Figure 1B). Although the precise molecular basis for mitochondrial permeability transition remains unclear [26], the adenine nucleotide translocator is a key component and cyclosporin A can act to suppress or delay this transition by binding cyclophilin D. The linkage between mitochondrial calcium dysregulation and excitotoxic cell death is further supported by observations that GABAergic neurons are relatively resistant to both mitochondrial calcium uptake and cell death compared to motor neurons following AMPA receptor stress [27]. Localized activation of caspases [28,29] or autophagy/mitophagy [16] have also been reported to cause neurite retraction at the sublethal or prelethal stage of excitatory injuries.

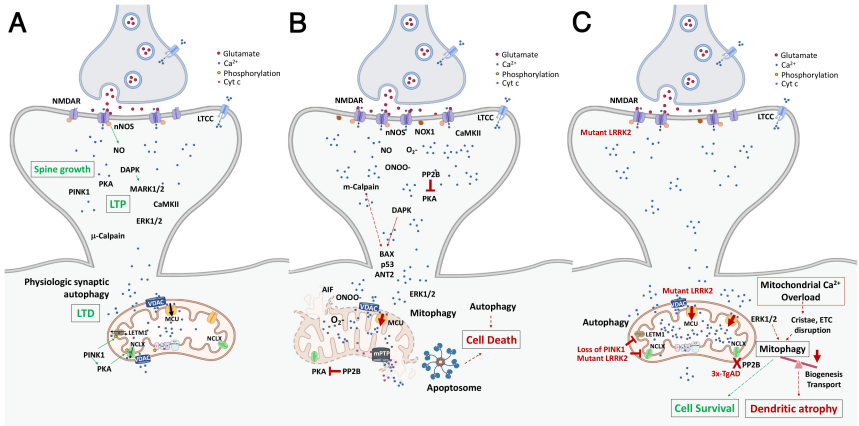

Figure 1. Calcium mishandling during excitatory neuronal injuries. (A) Under normal circumstances, the NMDA receptor (NMDAR) exist in a balance between open and closed states. The regulated influx of calcium serves to activate nNOS to produce nitric oxide (NO) and to activate kinases including CAMK2A/CaMKII, MAPK/ERK1/2, PKA, DAPK and MARK1/2, to regulate synaptic plasticity and long-term potentiation (LTP) or long-term depression (LTD). Mitochondrial calcium taken up by MCU and LETM1 is balanced by calcium release via SLC8B1/NCLX and LETM1. PINK1 directly or indirectly promotes phosphorylation and activation of PKA, SLC8B1/NCLX, LETM1, and complex I subunits, which may partially explain its ability to increase dendritic spine density and maturation [15]. Normal release of mitochondrial calcium from SLC8B1/NCLX plays a permissive role for PINK1-mediated mitophagy. CAPN1 (μ-calpain) is neuroprotective and required for synaptic plasticity. Among other effects, μ-calpain mobilizes Atg9 to facilitate autophagy [154]. Calcium-dependent activation of physiological synaptic autophagy mediates LTD [129,130]. (B) During classic excitotoxicity elicited by excessive glutamatergic signaling following agonist treatment or hypoxic-ischemic injury, overactivation of NMDARs results in massive influx of calcium, activating CAPN2 (m-calpain) and death associated protein kinase (DAPK) to promote cell death. Activation of neuronal nitric oxide synthase (NOS1/nNOS) by NMDAR and increased oxidative stress from plasma membrane NOX1/NADPH oxidase promote formation of peroxynitrite (ONOO-), which can diffuse to elicit mitochondrial electron transport chain (ETC) dysfunction and further production of superoxide (O^{2-}). At the mitochondrion, large post-synaptic calcium fluxes drive increased MCU-mediated calcium uptake into the mitochondrial matrix. Activation of PP2B/calcineurin inactivates PKA with loss of mitochondrial NCLX-mediated calcium efflux. These factors potentiate formation of the mitochondrial permeability transition pore (mPTP), with mitochondrial swelling and release of cytochrome c (Cyt c) and apoptosis-inducing factor (AIF) to elicit apoptotic or necrotic cell death depending upon metabolic status. In addition, autophagic degradation of ferritin potentiates ferroptotic cell death [117]. (C) It is increasingly recognized that excitatory injuries can also be triggered by predisposing conditions even under more physiological levels of excitatory activity. These conditions include upregulation of spontaneous excitatory synaptic activity as observed with LRRK2 mutations [10]. Voltage-gated calcium channels such as L-type calcium channels (LTCC) further increase calcium influx. Increased mitochondrial calcium uptake due to MCU upregulation downstream of LRRK2 mutations combined with dysregulated mitochondrial calcium efflux promote mitochondrial calcium overload. Decreased mitochondrial calcium efflux is caused by reduced SLC8B1/

IB. Excitatory mitochondrial calcium toxicity (EMT)

Whereas elevated calcium levels in classic excitotoxicity activates cytosolic enzymes that elicit cell death, in neurodegenerative diseases such as AD and PD, emerging evidence highlights disrupted post-synaptic mitochondrial calcium handling as a key injury mechanism [6] (Figure 1C). In these chronic neurodegenerative diseases, synaptic loss and shrinkage of the neuronal arbor are dominant features in human post-mortem brain tissues and in genetic models of familial neurodegeneration [14,15,30,31], with little evidence of active cell death. Nevertheless, glutamate hyperexcitability clearly contributes to synaptodendritic atrophy and cognitive dysfunction as evidenced by the protective effects of glutamate receptor antagonists [8,10,30,32].

Following excitatory synaptic stimuli, calcium floods into dendritic spines and adjacent shafts through ionotropic glutamate receptors. The magnitude of this change regulates the magnitude of learning-related changes of long-term potentiation and long-term depression in a U-shaped curve [33]. The mitochondrion plays an important role in the rapid, recovery of intracellular calcium levels after synaptic stimulation [34], taking up calcium into its matrix via the mitochondrial uniporter (MCU) complex, a low affinity, high-capacity calcium transporter [35]. This rapid mitochondrial uptake serves to buffer cytosolic calcium levels, and is balanced by a slower re-release of calcium back into the cytosol (Figure 1A). In excitable cells such as neurons and muscle, mitochondrial calcium release is primarily mediated by SLC8B1/NCLX, the mitochondrial sodium calcium lithium exchanger [36].

The process of EMT results from dysregulation of mitochondrial calcium uptake and release during spontaneous neuronal activity (Figure 1C). It was initially described in culture models of familial Parkinson's disease. Dominant genetic mutations in *LRRK2* were long understood to cause shortening and simplification of the dendritic arbor [37]. This dendritic atrophy is prevented by administration of

NCLX and LETM1 phosphorylation secondary to PINK1-deficiency, or due to indirect impacts of expressing mutant LRRK2 and familial AD mutations (3x-TgAD). Mitochondrial calcium overload alters matrix pH and disrupts cristae structure and ETC function [156], resulting in mitochondrial depolarization, calcium dysregulation and mobilization of cardiolipin to promote multiple mitophagy pathways [16,17,79] for removal of damaged mitochondria. Low levels of mitophagy result in cell survival, but mutations in PINK1, PRKN, MAPT or the age- or disease-related loss of PPARGC1A/PGC-1α result in impaired mitochondrial biogenesis [155]. Unopposed mitophagy causes net mitochondrial loss from dendrites, eliciting dendritic atrophy and pathological synaptic loss. Created in BioRender. Chu, C. (2025) https://BioRender.com/q54m591, and using Microsoft Powerpoint 16.77.1.

partial NMDA receptor antagonists [10] or by inhibiting L-type calcium channels [16], implicating calcium dysregulation. Moreover, expression of either LRRK2^{G2019S} or LRRK2^{R1441C} elicit selective depletion of dendritic, but not axonal, mitochondria through a mechanism involving elevated cytosolic calcium and autophagy [16]. As discussed in section IV, autophagy and mitochondrial loss contribute pathologically to dendritic retraction.

Fibroblasts from familial PD patients with LRRK2^{G2019S} or LRRK2R1441C show increased message and protein expression for MCU and one of its accessory proteins MICU1 [17]. Chemical inhibitors of MCU and RNAi targeting MCU prevents an increase in autophagic mitochondrial degradation and protects against dendritic atrophy, whereas blocking the activity of SLC8B1/NCLX exacerbates injury [17]. LRRK2 deletion or mutation also impairs the activity of SLC8B1/NCLX, lowering the threshold for secondary insults that elicit mitochondrial permeability transition [38]. These data show that mitochondrial calcium stress enhances susceptibility to neuron cell death, triggering mitochondrial degradation through mitophagy [17]. The balance of these processes may regulate the lethality of EMT-inducing stressors.

Loss of function mutations in the recessive PD-linked gene *PINK1* also elicit dendritic simplification [39-41], accompanied by loss of dendritic spines in the hippocampus *in vitro* [42] and in cortical neurons *in vitro* and *in vivo* [15]. In particular, the mature mushroom spines implicated in LTP are affected to a greater extent than less mature spines [15]. Inhibitors of MCU that are protective in the mutant LRRK2 model also confer protection in zebrafish models of PINK1-linked PD, which also show an upregulation of MICU1 [43]. In contrast, PINK1 knockout systems exhibit a deficit in mitochondrial calcium release, due to reduced SLC8B1/NCLX activity [44,45]. PINK1 binds to and promotes the phospho-activation of PKA [41]. PKA directly phosphorylates and activates SLC8B1/NCLX at S258D [44]. The SLC8B1/NCLX^{S258D} phosphomimic confers protection in both PINK1-deficient [44] and mutant LRRK2 models [17]. The ability of both PKA and SLC8B1/NCLX^{S258D} to prevent excitatory toxicity has been replicated following LRRK2 deletion, mutation or inhibition [38]. Although the role of LETM1, another mitochondrial calcium transporter, in extruding mitochondrial calcium is controversial [46,47], phosphorylation of LETM1 by PINK1 at T192 protects against toxicity mediated by the PD toxin 1-methyl-4-phenylpyridinium [48]. Taken together, these studies implicate mitochondrial calcium overload due to unbalanced mitochondrial calcium import and export in sublethal EMT as well as in cell death triggered by the mitochondrial permeability transition.

In addition to Parkinson's disease, EMT has been observed in amyloid precursor protein models of Alzheimer's disease *in vitro* and *in vivo* [49]. The power of Drosophila genetics was harnessed to study the role of EMT-related processes in multiple Drosophila models of neurodegenerative

diseases, including *Pink1* knockout for PD, *Abeta*$_{42}$ transgenic for AD, *HTT93Q* transgenic for HD and *Tau*^{R406W} transgenic for FTD [50]. In each of these Drosophila models, suppressing MCU activity or enhancing SLC8B1/NCLX expression proved to be beneficial [50]. This *in vivo* study elegantly shows the therapeutic possibilities of modulating EMT for multiple neurodegenerative diseases.

II. Macroautophagy and mitophagy in neurons

Macroautophagy is a homeostatic process conserved across eukaryotic cells by which effete, unneeded or damaged intracellular macromolecules or organelles are targeted into acidic lysosomal compartments for degradation, allowing for cellular renewal and replacement by newly synthesized components. While avian and mammalian cells also undergo HSC70-mediated forms of autophagy termed chaperone-mediated autophagy and endosomal microautophagy [51], there is not much literature on these forms of autophagy in neuronal excitotoxic injury. The term "autophagy" is used herein to indicate macroautophagy.

Neurons are post-mitotic cells with extremely extended cytoplasmic arbors, the tips of which support synaptic contacts with other cells. It is thus not surprising that neurons show highly efficient basal autophagy [52], along with distinct and redundant mechanisms to upregulate stress- or damage-induced autophagy. In contrast to fibroblasts and cancer cells, basal neuronal autophagy is less focused on proteasomal components and more focused on synaptic proteins and mitochondria [53]. Regulation and outcomes of neuronal autophagy are influenced by the extreme compartmentalization of functions within different parts of a neuron [54].

Tremendous advances have been made regarding the molecular regulation of macroautophagy over the past 25 years, with many excellent reviews (For example, [55]). In the following two subsections, these mechanisms will be briefly summarized in context of the molecular markers that are most commonly used to monitor and/or infer changes in autophagic and mitophagic activity (See also [56]).

IIA. Monitoring autophagy in culture and in vivo

Autophagy can be divided into several major stages: 1) initiation, 2) membrane extension and formation of autophagosomes, 3) maturation, trafficking, and fusion steps that culminate in delivery of cargo into lysosomes, and 4) completion of lysosomal degradation and release of degradation products for recycling (Figure 2A). In addition, there are cargo-targeting

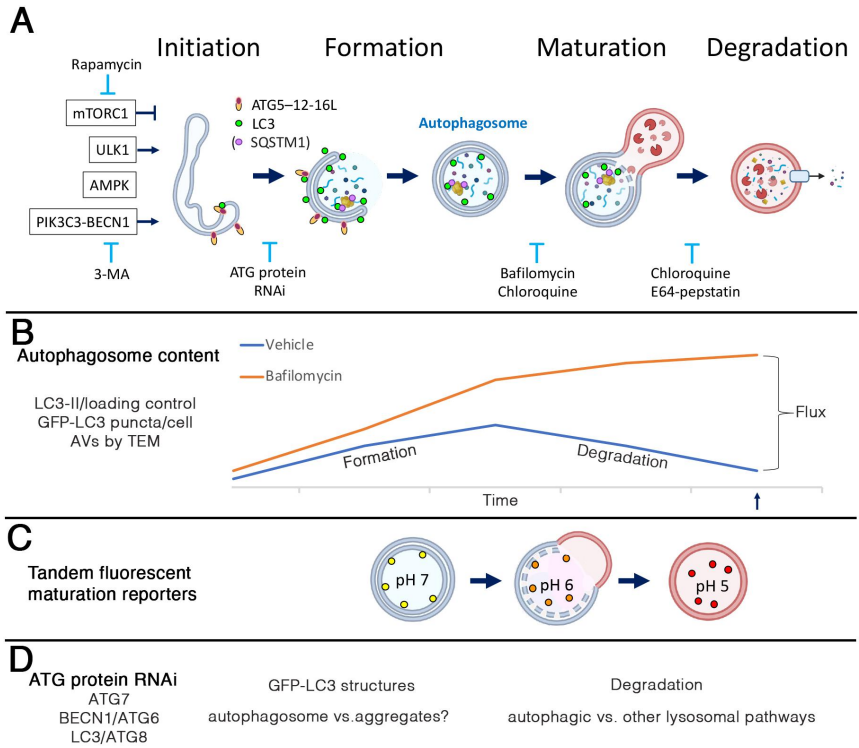

Figure 2. Monitoring the dynamic process of autophagy. (A) Simplified schematic of the major steps of autophagy with selected regulatory proteins and chemicals. Integration of mTORC1, ULK1 and AMPK signaling inputs results in PIK3C3-BECN1 complex-mediated phosphatidylinositol phosphorylation and deposition of ATG proteins including LC3/ATG8. This leads to membrane extension and autophagosome formation. While the cargo receptor SQSTM1/p62 is not essential for autophagosome formation, it is degraded with lipidated LC3-II and other autophagosome contents following maturation through fusion with acidic lysosomes containing degradative enzymes (red toned structures). SQSTM1 levels are often inversely correlated with autophagic activity. (B) Autophagosome content can be assessed by western blot for LC3-II, visualization of fluorescently labeled LC3 puncta or autophagic vacuoles (AVs) by TEM. Autophagy activity or flux cannot be inferred simply from the amount of autophagic markers observed. The amount of autophagic structures observed at any given time reflects the balance between rates of autophagosome formation and degradation. Bafilomycin, chloroquine or lysosomal protease inhibitors such as E64-pepstatin can be used to arrest autophagic degradation. Treatment with degradation inhibitors is used to measure autophagic flux at specific times points, calculated as the difference between autophagosome content with and without degradation inhibitors. (C) Tandem fluorescent reporters such as RFP-GFP-LC3 are used to track autophagosome maturation to autolysosomes. At neutral pHs, both red and green fluorescence are observed, giving rise to colocalized yellow puncta. As pH decreases, the green fluorescence diminishes. Time-dependent transitions to red only puncta is frequently quantified as the red:green fluorescence ratio. (D) To definitively establish a role for the autophagy machinery in autophagosome formation and degradation, these processes should be inhibited by

mechanisms to enrich for specific types of cargo in a process termed selective autophagy [57,58].

Initiation involves the integration of trophic and stress signaling pathways to produce membranes marked by PI3P, upon which the membrane extending machinery will dock. The mammalian target of rapamycin (mTOR) is a major pro-growth kinase that acts to suppress both the Unc-51-like kinase 1 (ULK1) and the PI3KC3/VPS34-BECN1 complexes involved in autophagy initiation [59], while also downregulating transcription of lysosomal and autophagy related genes by phosphorylating TFEB [60]. Thus, inactivation of mTOR is a major driver for nonselective, bulk autophagic degradation. AMPK (adenosine monophosphate-activated protein kinase) is another regulator of autophagy, acting to phosphorylate ULK1, although the impact of AMPK on ULK1 activation [61,62] or mitophagy [63] is nuanced and context-dependent.

Monitoring the phosphorylation state of mTOR or its downstream targets may offer useful information concerning whether the excitatory insult results in a more or less permissive environment for autophagy. However, simple changes in the expression of autophagy proteins are not informative as to whether autophagic activity itself is changed [56]. Neither mRNA nor routine immunoblot analysis of BECN1, for instance, can show whether it is assembled into pro-autophagic VPS34 complexes or is bound up in anti-autophagic Bcl2 complexes [64]. To measure changes in autophagic activity or flux, it is necessary to demonstrate both increased autophagosome formation and increased autophagosome degradation.

PI3P-labeled membranes resulting from the initiation complexes serve to attract proteins such as DFCP1 (zinc-finger FYVE domain-containing protein 1) and WIPI2 (WD repeat domain phosphoinositide-interacting protein 2), which in turn recruits the ATG16L1-ATG12~ATG5 complex that serves an E3-like role in facilitating the covalent, ubiquitin-like conjugation of ATG8 family members to phosphatidylethanolamine [55]. The ATG8-ylation of membranes plays a key role in growth of autophagic membranes to form the autophagosome, a double membrane neo-organelle that functions to deliver its contents to the lysosome (Figure 2A). Human ATG8 proteins include three isoforms of microtubule-associated protein 1 light chain 3 (LC3A, LC3B, LC3C) and three isoforms of the GABARAP family (GABARAP, GABARAPL1, GABARAPL2). ATG8-ylation

RNAi targeting essential ATG proteins. Non-autophagic processes such as GFP aggregation or ubiquitin-mediated recruitment of SQSTM1 to small protein aggregates can be confused with autophagic puncta. Likewise, reduced levels of autophagy markers or cargoes can also be due to impaired biosynthesis or engagement of other proteolytic pathways. Created in BioRender. Chu, C. (2025) https://BioRender.com/q54m591, and using Adobe Photoshop 22.4.3 and Microsoft Powerpoint 16.77.1.

requires an E1 ATG8-activating enzyme called ATG7 and the E2 ATG8-conjugating enzyme ATG3. Ubiquitin-like conjugation of ATG12 to ATG5 also requires ATG7 with ATG10 acting as the E2 enzyme. RNAi targeting ATG5, ATG7 or LC3 are useful for determining whether autophagy induction ameliorates or exacerbates excitotoxic injuries (Figure 2D).

Given that LC3 is covalently attached to both the outer and inner membranes of the autophagosome [65], and some LC3 remains inside the autophagosome until degraded in the lysosome, it is the most commonly-used marker of autophagosomes. When LC3 is covalently coupled to PE, its electrophoretic migration through SDS-polyacrylamide gels is enhanced compared to the unconjugated LC3-I, giving rise to an LC3-II band. Notably, LC3-II is more accurately normalized to a general housekeeping loading control and not to LC3-I [56]. In addition, fluorescently tagged GFP-LC3 undergoes a transition from a light diffuse distribution to bright puncta upon recruitment to autophagosomes [66]. Early and late autophagic vacuoles can also be observed using transmission electron microscopy (TEM), but morphological expertise is needed to distinguish autophagic structures from edematous vacuolation and other membrane-bound organelles.

Importantly, the numbers of autophagic structures as detected by any of the above methods do not necessarily reflect changes in autophagic activity (Figure 2B). Both increased formation and/or decreased degradation of these structures can lead to increased LC3-II levels, increased LC3 puncta or increased autophagic structures by TEM. The highest levels of accumulation are often observed under conditions of impaired degradation. Thus, increased expression of autophagosome markers alone cannot be used to infer changes in autophagic activity.

A direct method to monitor autophagic flux is to apply inhibitors of lysosomal degradation (such as bafilomycin, which inhibits fusion of autophagosome-lysosome fusion and raises the pH in lysosomes, and lysosomal protease inhibitors) to determine the amount of LC3 and/or cargo that would have been degraded in the absence of inhibitors [66] (Figure 2B). There are pH sensitive fluorescent tags that can be used to monitor the delivery of autophagosome contents into an acidic environment [67]. These include tandem RFP-GFP-LC3 or mCherry-GFP-LC3 constructs that show loss of the green fluorescence upon delivery to acid environments (Figure 2C). The assumption is that a normally acidified lysosome should act to complete degradation, but this may not always be the case in pathological situations involving lysosomal enzyme deficiencies. Thus, these reporters are more accurately used to monitor maturation rather than flux. A new generation of reporters that involves lysosomal cleavage and release of HaloTag from Halo-LC3 has recently been reported for monitoring autophagic flux [68].

For selective autophagy, adapter proteins, such as SQSTM1/p62 (sequestosome 1) and other LC3-interacting receptors, bind to and deliver

ubiquitinated cargos into the growing autophagosome and are degraded with the cargo. For this reason, SQSTM1 has been used as a crude inverse proxy to autophagic flux, particularly for *in vivo* studies. Specifically, inhibition of autophagic flux is associated with increased SQSTM1, while increased flux is associated with decreased SQSTM1. While this relationship holds up reasonably well in brain tissues (personal observation), SQSTM1 levels are not always a reliable index of flux in other systems [56], possibly due to additional points of transcriptional and/or proteasomal regulation.

IIB. Mitophagy mechanisms and methods

Just as SQSTM1 binds to ubiquitinated aggregates and links them to LC3, there several major pathways that act to selectively target mitochondria to the autophagic machinery under developmental or stressed conditions [57] (Figure 3A,E). However, engagement of these pathways do not necessarily correlated with induction and completion of mitophagy.

To demonstrate changes in autophagic degradation of mitochondria [69], it is not strictly necessary to define the exact pathway leading to mitophagy. As with autophagic flux, the minimal criteria would be to demonstrate both increased formation (Figure 3B) and successful degradation of mitochondrially-loaded autophagosomes (mitophagosomes). Colocalization of mitochondrial markers with LC3 can be quantified to assess levels of mitophagosomes. While it is not typically possible to identify partially degraded autophagic contents by TEM, bafilomycin can be employed to trap newly formed autophagosomes to visualize the cargo and quantify numbers of new autophagosomes formed in a particular time period [69,70].

There are several methods to demonstrate mitophagosome maturation and delivery to lysosomes (Figure 3C). These include colocalization of mitochondrial markers with lysosomal markers, which is inhibited by ATG protein RNAi. Alternatively, fluorescent mitophagy flux markers, including mito-Keima, mito-SRAI and mito-QC, have been developed to monitor delivery of mitochondria into acidic compartments [56,71,72]. Notably, a range of fluorescent reagents have been introduced into transgenic mice for monitoring autophagy or mitophagy *in vivo*, although they can be difficult to interpret [73]. Changes in mitochondrial content as measured by biochemical or image-based methods can also be useful, but the potential contribution of non-lysosomal degradation systems should be ruled out. The bafilomycin flux assay described above can be used to study mitophagic flux using fluorescent or western blot methods to monitor mitochondrial content [66]. Documenting a decrease in mitochondrial content that is inhibited by bafilomycin or other lysosomal inhibitors, along with the use of RNAi targeting core autophagy proteins serve to confirm increased mitophagy (Figure 3D).

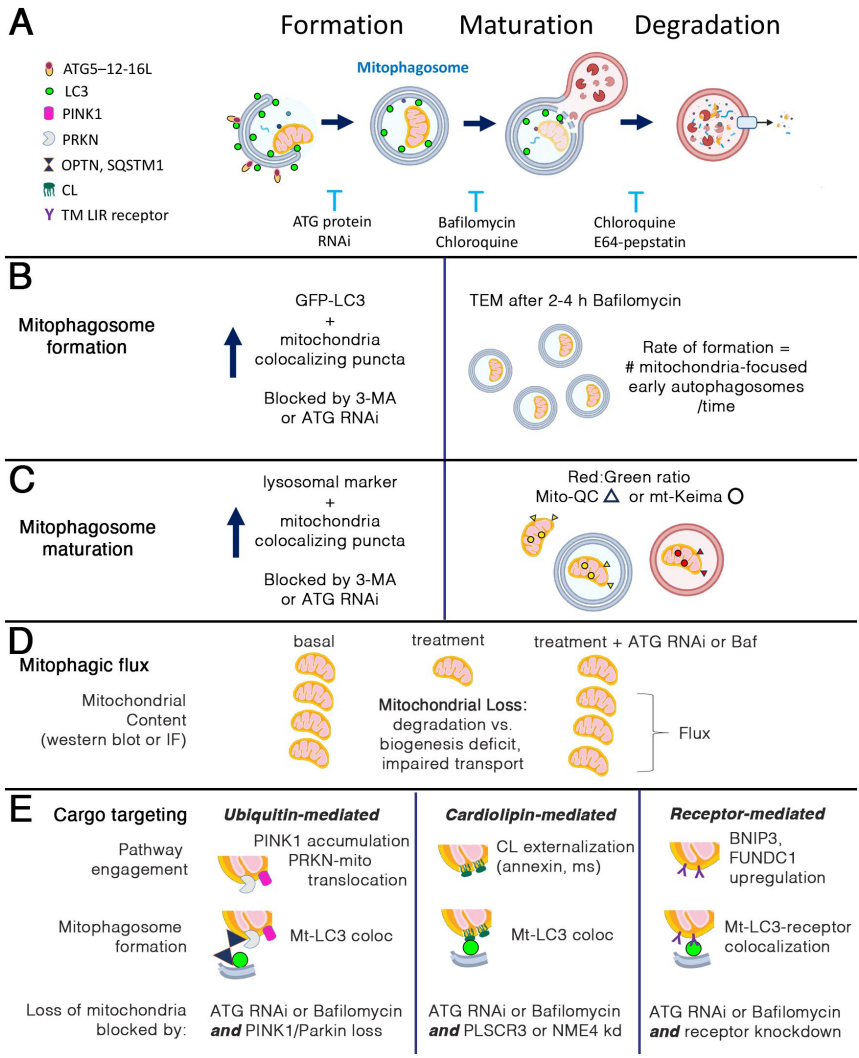

Figure 3. Studying mitophagy and involvement of selected cargo recognition pathways. (A) The general steps of autophagic mitochondrial degradation can be modulated in a parallel manner as general autophagy. (B) Mitophagosome formation is monitored by quantifying recruitment of mitochondria to GFP-LC3 puncta (left). Rates of formation can also be calculated using the bafilomycin clamp method (right). In the presence of bafilomycin, newly formed mitophagosomes are arrested and the numbers formed during a specific time period can be quantified under control and treatment conditions [70]. (C) Maturation of mitophagosomes to acidic lysosomes can be followed by quantifying delivery of mitochondrial antigens to lysosomes using either Lysotrackers or LAMP protein immunofluorescence (left) [79]. As multiple pathways can lead to lysosomal degradation, verifying a requirement for the autophagy machinery using RNAi is helpful. Alternatively, several fluorescence indicators have been developed to track the movement of mitochondrial surface or matrix proteins from a neutral pH environment to the acidic lysosomal

In terms of cargo targeting mechanisms, both protein- and lipid-based signals for mitophagy are triggered by mitochondrial damage (Figure 3E). Some mitophagy receptors, such as FUNDC1 and BNIP family members, are transcriptionally regulated transmembrane receptors of the outer mitochondrial membrane. Others, such as OPTN/optineurin, SQSTM1 or NDP52 are implicated in ubiquitin-mediated mitophagy [74-76]. In this process, PINK1 and PRKN, which interestingly are both linked to recessive PD, accumulate on the surface of depolarized mitochondria. PINK1 phosphorylates ubiquitin and the UBL-domain of PRKN to active its E3 ligase function [77]. Polyubiquitination of outer mitochondrial proteins then lead to their recognition for autophagic clearance. AMBRA1 is another mitophagy adapter implicated in both PRKN-dependent and -independent mitophagy [78]. A third class of selective autophagy mechanisms involves mitochondrial lipids such as cardiolipin or ceramide [79-81]. Cardiolipin is enzymatically externalized to the surface of mitochondria in primary neurons subjected to parkinsonian stressors [79,80], where it interacts with the N-terminal helices of LC3 to mediate mitophagy [79]. Interestingly, ceramide acts to promote LC3-cardiolipin interaction [82]. Knockdown of both LC3A and LC3B is necessary to inhibit cardiolipin-mediated mitophagy [83]. Changes in mitochondrial lipid composition also act to promote PINK1 stabilization [84]. The redundancy of mitophagy targeting mechanisms confirms the importance of normally regulated mitophagy for neuron health and function [57,85].

Importantly, it is not sufficient to use simple changes in mRNA or protein levels of PINK1, PRKN or mitophagy receptors/adapters to infer changes in mitophagy. Translocation to damaged mitochondria is a key requirement for activation of ubiquitin-mediated mitophagy. Moreover, both PINK1 and PRKN are implicated in non-mitophagy processes important to mitochondria and to neurons. These include mitochondrial biogenesis [86], dendritic branching [15,41], and regulating the density, remodeling and function of dendritic spines [15,87]. Similarly, a lack of PRKN translocation or mitochondrial ubiquitination suggests that the PINK1-PRKN pathway has not been activated, but mitophagy may nevertheless be occurring through another mitophagy pathway [53].

compartment (right). (D) A given experimental condition may result in mitochondrial loss by multiple mechanisms. Demonstrating mitophagic flux requires providing evidence of mitochondria loss that can be blocked by inhibiting autophagy. (E) Engagement of specific mitophagic cargo targeting pathways can be assessed for ubiquitin, cardiolipin and transmembrane LIR-domain mitophagy receptors. However, engagement does not necessarily reflect successful mitophagosome formation and degradation, and more than one pathway may be triggered by a given stressor. For these reasons, it is also necessary to establish mitophagosome formation and loss of mitochondrial content that can be blocked by inhibiting autophagy and the implicated pathway. Created in BioRender. Chu, C. (2025) https://BioRender.com/q54m591, and using Adobe Photoshop 22.4.3 and Microsoft Powerpoint 16.77.1.

III. Impact of excitatory injuries on autophagy and mitophagy

Calcium plays an important role in regulating a large number of cellular processes that include autophagy. As with many calcium-regulated processes, too little or too much calcium may produce unpredictable results that are likely context-dependent. Cells, particularly polarized cells like neurons, depend upon proper temporal and spatial regulation of calcium. Moreover, the regulation of autophagy and mitochondrial content varies depending upon the neuronal subcompartments involved. These include the soma or cell body, axon and axon terminals and the dendritic tree. Indeed, the dendritic spine itself functions to concentrate and localize postsynaptic calcium responses [88]. Nevertheless, there is general agreement that injurious excitatory stimuli elicit increases in autophagy markers. Studies that experimentally address changes in autophagic or mitophagic flux elicited by excitatory injury are summarized below (Table I).

IIIA. Autophagy regulation during excitotoxicity

Due to the homeostatic nature of autophagy, mechanisms that promote autophagy are often co-activated with mechanisms that reduce autophagy. These opposing mechanisms presumably serve to prevent harmful overactivation of autophagy [89]. For example, nutrient sensing mTOR complexes on the surface of lysosomes respond to calcium through calmodulin-dependent activation of mTOR to suppress autophagy [90]. On the other hand, calcium also acts to activate AMP-activated protein kinase (PRKAA/AMPK) through phosphorylation by calmodulin-dependent protein kinase to promote autophagy [91]. As both mTOR and PRKAA/AMPK function at the lysosomal membrane, they are likely exposed to similar levels of peri-lysosomal calcium with antagonistic effects on autophagy induction [92]. Therefore, changes in autophagic flux should be experimentally determined using methods outlined above to demonstrate both successful degradation and involvement of the autophagy machinery.

Autophagic flux is increased during excitotoxic injury to rat primary cortical neurons in a KA-hypoxia model of stroke as evidenced by flux analysis of LC3-II and SQSTM1 immunoblots in the presence and absence of lysosomal inhibitors [93]. Although the authors did not report the numbers of red-only mRFP-GFP-LC3 puncta indicative of autophagosome-lysosome fusion, this strong study employed multiple methods of analysis including time course studies of LC3 puncta and two lysosomal markers. In SH-SY5Y cells treated with high 10 mM doses of glutamate for 24 h, increased LC3-II and SQSTM1 flux was demonstrated using chloroquine [94]. Increased autophagic flux was also demonstrated *in vivo* following injection of a glutamate analogue into the brains of rat pups to model perinatal hypoxic-ischemic injury [95]. The

Table I. Papers Examining Autophagy or Mitophagy in Excitatory Injuries. The last column summarizes correlative or definitive evidence supporting beneficial vs. detrimental roles of autophagy/mitophagy.

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Kulbe [95]	2014	Cerebral ischemia	1° rat hippocampal neurons	Glutamate (100 μM x 5 min)	Inhibits autophagic flux (LC3 II, SQSTM1 western)	cell death at 24 h (ethidiumhomo-dimer)	CaMKII inhibitor protected but did not affect autophagy. Pre-treatment with rapamycin & trehalose conferred protection (unknown if these treatments corrected the flux problem or if their protection required ATG machinery).	Protective
Xiong [90]	2022	not stated	human SH-SY5Y cells	Glutamate (10 mM x 24 h)	Increases autophagic flux (LC3-II and SQSTM1 westerns, chloroquine flux assay)	cell death at 24 h (cell counting kit)	Chloroquine confers neuroprotection. Protection by extracellular ATP correlates with suppression of autophagy.	Pathogenic

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Yue [120]	2019	not stated	1° mouse cortical neurons	Glutamate (200 μM x 30 min)	Increases autophagic flux (BECN1, LC3II, SQSTM1 Westerns, MDC). Confusing bafilomycin flux assay that reduced p62 levels.	cell death at 24 h (MTT)	GPR30 agonist protection is correlated with downregulation of autophagy. Protection is blocked by both inhibitors and activators of autophagy (wortmannin & rapamycin), raising possibility of autophagy-independent functions of PI3K/AKT/mTOR signaling.	Pathogenic
Vucicevic [96]	2020	not stated	human SH-SY5Y, rat PC12 cells	Glutamate (2.5 mM x 24 h) after 24 h of HBSS nutrient deprivation. Also D-aspartate & ibotenate	Suppresses starvation-induced autophagy by transcriptional repression (TEM, LC3 puncta, acidic puncta, multiple westerns, including an LC3-II bafilomycin/ pepstatin flux assay)	cell death at 24 h (MTT, annexin, membrane damage)	Constitutively active AMPK, metformin, overexpression of TFEB, or lithium all confer partial protectionn with unknown impact in flux assay. 3-MA and wortmannin exacerbate cell death.	Protective

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Descloux [91]	2018	Periventricular leucomalacia; neonatal hypoxia-ischemia	rat pups in vivo	Ibotenate injection (10 µg)	Increases autophagic flux (LC3, SQSTM1 western; LC3 puncta, TEM; LAMP1 puncta, CTSB puncta)	ventricular dilatation and loss of brain volume	3-MA reduces LC3, increases SQSTM1 and protects.	Pathogenic
			1° rat cortical neurons	Ibotenate (50 µM x 6 h)	Increases autophagic flux (LC3, SQSTM1 western; LC3 puncta; pepstatin/ E64 flux assay)	cell death at 6 h (LDH)	3-MA, E64/PepA, Atg7 RNAi and Becn1 RNAi all reverse flux markers and confer protection	Pathogenic
Ginet [89]	2014	Neonatal hypoxia-ischemia	1° rat cortical neurons	Kainate-hypoxia (30 µM Ka at 6% oxygen x 30 min)	Increases autophagic flux (LC3, SQSTM1 western; LC3 and lysosomal puncta; pepstatin/E64 flux assay)	cell death at 6 h (PI, LDH)	Becn1 RNAi, Atg7 RNAi and 3-MA protect; upregulation of Becn1 or Atg7 sensitizes to injury. Caspase inhibitors did not protect.	Pathogenic
			rat pups in vivo	Carotid ligation plus 2 h systemic hypoxia	Increases autophagic flux (LC3, SQSTM1 western; LC3 puncta)	lesion volume at 24 h	Downregulation of Becn1 in vivo protects, accompanied by decreased LC3-II and increased SQSMT1.	Pathogenic
Dong [119]	2012	not stated	1° rat striatal neurons	Kainate (100 µM x 4 h)	Increases autophagic flux (LC3, BECN1, SQSTM1 western; LC3 puncta, TEM)	cell death at 4 h (LDH)	Protection by p53 inhibitors are correlated with reduced autophagy	Pathogenic

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Yang [123]	2022	Epilepsy	Adult mice in vivo, hippocampal brain slices, patient brain tissue	Kainic acid injection (1 nmol) or pentylenetetrazol (35 mg/kg qod x 30 d)	Increases autophagic flux (LC3, SQSTM1). BECN1 increased in epileptic patients, KA-Induced and pentylenetetrazol-kindled mice.	Nissl, Golgi-Cox, seizure activity.	Becn1 deficient mice showed neuron loss *in vivo* and decreased spine density in slices, but were protected from seizures.	Mixed
			1° rat hippocampal and cortical neurons	None	Increases autophagic flux (LC3, SQSTM1)	cell death (MTT)	Becn1 deficient neurons showed decreased viability	Protective
Liu [124]	2023	Epilepsy	Adult mice in vivo, 1° mouse hippocampal neurons	Kainic acid injection (1 nmol); for status epilepticus 20 mg/kg ip.	Chronic seizures increase TLR76-dependent autophagic flux (BECN1, LC3BII/I, SQSTM1 western, LC3 puncta, TEM) and decrease KIF5A expression, leading to impaired GABAAR trafficking to the synapse.	Seizure activity, neuronal hyperexcitability	Deletion of Tlr7 or 3-MA prevents degradation of p62 and KIF5A, while rapamycin increases KIF5A degradation. Deletion of Tlr7 or restoring KIF5A levels reduces seizure susceptibility.	Pathogenic

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Liu [148]	2021	not stated	Adult mice in vivo	Kainic acid injection (0.625 nmol)	Increases autophagic flux (LC3, SQSTM1 western). PINK1 increases in mt fraction at 6-12 h, decreases from cytoplasmic fraction at 6-24 h.	Neuron loss (Nissl), behavior at day 14	Protection by NADPH is correlated with suppression of autophagy activation *in vivo*. Rapamycin negates protection by NADPH in cortical neurons. Combined NADPH and mito-apocynin enhances motor recovery and suppresses autophagy.	Pathogenic
Nishiyama [94]	2010	not stated	human HEK293 cell, 1° nouse hippocampal neurons	*lurcher* mouse (GluD2 mutation)	Increases AMPK phosphorylation and LC3-II/I ratio	cell death (PI, # surviving neurons, axonal damage)	AMPK dominant negative had no effect on cell death.	Neutral
			mouse cerebellum in vivo		Not studied	behavior, # surviving neurons and axon swelling of Purkinje cells	Autophagy reduces death but is detrimental to axons. Crossing with *Atg5* conditional KO did not rescue ataxia or cell death, but further reduced Purkinje cell numbers while rescuing axon swelling.	Mixed

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Yue [92]; Wang [93]	2002; 2006	not stated	human HEK293 cell, mouse cerebellum in vivo	*lurcher* mouse (GluD2 mutation)	Yue: increases punctate BECN1 staining. Increased autophagosomes by TEM in vivo. Wang: increases puncta when GFP-LC3 mouse is crossed with lurcher.	Yue: PI in HEK cells	Yue: 3-MA protects in HEK cells, but impact on flux not studied. Wang: Role of autophagy not experimentally addressed.	Pathogenic
Plowey [116]	2008	Parkinson	neuron-differentiated SH-SY5Y	LRRK2-G2019S	Increases autophagy in both somatic and neuritic compartments by LC3 puncta and TEM.	Neurite retraction	Protection by LC3 RNAi, ATG7 RNAi and ERK1/2 inhibitor, with confirmation of decreased neuritic LC3 puncta. Rapamycin enhances injury.	Pathogenic
Cherra [117]	2010	Parkinson	1° mouse cortical neurons, neuron-differentiated SH-SY5Y	LRRK2-G2019S, MPP+	PKA suppresses autophagy through phosphorylation of LC3 (GFP-LC3 puncta density, tandem flux reporter, western)	Neurite retraction	LC3 phosphomimic suppresses autophagy and protects against dendritic simplifcation	Pathogenic
Cherra [16]	2013	Parkinson	1° mouse cortical neurons, neuron-differentiated SH-SY5Y	LRRK2-G2019S or -R1441C	Increased mitophagic flux (GFP-LC3 puncta, loss of mitochondria blocked by bafilomycin and ATG7 RNAi)	Neurite retraction	Blocking calcium uptake inhibits autophagy, the loss of dendritic mitochondria, and dendritic retraction	Pathogenic

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Plowey [10]	2014	Parkinson	1° rat cortical neurons	LRRK2-G2019S or -R1441C; AMPA 100 µM, NMDA 10 µM, endogenous glutamate release by K+ x 12 h	Not studied	Neurite retraction; cell death after K+ (apoptosis by DAPI)	Mutant LRRK2 elicits increased spontaneous electrical activity that triggers excitatory neurite retraction, and increased agonist responses associated with excitatory cell death. Role of autophagy not studied.	Unknown
Verma [17]	2017	Parkinson	1° mouse cortical neurons, neuron-differentiated SH-SY5Y, patient fibroblasts, post-mortem patient brain	LRRK2-G2019S or -R1441C	Increased autophagy and mitophagy	Neurite retraction	Mitochondrial calcium uptake and ERK1/2 mediated mitophagy contributes to dendritic retraction. NCLX phosphomimic acting downstream of PINK1-PKA protects	Pathogenic
Van Laar [106]	2015	Parkinson	1° rat cortical neurons	Glutamate (100 µM or 500) µM and glycine (1 µM) x 10 min	Suppresses mitophagy due to oxidative stress. Treatment with pepstatin/E64 revealed no evidence of mitophagosome formation despite parkin translocation (# of neurons with colocalizing structures)	Not studied	Not studied	Unknown

(*Continued*)

Table I. (Continued).

Author [Ref #]	Year	Disease Modeled	Experimental System	Treatment/Mutation	Impact on Autophagy/ Mitophagy	Injury/Protection Readout	Pathogenic, Neutral or Protective?	Impact
Zhuang [128]	2022	Glaucoma	1° rat retinal ganglion cells	NMDA (100 μM x 24 h)	Basal effect of NMDA on mitophagy markers was not statistically compared	Cell death (LDH)	S3 protection requires parkin and is correlated with increased parkin expression and increased mitochondrial-parkin colocalization. Unknown if core autophagy machinery is necessary for protection.	Protective
Lian [129]	2023	Glaucoma	1° rat retinal ganglion cells	Glutamate (100 μM x 72 h)	Impact of glutamate not studied.	Cell death (apoptosis by Hoechst, LDH)	Protection by fucoxanthin correlated with RNA and protein upregulation of parkin, downregulation of BAX, and upregulation of optineurin protein, increased fluorescent autophagy markers and colocalization of LC3 and MitoTracker. Did not examine parkin translocation to mitochondria.	Protective

glutamate-treated brain tissues exhibited time-dependent increases in LC3-II and decreases in SQSTM1, increased autophagic and lysosomal puncta, and increased autophagic structures by electron microscopy.

In the Lurcher mouse, cerebellar Purkinje and granule neurons degenerate through excitotoxic mechanisms. Lurcher is caused by gain of function mutation in the delta2 glutamate receptor, which results in constitutive cation uptake. Autophagy is activated in this model due to interaction of the glutamate receptor with BECN1 via the PDZ domain protein nPIST, and most dying cells exhibit an autophagic morphology [96]. Elevated levels of autophagosomes, have also been confirmed by crossing Lurcher mice with GFP-LC3 transgenic mice [97]. Given the absence of SQSTM1 elevation in Lurcher brains, these data are compatible with increased autophagy. An increase in PRKAA/AMPK activity may also contribute to autophagy induction in this model [98].

In contrast, a study of dissociated hippocampal neurons treated with excitotoxic doses of glutamate for 5 min demonstrated decreased autophagic flux [99]. Although the study examined LC3-II levels as normalized to LC3-I instead of the recommended housekeeping protein [56], the inclusion of parallel SQSTM1 flux studies showed impaired autophagic flux by immunoblot analysis. In nutrient-deprived SH-SY5Y cells, glutamate elicited transcriptional downregulation of autophagy proteins and cell death that could be reversed by a constitutively active PRKAA/AMPK [100]. A transient interruption in autophagic flux can be observed in the early phases after NMDA injection due to LAMP2 degradation, with flux being re-established by 24 h [101]. Given that the autophagy response to KA is transient, with upregulation of phospho-mTOR acting in a negative feedback loop [102], differences in severity and duration of the excitotoxic insult may yield different effects on autophagy.

IIIB. Calcium dysregulation and mitophagy during excitatory injury

Mitophagy can be triggered by mitochondrial calcium toxicity during EMT [16,17]. Several mechanisms may account for this. Calcium overload during EMT may lead to mitochondrial membrane depolarization, triggering enrichment of PINK1-PRKN and initiation of ubiquitin-dependent mitophagy [103]. PRKAA/AMPK and extracellular signal regulated protein kinase (MAPK3,1/ERK1/2) are both activated through calcium-dependent mechanisms [104,105], serving to drive mitophagy even in the absence of mitochondrial injury [105,106]. Calcium also regulates Miro1 to elicit changes in mitochondrial shape that facilitate mitophagy [107]. On the other hand, deficits in the release of calcium from mitochondria, such as those observed in genetic models of AD

and PD [38,44,49], have been linked to a failure to elicit FCCP-mediated mitophagy due to impaired calcium-dependent activation of the MAPK3,1/ERK1/2 pathway [108].

While mitochondrial calcium overload acts to promote dendritic mitophagy during EMT, in uninjured HEK293 cells subjected to a variety of autophagy inducers, MCU-mediated calcium uptake is increased to limit mitophagy triggered by cytosolic calcium [109]. The authors propose that the brisk MCU-mediated calcium influx triggered during autophagy induction in $SNCA^{A53T}$ PD patient cells may serve to prevent harmful overactivation of mitophagy. Given that mitochondrial calcium efflux was not impaired in the cells treated with rapamycin or CCCP [109], it is likely that cytosolic pathways such as MAPK/ERK-mediated mitophagy are downregulated by enhanced MCU-mediated uptake. However, if mitochondrial calcium rises to injurious levels during EMT in cells with impaired mitochondrial efflux [38,44], additional mechanisms are triggered to promote mitophagic clearance of calcium-overloaded mitochondria.

Interestingly, in primary neurons exposed to glutamate, mitophagy as measured by recruitment of LC3 to mitochondria did not occur, despite PRKN-translocation to mitochondria [110]. Co-administration of N-acetyl cysteine along with glutamate acted to de-repress mitophagy downstream of PRKN translocation. These data suggest that reactive oxygen species play a role in interrupting the formation of mitophagosomes downstream of PRKN recruitment.

IV. Impact of autophagy and mitophagy on the outcome of excitatory neuronal injuries

Autophagy and mitophagy play important basal and stress-induced homeostatic functions, and are generally regarded as neuroprotective responses. Certainly, mice that are deficient in brain autophagy show neurodevelopmental and neurodegenerative phenotypes [111,112]. While markers of autophagy are frequently increased in disease or injury models, the role of autophagy in modulating injury, whether beneficial, irrelevant or harmful, needs to be experimentally determined. The majority of studies implicate autophagy in a harmful role during excitotoxic cell death (Table I). Autophagy initiation that does not successfully elicit successful degradation (or re-use of degradation products) defines a harmful state of autophagic stress [113].

IVA. Autophagy and excitatory neuronal injuries

Surprisingly, the majority of studies that experimentally address causality conclude that autophagy plays an injurious role during excitotoxic neuron cell death. In primary neurons injured by KA-hypoxia, both pharmacological and molecular knockdown of BECN1 or ATG7 conferred protection in culture and *in vivo* [93]. In this study, there was no evidence of caspase activation, and apoptotic inhibitors showed no effect. In another study, 3-methyladenine (an inhibitor of PI3K) prevented the elevation of both autophagy and apoptotic markers *in vivo*. Moreover, either 3-methyladenine or RNAi targeting BECN1 and ATG7 conferred protection in primary neuronal cultures [95]. RNAi targeting Atg7 also demonstrates a pro-death role for autophagy in neonatal Purkinje and cortical neuronal cell death following hypoxia-ischemia *in vivo* [114]. Autophagy-dependent activation of apoptotic pathways is described in models of neonatal hypoxia-ischemia [115].

An alternative mechanism operating in KA-induced excitotoxicity involves the activation of ferroptosis [116], a form of autophagy-dependent or autophagy-potentiated cell death [117,118]. The ferritin heavy chain 1 (FTH1) can be degraded through selective autophagy employing the nuclear receptor coactivator 4 (NCOA4) as an autophagy receptor [118]. This results in a diminished capacity to bind and neutralize reactive states of iron, leading to lipid peroxidation and induction of ferroptosis. Injecting 3-MA into the striatum of mice prior to KA injection conferred protection, reversing the increase in NCOA4 and the decrease in FTH1 [116]. Interestingly, the molecular signature of ferroptosis includes peroxidized phosphatidylethanolamine (15-HpETE-PE) [119], raising the possibility that ATG8-ylation, and therefore autophagy, may be impaired during ferroptotic cell death. Ferroptotic cell death has also been implicated in glutamate excitotoxicity in models of sepsis-associated encephalopathy [120]. In this study, the causal role of autophagy was not directly tested, but treatment with ferrostatin-1 to block ferroptosis prevented sepsis-induced nucleophagy, stabilized expression of PSD95 and other synaptic markers, and improved cognitive outcomes.

In excitatory injuries related to neurodegenerative disease models such as LRRK2, RNAi inhibition of autophagy ameliorates neuritic retraction, also implicating autophagy in a detrimental role [121,122]. Compartmentalized activation of necroptotic and apoptotic pathways may contribute to neuritic retraction in other excitatory models [20,29].

There are several papers demonstrating that neuroprotective treatments are associated with decreased autophagy markers, supporting but not proving that autophagy may potentiate injury [123,124]. These papers typically show an increase in autophagy markers with injury, and neuroprotective

interventions are associated with a decrease in autophagy markers. Unfortunately, without examining the direct effects of inhibiting or stimulating autophagy, it is not possible to determine causality. While it is possible that the neuroprotective agent suppresses autophagy to mediate protection, it is also possible that the neuroprotective agent simply removes the upstream trigger for autophagy by preventing cellular damage through other mechanism(s).

The ability of rapamycin or trehalose to confer protection have been used to support a neuroprotective role for autophagy [99]. However, given the nonselective nature of chemical reagents, affecting not only autophagy, but also protein synthesis, glucose handling, oxidative stress and stress signaling pathways, it is possible that protection occurs through other mechanisms [125]. To move beyond correlation, it would be necessary to determine if protection can still occur if autophagy induction is blocked. For example, disruption of Atg5 was used to prove that while autophagy is associated with dying neurons in the Lurcher model, it does not contribute to Purkinje cell death [98]. Instead, RNAi for ATG5 aggravated cell death, indicating a protective role for autophagy. Likewise, conditional deletion of *Atg7* or heterozygous disruption of *Becn1* in seizure models increases susceptibility to seizures [126,127]. While these studies implicate a possible role for autophagy in suppressing excessive excitatory synaptic activity, the anti-seizure effects could also be due to non-autophagy functions of ATG proteins. For example, GABA receptors mediate inhibitory neurotransmission, and the balance of excitatory and inhibitory inputs regulates seizure thresholds. Some of the effects attributed to autophagy may be due to regulation of the kinesin-dependent trafficking of GABA A receptor subunits via interaction with the GABARAP branch of the ATG8 family [128].

Another consideration in studying the role of autophagy in mediating protection or degeneration relates to chronicity. Autophagy plays a normal role in downregulating AMPA and NMDA receptors during long term depression [129,130] (Figure 1A). In the short term, this downregulation serves to limit excitatory input and protect against dendritic injury. However, the impact of excessive or prolonged autophagy may lead to dendritic shrinkage and permanent loss of synapses [131].

IVB. Mitophagy and excitatory neuronal injuries

Given the important role of mitochondrial calcium overload in mediating both excitotoxic cell death and sublethal EMT, it would be reasonable to predict that mitophagy ought to play a neuroprotective role in both acute

and chronic excitatory injuries. However, the few studies identified in the literature do not present a clear answer.

In two studies of retinal ganglion cell excitotoxicity, neuroprotective treatments were associated with increased PRKN expression. In the first, an inhibitor of the USP30 deubiquitinase increases PRKN colocalization with mitochondria [132]. While PRKN knockdown indicated a role for PRKN in reducing NMDA-mediated injury, the investigators did not investigate the impact of PRKN knockdown on mitophagy, nor whether the core autophagy machinery is necessary for neuroprotection. In the second, a carotenoid that protects against glutamate exposure upregulates mRNA and protein levels of PRKN, optineurin and LAMP1 [133]. While there were increased mitophagosomes, colocalization studies were not performed to determine parkin mitochondrial translocation. No experiments were shown to determine whether parkin, optineurin or autophagy proteins are required for neuroprotection.

In an *in vivo* model of human temporal lobe epilepsy elicited by intracranial KA injection, upregulation of phosphoglycerate mutase 5, a mitochondrial phosphatase, increases PINK1 mRNA and protein levels and LC3B, with nonsignificant decreases in TIMM23 and TOMM20 [134]. Knockdown of phosphoglycerate mutase 5 was correlated with reversal of these findings, prompting an unsupported conclusion that neuroprotection was due to a reduction in mitophagy. Due to the contradictory and correlative nature of these studies, the role of mitophagy in regulating excitotoxic cell death remains unresolved.

EMT is defined by mitochondrial calcium overload, which leads to atrophy and loss of dendritic and post-synaptic structures. In the mutant LRRK2 model, autophagy has been implicated in mediating neuritic retraction [121]. While the pathway triggering mitophagy remains undefined, transient transfection with mutant forms of LRRK2 elicits selective loss of mitochondria from dendrites [16,17] that precede subsequent dendritic shortening by 22-23 days *in vitro* [10,37]. It is known from mitochondrial dynamics studies, that a sufficient density of mitochondria is required for dendritic extension [135] and spine maturation [136].

In addition to excessive mitochondrial injury and degradation, a decreased ability to replace degraded mitochondrial through mitochondrial biogenesis may contribute to spine loss and dendritic degeneration. Under normal circumstances, the rapid loss of spines that occurs following brief, sublethal hypoxic or excitotoxic exposures recovers within a few hours [137]. However, ablation of mitochondria from dendrites prevents new protein synthesis in spines [138]. NMDA receptor ligation normally triggers calcium dependent activation of nuclear MAPK3,1/ERK1/2 signaling [139] and localized mitochondrial biogenesis [140]. Yet, pathological elevations in cytosolic calcium

can inhibit MAPK3,1/ERK1/2 nuclear translocation [141]. Whereas nuclear MAPK3,1/ERK1/2 signaling is essential for neurite outgrowth and spine plasticity [142,143], cytosolic MAPK3,1/ERK1/2 promotes mitophagy [106] and downregulates mitochondrial biogenesis [144,145]. Interestingly, both PINK1 and PRKN are involved in promoting mitochondrial biogenesis [146,147], whereas deficits in mitochondrial biogenesis are frequently observed in human disease [148,149]. Thus, a combination of factors triggered by EMT-mediated mitochondrial calcium overload results the loss of functional mitochondria necessary to support synaptic structure and function (Figure 1C).

V. Concluding remarks

Given the importance of calcium signals, autophagy and mitophagy in regulating neuron structure and function, it is perhaps not surprising that dysregulation of these processes results in neuronal atrophy and cell death. Excess excitatory input to neurons can be triggered by a variety of acute stressors such as stroke, trauma and hypoglycemia, resulting in elevations in autophagy and mitophagy. Likewise, genetic mutations related to PD, AD, HD and FTD elicit changes in synaptic reactivity and mitochondrial calcium uptake and/or release to promote synaptic loss, circuit dysfunction, and dendritic atrophy. While increased autophagy and mitophagy are frequently observed in acute and chronic models associated with excitatory injury, their role(s) in mediating or protecting against injury is context-dependent and must be experimentally established rather than inferred.

Autophagy typically is thought to play a cytoprotective role, although it can also contribute to acute neuronal cell death. Interestingly, in excitotoxic injuries, the preponderance of studies implicates autophagy in a detrimental role (Table I). Importantly, studies showing a correlation between a neuroprotective treatment and decreased autophagy/mitophagy do not distinguish between a harmful role for autophagy versus upstream amelioration of injury such that an autophagic/mitophagic response is no longer needed. Autophagy mediates long term depression, protects against excitatory overstimulation and prevents axon degeneration under physiological conditions [129,130,150]. Nevertheless, autophagy is also known to mediate dendritic shortening and synaptic downregulation [121,122,151,152]. Whereas the morphology of dying neurons observed following excitotoxic insults is typically necrotic in appearance, inhibitors of apoptotic, autophagic and ferroptotic pathways can rescue cell death and neurite retraction. *In vivo*, autophagy sometimes shows mixed results, ameliorating neuron loss, but

exacerbating disease-related phenotypes such as seizures and ataxia. Reducing neuron cell death may nevertheless fail to correct synaptodendritic pathology, particularly as autophagy and mitophagy contribute to retraction and simplification of the axodendritic arbor.

Conditions that promote mitochondrial calcium overload contribute to both excitotoxic cell death and EMT. The mechanisms that promote calcium overload vary from increased excitatory neurotransmission and mitochondrial calcium uptake to deficits in mitochondrial calcium release and potentially dysregulation of other intracellular calcium stores. Irregardless of the mechanism(s) leading to mitochondrial calcium dysregulation, inhibiting MCU and promoting SLC8B1/NCLX activity are neuroprotective in a wide range of injury models [6,11,17]. Furthermore, combination strategies that target autophagic and non-autophagic mechanisms simultaneously are efficacious in animal models of KA toxicity [153]. In addition to strategies that reduce mitochondrial calcium uptake or enhance mitochondrial calcium release, addressing deficits in mitochondrial biogenesis or trafficking may show promise for treating diseases involving excitatory dysregulation.

Table of Contents

Abbreviations

AD	Alzheimer disease
ALS-FTD	amyotrophic lateral sclerosis-frontotemporal dementia spectrum
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
PRKAA/AMPK	AMP-activated protein kinase
ATG7	autophagy related 7
ATG8	autophagy related 8 family member, including GABARAP and LC3
BECN1	beclin 1

CAMK2A/CaMKII	Ca2+/calmodulin-dependent protein kinase-II
EMT	excitatory mitochondrial calcium toxicity
GABARAP	Gamma-aminobutyric acid receptor-associated protein
HD	Huntington disease
KA	kainic acid
MAP1LC3/LC3	microtubule associated protein 1 light chain 3
LRRK2	leucine-rich repeat kinase 2
MAPK3,1/ERK1/2	extracellular signal regulated protein kinase
MCU	mitochondrial uniporter
mTOR	mammalian target of rapamycin
NMDA	N-methyl-D-aspartate
PD	Parkinson disease spectrum, including PD with dementia, Lewy body dementia
PINK1	PTEN-induced kinase 1
PPARGC1A/PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1α
PRKN	parkin RBR E3 ubiquitin protein ligase
SLC8B1/NCLX	mitochondrial sodium calcium lithium exchanger
SQSTM1/p62	sequestosome 1
TEM	transmission electron microscopy
ULK1	Unc-51-like kinase 1

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ORCID

Charleen T. Chu http://orcid.org/0000-0002-5052-8271

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