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REVIEW

Autophagy and ALS: mechanistic insights and therapeutic implications

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ABSTRACT

Mechanisms of protein homeostasis are crucial for overseeing the clearance of misfolded and toxic proteins over the lifetime of an organism, thereby ensuring the health of neurons and other cells of the central nervous system. The highly conserved pathway of autophagy is particularly necessary for preventing and counteracting pathogenic insults that may lead to neurodegeneration. In line with this, mutations in genes that encode essential autophagy factors result in impaired autophagy and lead to neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS). However, the mechanistic details underlying the neuroprotective role of autophagy, neuronal resistance to autophagy induction, and the neuron-specific effects of autophagy-impairing mutations remain incompletely defined. Further, the manner and extent to which non-cell autonomous effects of autophagy dysfunction contribute to ALS pathogenesis are not fully understood. Here, we review the current understanding of the interplay between autophagy and ALS pathogenesis by providing an overview of critical steps in the autophagy pathway, with special focus on pivotal factors impaired by ALS-causing mutations, their physiologic effects on autophagy in disease models, and the cell type-specific mechanisms regulating autophagy in non-neuronal cells which, when impaired, can contribute to neurodegeneration. This review thereby provides a framework not only to guide further investigations of neuronal autophagy but also to refine therapeutic strategies for ALS and related neurodegenerative diseases.

Abbreviations: ALS: amyotrophic lateral sclerosis; Atg: autophagy-related; CHMP2B: charged multivesicular body protein 2B; DPR: dipeptide repeat; FTD: frontotemporal dementia; iPSC: induced pluripotent stem cell; LIR: LC3-interacting region; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; MTOR: mechanistic target of rapamycin kinase; PINK1: PTEN induced kinase 1; RNP: ribonuclear protein; sALS: sporadic ALS; SPHK1: sphingosine kinase 1; TARDBP/TDP-43: TAR DNA binding protein; TBK1: TANK-binding kinase 1; TFEB: transcription factor EB; ULK: unc-51 like autophagy activating kinase; UPR: unfolded protein response; UPS: ubiquitin-proteasome system; VCP: valosin containing protein.

Introduction

The diverse cellular processes involved in protein homeostasis are critical for maintaining neuronal health and preventing neurodegenerative disease. Genetic, biochemical and pathophysiological evidence in model systems have repeatedly shown that dysfunction of these homeostatic mechanisms leads to the accumulation of misfolded proteins and neurodegeneration in multiple contexts. Of particular interest among proteostasis pathways that have been characterized to date is the process known as autophagy, the term for which was first coined by Christian de Duve in 1963 [1,2] from the Greek αὐτός (the reflexive pronoun, for the "self") and φαγεῖν (to eat). This self-consumptive pathway maintains cellular and organismal integrity in the setting of various developmental events or stressors by degrading cellular components and organelles for reuse or redistribution. The ability of autophagy to address disparate needs of the cell arising during stress and development is achieved through specialization among the major autophagy subtypes – designated macroautophagy, microautophagy, and chaperone-mediated autophagy –

together with additional organelle- or compartment-specific mechanisms (e.g., mitophagy, granulophagy, pexophagy, reticulophagy, aggrephagy, nucleophagy, and xenophagy, among others). Moreover, autophagy is an essential pathway, as illustrated by embryonic lethality in animals missing key components of the autophagic machinery [3–9], and the high degree of evolutionary conservation across eukaryotes [10].

The individual stages of autophagy, each with its own set of regulatory factors, provide numerous potential points of dysfunction that can confer vulnerability to disease. Furthermore, mounting evidence shows that autophagy is regulated by distinct mechanisms in different cell types [11–26], and these cell type-specific mechanisms underlie the differential vulnerability of separate tissues to autophagy mutations, resulting in heterogenous manifestations of disease. Accordingly, to fully understand the implications of autophagy regulation and autophagy disruption in amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases, we will review the current understanding of mammalian autophagy, with a particular focus on pivotal regulatory features that

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differ between specific cell types. The clinicopathological features of ALS are beyond the scope of this review and are described elsewhere [27–30]. Here, we will first provide an overview of the autophagy process and describe essential components of each stage of the pathway. Second, we will focus on specific pathway components for which there are ALS-related mutations and describe the physiologic sequelae of these mutations. We will also delineate the extent to which autophagy is affected by common ALS-related genes, as demonstrated by cell and animal models of ALS. Third, we will explore autophagy machinery in the nervous system on a cell type-specific basis to highlight tissue-specific phenotypes caused by autophagy disruption, how these relate to ALS and neurodegeneration, and identify potential targets for selective modulation of autophagy in each cell type. Finally, we will conclude by integrating the concepts examined in this review to provide frameworks for investigating remaining critical questions surrounding autophagy and neuronal proteostasis, thereby informing strategies for developing effective autophagy-based therapies in neurodegenerative disease.

Overview of autophagy

Macroautophagy, often referred to simply as autophagy, is an evolutionarily conserved eukaryotic pathway for bulk and selective degradation of proteins and organelles [31]. It is characterized by a multi-step process in which cytoplasm and its contents are sequestered within double-membraned autophagosomes (Figure 1). The first step in autophagosome formation involves *induction* of a nascent autophagosome, or phagophore, which is regulated by a multimeric structure known as the phagophore assembly site (PAS) [32–35]. This complex is composed of three ULK (unc-51 like autophagy activating kinases; ULK1, ULK2, and ULK3), ATG13 (autophagy related 13), ATG101, and RB1CC1/FIP200 [36]. The initiation step begins with the dissociation of MTOR (mechanistic target of rapamycin kinase) from the PAS, cued by nutrient starvation or treatment with rapamycin or its various derivatives [36,37]. Next, *nucleation* of the nascent phagophore is orchestrated by a class III phosphatidylinositol 3-kinase (PtdIns3K) complex containing BECN1, PIK3C3/VPS34, PIK3R4/p150, NRBF2 and either ATG14 or UVRAG [38,39]. *Expansion* then occurs through maturation of the
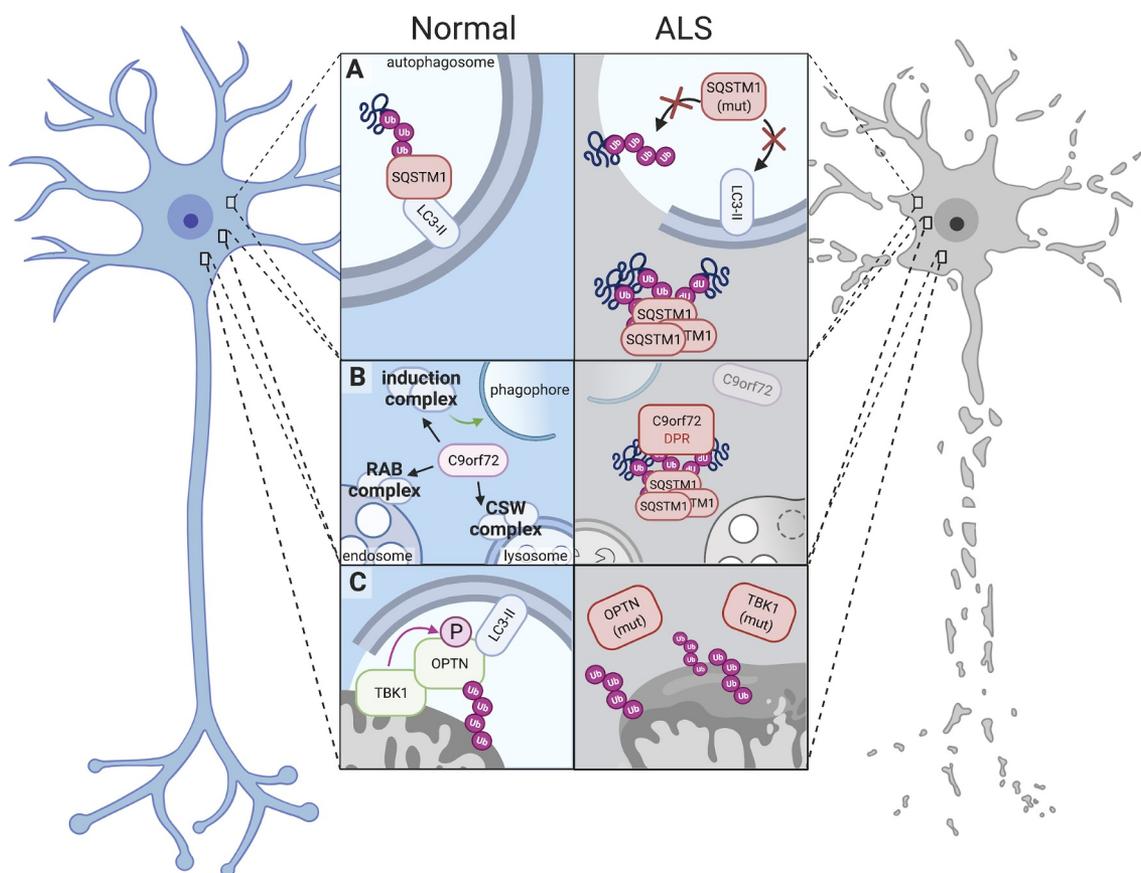

Figure 1. Dysfunction of autophagy-related proteins impairs proteostasis and leads to neurotoxicity in ALS. (**A**) Under normal conditions, SQSTM1 serves as a receptor protein in selective autophagy and binds both LC3-II and polyubiquitinated proteins, thereby targeting ubiquitinated substrates to phagophores (*left*); Mutations in *SQSTM1* abrogate SQSTM1's binding activities (*right top*) or result in the aggregation of SQSTM1 into ubiquitin-positive inclusions (*right bottom*). (**B**) The C9orf72 protein participates in several autophagy-related complexes, including the autophagy **induction complex** (ULK1-RAB1A) that promotes autophagosome biogenesis, the RAB7-RAB11 complex (**RAB complex**) that regulates endosome maturation, and the C9orf72-SMCR8-WDR41 (**CSW) complex** that regulates lysosomal dynamics and autophagic flux (*left*). Disease-associated *C9orf72* mutations reduce C9orf72 protein levels (*right*), while dipeptide repeat proteins generated from the *C9orf72* expansion localize to SQSTM1- and ubiquitin-positive inclusions (*right*). (**C**) In normal mitophagy, TBK1 binds and phosphorylates OPTN, enhancing its affinity for polyubiquitinated mitochondria and LC3-II (*left*). *TBK1* and *OPTN* mutations perturb these functions and compromise efficient mitophagy, leading to failed mitochondrial clearance and dysfunctional mitochondria.

extending phagophore membrane guided by the ATG12–ATG5-ATG16L1 complex, catalyzing insertion of MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) into the phagophore membrane [40,41]. Before membrane insertion, the unbound and immature LC3 precursor protein (LC3-I) undergoes proteolytic processing by ATG4, and conjugation to phosphatidylethanolamine through ATG7 and ATG3. The resulting mature, lipidated isoform (LC3-II) is incorporated into the phagophore membrane [41].

In the *sequestration* phase, the extending ends of the phagophore fuse with one another, forming a double membrane-bound vesicle called an autophagosome that surrounds a segment of cytoplasm, together with its macromolecular and organellular constituents [42]. Molecular motors transport autophagosomes along microtubules to coordinate fusion with lysosomes and form autolysosomes, within which the vesicular constituents are exposed to hydrolytic proteases for degradation [43,44]. Fusion of the autophagosome and lysosome is mediated by the homotypic fusion and protein sorting (HOPS) complex, whose subunits include VPS11, VPS16, VPS18, VPS33A, VPS39, and VPS41 [45,46]. Dismantled cellular components in the autolysosome lumen are actively exported for reconstitution into new macromolecules or for energy metabolism [42]. Global regulation of the myriad effectors and phases of autophagy is overseen by TFEB (transcription factor EB), a basic helix-loop-helix leucine zipper that directs expression of autophagy and lysosomal genes comprising the coordinated lysosomal expression and regulation (CLEAR) network by binding to a conserved promoter motif [47,48]. Further details regarding TFEB activity and regulation are reviewed elsewhere [49–52].

Although autophagic cargo can be trapped passively within the autophagosome in nonselective autophagy, direct substrate recruitment to phagophores in selective autophagy is mediated by a series of receptor proteins (e.g., SQSTM1/p62 [sequestosome 1], NBR1, AMBRA1) that contain both an LC3-interacting region (LIR) and separate motifs for binding polyubiquitinated proteins [41]. These receptor proteins therefore serve as molecular scaffolds for targeting ubiquitinated proteins to phagophores for degradation via autophagy rather than the proteasome.

Additional autophagic specialization is achieved through **chaperone-mediated autophagy** (CMA), **microautophagy**, targeted degradation of mitochondria in **mitophagy**, and degradation of ribonucleoprotein particles (RNPs) in **granulophagy**. Client proteins that harbor an internal or masked amino acid motif (lysine-phenylalanine-glutamate-arginine-glutamine, or KFERQ) are recognized by the molecular chaperone HSPA8/HSC70 upon partial protein unfolding, enabling CMA. Once bound by HSPA8, the protein is targeted to the lysosome via interaction between HSPA8 and the lysosomal protein LAMP2A [53]. The substrate protein is fully unfolded by HSPA8, LAMP2, and additional co-chaperones as it translocates into the lysosomal lumen for degradation [54]. In microautophagy, lysosomes directly capture cargo from the cytoplasm by membrane invagination to form intraluminal "autophagic tubes," which expand and pinch off to form microvesicles within the lysosome that are ultimately degraded by lysosomal hydrolases [55].

Physiologic and chemical insults to mitochondria – via reactive oxygen species, disruption of the inner membrane potential in disease models, or treatment with protonophores such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) – triggers mitophagy. Upon mitochondrial damage, PINK1 (PTEN induced kinase 1) is recruited to the outer mitochondrial membrane [56], where it phosphorylates and activates the E3 ubiquitin ligase PRKN/parkin, which in turn ubiquitinates mitochondrial surface proteins [24]. These polyubiquitin chains are also phosphorylated by PINK1, and the resultant phospho-ubiquitin moieties not only bind the receptor proteins OPTN (optineurin), CALCOCO2/NDP52, and SQSTM1, but also activate TBK1 (TANK-binding kinase 1) [57]. OPTN, CALCOCO2, and SQSTM1 contain ubiquitin-binding domains and LIR motifs, thereby providing a means for delivering ubiquitinated mitochondria to autophagosomes [58]. Additionally, TBK1 amplifies the signaling cascade for mitophagy by phosphorylating OPTN and SQSTM1 (which enhances binding to ubiquitin and LC3) and promoting TBK1 autophosphorylation in a positive-feedback manner [58,59]. Interestingly, degradation of neuronal mitochondria can occur in a non-cell autonomous fashion, as demonstrated by the astrocytic engulfment and degradation of mitochondria excreted from retinal ganglion cells [60].

Essential processes such as translation may stall during specific phases of development or when cells are exposed to certain stressors (heat shock, ER perturbation, proteasomal dysfunction, or hyperosmolar exposure). To prevent potentially toxic errors in translation and facilitate mRNA degradation, cells upregulate granulophagy, a distinct subtype of autophagy that acts on ribonuclear protein (RNP) granules [61]. Several different RNPs are granulophagy substrates, including cytoplasmic stress granules and processing bodies, which dynamically assemble to sequester non-translating mRNA-ribonuclear protein complexes (mRNPs), translation initiation factors, translational repressors, and mRNA decay machinery until the stress resolves or is removed [62]. In granulophagy, RNP granules that contain RNA-binding proteins and their associated mRNAs are targeted for degradation via VCP/p97 (valosin containing protein), a chaperone-like, type II AAA+ ATPase that binds and delivers large ribosomal subunits to autophagosomes [63–66]. Granulophagy can also serve a compensatory role in the setting of dysfunctional macroautophagy [67].

Key autophagy factors and disease-causing mutations in ALS

The long-term health of neurons is inextricably reliant on intact proteostasis. This notion is underscored by the fact that nearly every neurodegenerative disorder is marked by the accumulation of misfolded proteins within or adjacent to affected neurons and glia, in spite of heterogeneous clinical and pathological characteristics among these diseases [68,69]. The abnormal and age-dependent, progressive formation of protein inclusions observed in disease-affected regions of the nervous system implies that proteostasis mechanisms become overwhelmed or dysfunctional with time [68–73]. Molecular

and histopathologic analyses of patient tissues demonstrate mislocalized or accumulated autophagy machinery, pathogenic mutations underlying familial neurodegenerative diseases disable known autophagy-related factors, and genetically ablating autophagy effectors produce neurodegeneration in cell and animal model systems [74–81], supporting a role for autophagy dysfunction in the development of age-related neurodegenerative diseases. Accordingly, precisely defining the functions performed by key autophagy effectors in each phase of autophagy, and the dysfunction conferred by genetic mutations in each factor in relation to disease phenotypes, is essential for the development of effective, autophagy-based therapies for neurodegenerative diseases. Furthermore, the degree to which autophagy is differentially regulated in a cell type-specific manner, and how each of the myriad autophagy effectors functions in distinct cell types, remains insufficiently explored, potentially contributing to the disappointing results of indiscriminate autophagy modulation in disease models to date. Ultimately, these are crucial considerations when deciding on discrete targets, maximizing clinically meaningful benefits, and minimizing off-target toxicity. Below, we consider the physiological and pathophysiological relevance of autophagy-related factors in the development of two neurodegenerative disorders, ALS and frontotemporal dementia (FTD), since these disorders often share a common background, and arise due to mutations in genes encoding fundamental components of the autophagy pathway. We will also discuss the impact of autophagy on ALS-FTD in a cell type-specific manner, with particular attention paid to autophagy effectors and their function in discrete cell types within the central nervous system (CNS).

SQSTM1/p62

Selective autophagy relies on autophagy receptor proteins that recognize ubiquitinated cargo and target them to the autophagy machinery [82]. The LC3-interacting region (LIR) enables autophagy receptors to interact with the Atg8 family of proteins within the nascent autophagosome. The ubiquitin-binding protein SQSTM1 harbors a well-characterized LIR [83,84]. Besides its LIR domain, SQSTM1 contains an N-terminal Phox1 and Bem1p (PB1) domain, a zinc finger (ZZ), a TRAF6 (TNF receptor associated factor 6)-binding (TB) motif, a KEAP1-interacting region (KIR), and a ubiquitin-associated (UBA) domain [85,86]. Homodimerization of the UBA domain maintains SQSTM1 in an inactive state by preventing its interaction with ubiquitin [87,88]. Upon cargo protein ubiquitination, the UBA domain of SQSTM1 is phosphorylated by ULK1 on serine 407, liberating the protein from dimeric inactivation [89]. Subsequently, TBK1, CSNK2 (casein kinase 2), or ULK1 phosphorylate the UBA domain on serine 403, increasing the affinity of SQSTM1 for ubiquitin chains [59,90]. The interactions between SQSTM1, ubiquitinated cargo proteins, and membrane-bound LC3-II are reinforced by homopolymerization of the SQSTM1 PB1 domain [91]. The resulting complex undergoes liquid-liquid phase separation (LLPS), facilitating assembly of the phagophore [92,93]. Incorporation of SQSTM1 into the autophagosome leads to its degradation upon fusion with lysosomes, along with its ubiquitinated targets. As such, SQSTM1 levels inversely correlate with autophagy efficiency, and are often used to estimate autophagy flux [84,94,95].

In addition to targeting ubiquitinated proteins, SQSTM1 also mediates the degradation of ubiquitinated bacteria and dysfunctional mitochondria [96]. As discussed above, the selective degradation of mitochondria by autophagy, or mitophagy, is regulated by the serine/threonine protein kinase PINK1 and the ubiquitin E3 ligase PRKN [97,98]. During PRKN-dependent mitophagy, SQSTM1 is recruited by TBK1 phosphorylation on serine 403 and is essential for the clearance of polyubiquitinated mitochondria [59,99].

SQSTM1 is also involved in several key physiological signaling pathways, including MTOR-dependent signaling, regulation of the ubiquitin-proteasome system (UPS), inflammation, the response to oxidative stress, and apoptosis [86,100–102]. When amino acids are abundant, SQSTM1 is phosphorylated and forms a signaling hub on the lysosomal membrane through its interaction with RPTOR, which in turn recruits and activates MTORC1 on the surface of the lysosome [103–105]. Additionally, SQSTM1 directly associates with the proteasome through its PB1 domain [106–108] and is itself a proteasome substrate [109]. Under conditions of proteasomal impairment, or when the UPS is overwhelmed, SQSTM1 delivers ubiquitinated substrates to the lysosome for autophagic degradation, thereby providing a crucial link between these two cellular degradation pathways.

SQSTM1 is also significantly upregulated during oxidative stress. The transcription factor NFE2L2/NRF2 (nuclear factor erythroid-derived 2-like 2) is an essential component of the oxidative stress response and is normally degraded by the UPS via its interactions with KEAP1 (kelch-like ECH-associated protein 1) [110]. Under oxidative stress, KEAP1 releases NRF2 to translocate to the nucleus and activate the transcription of genes involved in the antioxidant response, including *SQSTM1* [110--110–112]. SQSTM1 interacts with KEAP1 and displaces NRF2, promoting its own transcription as well as other genes involved in the antioxidant response [110]. This positive feedback loop significantly enhances the cellular capacity for selective autophagy, contributing to the clearance of damaged organelles and proteins, and eventually recovery from stress [111,113].

SQSTM1/p62 in disease

SQSTM1 is a major component of neuronal and glial cytoplasmic inclusions that characterize many neurological disorders such as Alzheimer disease and Parkinson disease [114,115]. Furthermore, SQSTM1-positive inclusions are found in the majority of patients with ALS and FTD, and often overlap with inclusions that stain for ubiquitin and TARDBP/TDP-43 (TAR DNA binding protein), an essential RNA-binding protein integrally connected with ALS-FTD [82,116–118] (Figure 1). In the most commonly inherited form of ALS and FTD due to mutations in the *C9orf72* gene (described below), SQSTM1 accumulates together with ubiquitin in neurons and glia [119–121] as well as muscle fibers [122]. In fact, individuals with disease-associated *C9orf72*

mutations display a significantly greater burden of SQSTM1-positive glial inclusions than those with sporadic ALS [82].

Consistent with the pivotal links between SQSTM1 and several distinct signaling pathways, *SQSTM1* mutations result in multiple overlapping disease phenotypes. *SQSTM1* mutations are not only a rare cause of ALS-FTD, accounting for ~1-3.5% of cases with or without family history [123–125], but also Paget's disease of bone (PDB), a chronic and progressive skeletal disorder characterized by disorganized bone turnover [126,127], and inclusion body myositis (IBM) [128]. Rather than being mutually exclusive, each of these conditions may co-exist with one another, resulting in a spectrum of overlapping clinical signs collectively referred to as "multisystem proteinopathy" [129]. In contrast to PDB-associated mutations that affect mainly the UBA domain [127], *SQSTM1* mutations identified in ALS-FTD affect several different regions. ALS-FTD-associated *SQSTM1* mutations frequently affect the UBA and LIR domains involved in autophagy and include both missense and nonsense mutations [123–125]. *SQSTM1* mutations affecting the UBA domain impair ubiquitin recognition [130], whereas mutations within the LIR domain impact LC3 recognition and substrate delivery to the autophagosomes [84,131]. Additionally, mutations detected in the *SQSTM1* promoter region are associated with reduced SQSTM1 protein levels in familial ALS-FTD [132,133].

Evidence from animal models strengthens the association between SQSTM1, autophagy impairment, and multisystem proteinopathy. *sqstm1*-knockout mice display reduced numbers of osteoclasts as well as cognitive impairment and anxiety, associated with hyperphosphorylated MAPT/tau and neurofibrillary tangles [134–136]. Knockdown of the *SQSTM1* ortholog in zebrafish leads to an ALS-like phenotype consisting of locomotor and motor neuron axonal abnormalities. Overexpression of wild-type human SQSTM1 or application of the autophagy activator rapamycin rescues these phenotypes [137]. On the other hand, overexpression of *SQSTM1* variants carrying disease-associated mutations in the UBA domain exhibit a PDB-like skeletal disorder [138,139] as well as impaired spatial learning and long-term memory [140]. Additional knock-in models involving the introduction of disease-associated mutations into endogenously expressed *SQSTM1* are needed to clarify its function in select cell types, and how pathogenic *SQSTM1* mutations result in neuronal dysfunction as well as disorders of bone and muscle.

C9orf72

In 2011, the seminal discovery of disease-associated expansion mutations within the *C9orf72* gene uncovered the most common genetic cause of inherited ALS and FTD, accounting for 23–47% of familial cases and 4–21% of sporadic disease. Unaffected individuals carry 2–30 repeated stretches of GGGGCC within the first intron of the gene, but patients with *C9orf72*-associated ALS-FTD harbor several hundred to thousands of repeats [141,142].

The C9orf72 protein belongs to the DENN (differentially expressed in normal and neoplastic cells) family [143,144] and binds the GTPases RAB7 and RAB11, which are involved in endosome maturation and recycling [145]. C9orf72 also forms a complex with SMCR8, a guanine nucleotide exchange factor, and WDR41, a WD40 protein essential for targeting the C9orf72-SMCR8-WDR41 complex to lysosomes [146–150]. Here, this complex interacts with RB1CC1-ULK1-ATG13-ATG101, which together are involved in autophagosome formation [149]. Separately, the C9orf72-SMCR8-WDR41 complex acts as a guanine nucleotide exchange factor for RAB8A and RAB39B, regulating vesicle trafficking and autophagic flux [148]. Additionally, through its interactions with ULK1 and RAB1A, a GTP-binding protein involved in vesicular protein trafficking and MTOR regulation [151,152], C9orf72 promotes autophagy initiation [153]. Together, these findings suggest that C9orf72 assists in the formation of nascent autophagosomes, consistent with the finding of impaired autophagy in murine cortical neurons [148] and human iPSC-derived neurons [154] upon *C9orf72* knockdown.

C9orf72 in disease

The *C9orf72* hexanucleotide repeat expansion exhibits substantial pleiotropy at the clinical and molecular levels. For instance, *C9orf72* expansion mutations are associated not only with ALS/FTLD, but also with symptoms reminiscent of Parkinson disease, Alzheimer disease, Huntington disease, and corticobasal syndrome, reinforcing the imperfect connection between clinical and molecular phenotypes and emphasizing the need for molecular diagnostics [155–164]. ALS-FTD due to *C9orf72* mutations displays TARDBP pathology, consisting of granular neuronal cytoplasmic inclusions, diffuse neuronal cytoplasmic staining, and glial cytoplasmic inclusions rich in TARDBP [120]. A unique hallmark of *C9orf72*-related disease is the presence of cytoplasmic star-like inclusions positive for SQSMT1 and ubiquitin, but not TARDBP, in cerebellar granule neurons and other cell types (Figure 1). Nevertheless, the impact of these inclusions is uncertain, as cerebellar degeneration and clinical phenotypes attributable to cerebellar dysfunction are lacking in *C9orf72*-related disease.

Although the *C9orf72* mutation occurs within a non-coding region of the gene, the unique secondary structure of the expansion triggers ribosome stalling and the initiation of translation through a process known as repeat-associated non-AUG (RAN) translation. As there is no true start codon, the GGGGCC expansion is translated in all three reading frames into repeating elements of two peptides: GP, GA and GR (sense), and PG, PR and PA (antisense) [165–165–168]. Antibodies specific for each dipeptide repeat (DPR) also stain SQSTM1-positive inclusions, including the star-like inclusions unique to *C9orf72*-related disease, suggesting that DPRs are targeted for clearance by the UPS and/or autophagy [119,169–171] (Figure 1). Furthermore, the loss of C9orf72 function and consequent impairment in autophagy may enhance DPR accumulation in *C9orf72* mutation carriers, acting synergistically to accentuate neurodegeneration [154,172,173]. On the other hand, DPR inclusions rarely co-label with TARDBP pathological inclusions and are mainly found in areas of the CNS that are not pathologically affected

[171,174–177], arguing against a direct role for DPRs in neurodegeneration.

C9orf72 mutations are associated with reduced basal autophagy and enhanced sensitivity to autophagy inhibitors in human iPSC-derived neurons [153,178]. Paradoxically, however, *c9orf72* knockout in mouse (which models the loss of function attributed to the hexanucleotide repeat expansion) enhances autophagy by de-repressing MTOR and, in turn, increasing TFEB nuclear translocation [179,180]. In addition, conditioned medium from astrocytes differentiated from patient-derived iPSC lines (two of which harbor *C9orf72* expansion mutations) applied to HEK293T cells reduces LC3-II while increasing SQSTM1 levels [181], suggesting non-cell autonomous inhibition of autophagy by astrocytes in ALS. Overall, further studies are needed to determine if disease-associated *C9orf72* expansions affect autophagy through additional means independent of loss-of-function mechanisms and whether the C9orf72 protein may have cell type-, species-specific, and non-cell autonomous effects that explain these discrepancies.

Deletion of the zebrafish and *C. elegans* homologs of *C9orf72* results in impaired locomotion and motor axon degeneration [182,183], implying that the C9orf72 protein is essential for motor neuron function and survival in these systems. In contrast, deletion of the mouse *C9orf72* ortholog leads to splenomegaly, lymphadenopathy, dysregulation of macrophages and microglia, and excess production of inflammatory cytokines [184–187]. While *c9orf72* knockout animals exhibit a shortened lifespan [184,185,187], no specific neuronal defects have been identified in these mice. On the other hand, loss of endogenous murine *C9orf72* results in glial activation, impaired autophagy and toxicity when combined with the *C9orf72* expansion mutation [173]. Further, neurons derived from iPSCs carrying the *C9orf72* repeat expansion recapitulate key features of cellular pathology associated with ALS, including hyperexcitability [154,188,189] and decreased viability upon exposure to excitotoxic agents such as glutamate [154,190,191]. These results are consistent with those in animal models, suggesting that the endogenously encoded *C9orf72* repeat expansion mutation leads to toxicity by both gain and loss of function mechanisms.

TBK1

TBK1/NAK/T2K (TANK binding kinase 1) is a cytoplasmically-localized homodimeric multidomain protein with an ubiquitin-like domain, a N-terminal serine/threonine kinase domain, and two coiled-coil domains [192]. TBK1 is expressed in all tissues, but at much higher levels in neurons of the hippocampus, cortex and lateral ventricle. Moderate levels of the protein have been detected in the glial cells of the cortex and cerebellum [193]. Through its phosphorylation of the autophagy receptors OPTN, SQSTM1, and CALCOCO2/NDP52 (calcium binding and coiled-coil domain 2) [58,59,194], TBK1 regulates several aspects of autophagy, including macroautophagy, xenophagy and mitophagy [59,90,148,194–196]. TBK1-dependent effects on autophagy and autophagy receptor proteins are spatially and functionally regulated by interactions with the ULK1 initiation complex [148,150,153,197]. TBK1 also phosphorylates SMCR8, which forms a complex with C9orf72 and WDR41, thereby enhancing autophagy initiation [148]. In neurons, TBK1 knockdown enhances the accumulation of SQSTM1, which can be rescued by phosphomimetic *SMCR8* mutations [148], suggesting that SMCR8 phosphorylation is crucial for TBK1's effects on autophagy.

In addition to its role in modulating autophagy, TBK1 regulates innate immunity by phosphorylating IRF3 (interferon regulatory factor 3), triggering nuclear localization of IRF3 and the subsequent production of IFNA2/IFNα (interferon alpha 2) and IFNB/IFNβ. These events, in turn, stimulate the expression of interferon-inducible genes, including *ISG15*, whose protein product interacts with SQSTM1 and HDAC6 [198,199]. Whether TBK1 acts on autophagy or innate immunity may partially depend on its associations with specific adaptor proteins such as NAP1L1 (nucleosome assembly protein 1), TANK and TBKBP1/SINTBAD that bind in a mutually exclusive manner [58,200,201].

TBK1 in disease

TBK1 mutations were initially described in conditions with prominent neuroinflammatory responses, including two forms of glaucoma [192,202,203] and herpes simplex encephalitis [204]. Whole exome sequencing studies revealed that mutations in *TBK1* are also responsible for ~1% of patients with familial or otherwise sporadic ALS [205,206]. Supporting the link between ALS and FTD, *TBK1* mutations have also been identified in FTD patients, and in fact occur more frequently in patients with combined ALS-FTD than in those with ALS alone [207–209]. Neuropathological analysis of CNS tissue from ALS patients with *TBK1* mutations shows SQSTM1- and TARDBP-positive inclusions in motor neurons and glia [206,210,211], along with reduced *TBK1* mRNA and protein, suggesting haploinsufficiency mechanisms [205,206]. Consistent with this, most ALS-associated *TBK1* mutations affect the kinase and the coiled-coiled domains, both of which are vital for TBK1 function [206].

In vitro, silencing of TBK1 or ALS-related TBK1 variants reduces the recruitment of OPTN and LC3B to damaged mitochondria [212,213], thereby impairing mitophagy and resulting in the accumulation of defective mitochondria (Figure 1). *Tbk1* haploinsufficiency impairs motor neuron autophagy and accelerates clinical phenotypes as well as the accumulation of mutant SOD1 in transgenic ALS mouse models [214,215]. Unexpectedly, however, TBK1 loss of function also prolongs the disease course by reducing neuroinflammation, suggesting that the dual functions of TBK1 in autophagy and inflammation independently and inversely impact ALS pathogenesis. In keeping with this, *Tbk1* deletion disrupts the migration of T-cells [216] that may exert anti-inflammatory effects in ALS models [192,217].

Other ALS-related mutations and their relation to autophagy

UBQLN2

UBQLN2 (ubiquilin 2) is an adaptor protein that binds polyubiquitinated substrates via a C-terminal ubiquitin-associated

(UBA) domain, and directs them to the UPS for degradation through an N-terminal ubiquitin-like (UBL) domain [218]. UBQLN2 can also act as an autophagy receptor by directly interacting with LC3 in autophagosomes [219,220]. Rare *UBQLN2* mutations are associated with familial forms of ALS and ALS-FTD [221–223], and UBQLN2 colocalizes with SQSTM1 and ubiquitin in neuronal aggregates characteristic of both ALS and ALS-FTD [221,224]. Overexpression of ALS-linked UBQLN2 mutants interrupts the interaction between UBQLN2 and ATG9-ATG16L1 [225], while also triggering intracellular inclusions [226,227] and motor defects *in vitro* and *in vivo* [226]. In rats, overexpression of ALS mutant UBQLN2 leads to motor neuron loss and accumulation of autophagy substrates such as SQSTM1 and LC3-II [228]. Consistent with a central role for ubiquilins in autophagy, UBQLN2 modulates MTORC1 activity, lysosomal acidification, and, in turn, autophagosome maturation [229–231].

FUS

FUS (Fused in Sarcoma) is a DNA/RNA-binding protein that participates in DNA damage, RNA transport, and RNA splicing. As with *TARDBP*, mutations in the gene encoding FUS cause familial ALS with FUS-positive pathology, but neuronal inclusions in sporadic disease show little FUS staining. The majority of disease-associated *FUS* mutations affect the C-terminal nuclear localization signal, resulting in cytosolic mislocalization and aggregation of mutant FUS [232–234]. In cultured neurons, mutant FUS impairs stress granules clearance; this effect is exacerbated by genetic loss of *ATG5*, enhancing stress granule accumulation and, ultimately, leading to cell death [235]. Conversely, rapamycin-mediated activation of autophagy ameliorates these features, suggesting that FUS-positive inclusions are autophagy substrates [235]. Supporting this, autophagy activation via PI3K, AKT or MTOR inhibition reverses stress granule accumulation caused by ALS-linked mutant FUS [236], and autophagy induction extends survival in a *Drosophila* model of FUS-ALS [236]. Intriguingly, FUS may exert a direct influence on autophagy initiation and autophagosome elongation by maintaining expression of factors including RB1CC1 and ATG16L1, which are diminished after CRISPR-mediated knockout of *Fus* but restored with re-introduction of wild-type FUS in N2A cells [237].

VCP

VCP/p97/Cdc48 (valosin containing protein) is involved in a wide range of essential cellular processes, including DNA replication and repair, cell cycle regulation, and protein clearance [238,239]. Mutations in *VCP* most commonly lead to the combined disorder of inclusion body myopathy, Paget disease of the bone, and FTD (IBMPFD) [240], but are also responsible for ~1-2% of familial ALS cases [241–243]. Thus, as with *SQSTM1, OPTN, HNRNPA2B1, MATR3*, and *TIA1*, *VCP* mutations result in pleiotropic phenotypes affecting muscle, nerve, bone and brain. Histopathological analyses consistently illustrate TARDBP, ubiquitin and SQSTM1-positive inclusions in VCP-related diseases [244–247]. Moreover, muscle from IBMPFD patients carrying *VCP* mutations [246] and transgenic mice expressing VCP mutants [244,248,249] display accumulations of SQSTM1- and LC3-positive autophagosomes and abnormal mitochondria [250,251]. Together, these observations suggest a conserved pattern of autophagy dysfunction in association with *VCP* mutations, implying a crucial role for VCP in autophagy and the maintenance of protein homeostasis.

Supporting this, several studies have highlighted key functions for VCP in autophagosome maturation. In IBMPFD murine muscle lysates, *VCP* mutations disrupt autophagosome maturation and fusion with lysosomes, but not the formation of early autophagosome precursors [246,249]. Similarly, enlarged and acidified autophagic vacuoles accumulate in cells expressing disease-associated VCP mutants, suggesting a late-stage block in the fusion of lysosomes with autophagosomes [249]. Motor neurons and astrocytes derived from iPSCs carrying pathogenic *VCP* mutations demonstrate cytoplasmic TARDBP accumulation, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and oxidative stress, ultimately leading to cell death [252]. Neurons may not be the only cell type affected by *VCP* mutations in these conditions – mutant *VCP* iPSC-derived astrocytes were unable to sufficiently support motor neuron survival, indicating the presence of non-cell autonomous mechanisms of neurodegeneration in VCP-related diseases [252].

OPTN

OPTN has emerged as a key player in the regulation of various cellular mechanisms, including vesicular trafficking, maintenance of the Golgi, NFKB/NF-κB signaling and immune response [253]. OPTN is an autophagy receptor that binds ubiquitinated cargo (via its UBAN domain) and LC3 (via its LIR domain) [194,254,255], although it can also act independently of target ubiquitination [254]. Upon mitochondrial damage, OPTN translocates to the surface of mitochondria, an interaction that is stabilized via TBK1-mediated phosphorylation, facilitating the engulfment of mitochondria into phagophores [256]. OPTN also directly interacts with SQSTM1 to form an autophagy receptor complex that accelerates autophagy flux [257]. Genetic analyses have uncovered >20 *OPTN* mutations in ALS patient cohorts, accounting for ~3% of familial disease and ~1% of sporadic disease [258]. Interestingly, the majority of pathogenic *OPTN* mutations are located in its UBAN domain and may impair ubiquitin binding [259], suggesting that defects in the targeting of ubiquitinated substrates could be important for ALS pathogenesis. In addition, ALS-associated *OPTN* mutations enhance microglial NFKB1 expression [260], which may accentuate neuronal loss secondary to neuroinflammation [261].

CHMP2B

CHMP2B (charged multivesicular body protein 2B) is a subunit of the endosomal sorting complex required for transport-III (ESCRT-III) [262]. ESCRT-III is one of the four multiprotein complexes required for multivesicular body formation, and is responsible for the membrane deformation that enables autophagosome initiation and endolysosomal trafficking [263]. *CHMP2B* mutations were initially associated with rare cases of chromosome 3-linked familial

FTD [264,265], but *CHMP2B* mutations were subsequently identified in ALS patients with a lower motor neuron predominant syndrome [266,267]. Although TARDBP is typically absent from neuronal inclusions found in *CHMP2B*-associated disease, SQSTM1-positive oligodendroglial inclusions are found in the motor cortex of ALS patients with *CHMP2B* mutations [267]. Motor neurons from *CHMP2B* mutant ALS patients show increased calcium concentrations, reduced ATP availability, downregulation of the MAPK/p38 signaling pathway and impaired autophagy initiation [266]. Furthermore, overexpression of CHMP2B mutants leads to the formation of large cytoplasmic vacuoles and increased LC3-II protein levels [266], suggesting that disease-associated *CHMP2B* mutations disrupt autophagy. In agreement with these observations, *CHMP2B* downregulation or expression of disease-associated CHMP2B mutants in *Drosophila* and mammalian cell lines results in aberrant endosomal structures, impaired autophagosome maturation, and cytosolic TARDBP aggregates [264,265,268,269].

Transgenic mice expressing mutant CHMP2B develop progressive neurological defects, axonal pathology, early mortality and neuronal loss reminiscent of FTD [270,271]. These mice also develop lysosomal storage pathology characterized by large neuronal aggregates derived from the endosomal system [272], and early microglial proliferation leading to a pro-inflammatory phenotype at later stages [270]. In contrast, few phenotypes were noted in *chmp2b* knockout animals [271], suggesting predominantly gain of function toxicity in association with pathogenic *CHMP2B* mutations.

Autophagy in the context of ALS

Disrupted autophagy and proteostasis are not only apparent in experimental models of familial ALS, with defined genetic mutations impairing autophagy effectors or imparting proteotoxic stress, but also likely play a key role in the pathogenesis of sporadic ALS (sALS). In humans with sALS, pathologic inclusions found in motor neurons are surrounded by LC3 and SQSTM1 [273], suggesting failed or stalled clearance via autophagy. SQSTM1-positive inclusions are also ubiquitin-positive, consistent with a disruption in the UPS [118]. Furthermore, biochemical and histopathologic examination of frontotemporal cortex and spinal cord in sALS patients reveals that ubiquitin-positive inclusions contain TARDBP [274,275], which is itself involved in the regulation of autophagy [276]. Markers of oxidative stress, ER stress, and the UPR are upregulated in spinal cords and anterior horn cells from human patients with sALS [277–280]. Accumulations of dysmorphic and enlarged mitochondria, many with deficiencies of critical oxidative enzymes, in anterior horn cells, intramuscular nerves, and skeletal muscle are characteristic of sALS [281–285]. Such mitochondrial abnormalities, together with observations of OPTN-positive inclusions and *de novo* *OPTN* mutations found in sALS [286–289], are suggestive of attenuated mitophagy. Collectively, these findings indicate autophagy dysfunction compounded by failures in additional proteostatic mechanisms, which may together lead to the degeneration of susceptible neuronal populations in sALS.

In familial ALS, apart from the ALS-associated mutations that directly affect autophagy-related genes, genetic mutations in *TARDBP* and *SOD1* deserve particular mention not only because they are responsible for some of the more common familial forms of ALS but also because of their intrinsic connections with autophagy. Mutations in *TARDBP*, which encodes the transactive response DNA binding protein 43 kDa (TDP-43), are the third most common cause of familial autosomal dominant ALS and account for rare cases of sporadic ALS [290–293]. Mutations in the *SOD1* gene, which encode the free radical/reactive oxygen species scavenger superoxide dismutase 1 [294,295], represent the second most common cause of familial ALS in Europeans and the most common cause in those of Asian descent [296]. Disease models involving mutations in either of these genes have been indispensable for uncovering fundamental mechanisms of ALS pathogenesis. Models of TARDBP proteinopathy range from transient transfections in primary rat cortical cultures [297], to motor neurons [298] and astrocytes [299] differentiated from patient-derived induced pluripotent stem cells (iPSCs) or murine embryonic stem cells [300], to transgenic or virally transduced rodents overexpressing wild-type [301–301–316], truncated [317,318], or mutant TARDBP harboring the A315T [301,308,319], Q331K [310], M337V [301,310,311,-311,320–322], G348C [308] mutations, knock-in mutant TARDBP^{N390D} [300], or *tardbp* knockouts [323–328] (these models are reviewed in further detail elsewhere [329–334]). SOD1 model systems include human and murine motor neuron cultures [335,336], and transgenic mice expressing SOD1 with the G93A [214,215,335–338], G86R [335,339], or H46R/H48Q [340] mutations (also reviewed elsewhere [341–344]).

Several studies employing these models point to cell-autonomous toxicity in both neurons and astrocytes in TARDBP- and SOD1-related ALS, in addition to non-cell autonomous mechanisms, implying that therapies should target both cell types for optimal effect. iPSC-derived motor neurons harboring the ALS-associated TARDBP^{M337V} mutation demonstrate an accumulation of insoluble mutant TARDBP at native expression levels [298], which in previous models has not only been closely linked to neurodegeneration [297], but also increases the risk of death in iPSC-derived astrocytes [299]. Furthermore, glial TARDBP inclusions predominantly reside in oligodendrocytes [345,346], and expression of TARDBP^{M337V} in astrocytes exerts cell autonomous toxicity [299]. Supporting the notion of non-cell autonomous contributions to disease, transplantation of SOD1^{G93A} astrocytes into the spinal cords of wild-type mice induced motor neuron loss [347]. In addition, transplantation of wild-type astrocytes into SOD1^{G93A} rat spinal cords was neuroprotective [348], and limited overexpression of NRF2 in astrocytes mitigated neurodegeneration in SOD1^{G93A} and SOD1$^{H46R/H48Q}$ mice [340].

These models also revealed key abnormalities in autophagy that occur in the context of ALS pathogenesis and suggest that autophagy modulation can dramatically influence ALS-related neurodegeneration. For example, loss of nuclear TARDBP leads to reduced ATG7 expression [349], and embryonic stem cell-derived motor neurons expressing TARDBP^{N390D} show increased levels of the autophagy suppressor, BCL2,

and reduced autophagic activity [300]. TARDBP downregulation promotes TFEB nuclear localization and TFEB target gene expression, but it also reduces DCTN1 (dynactin subunit 1) expression, thereby blocking autolysosome formation and impairing autophagic flux [350]. Transgenic expression of a C-terminal TARDBP fragment commonly associated with ALS and FTD leads to decreases in several autophagy effectors, including ATG3, ATG7, LC3, SQSTM1, and BECN1 [351]. Together, these findings link autophagy dysfunction to TARDBP pathology in ALS and suggest that correcting autophagy dysregulation or promoting the autophagic degradation of TARDBP may be of therapeutic benefit in ALS.

To this end, inducing autophagy with rapamycin reduces TARDBP accumulation and mislocalization *in vitro* [352] and *in vivo* [353], whereas inhibiting autophagy with 3-methyladenine impairs TARDBP degradation [352]. Autophagic degradation of TARDBP is enhanced by downregulation of HSP90AA1 and CDC37, suggesting that these chaperones are key suppressors of autophagy and may accentuate TARDBP-related toxicity in ALS [354]. HSPB8 overexpression also enhances autophagic clearance of TARDBP via elevations in TFEB, SQSTM1, and LC3 [355,356]. Importantly, autophagy induction rescues relevant outcomes in models that do not involve protein overexpression, but rather native mutant forms of TARDBP associated with familial disease. For example, inducing autophagy using novel compounds belonging to the benzoxazine class of molecules, including 10-NCP, enhance TARDBP^{M337V} degradation and extend survival in rodent and human neurons [357]. Inhibition of MTOR likewise reduces TARDBP aggregation and toxicity in multiple cell and animal models of ALS [353,358,359]. Similarly, the phosphodiesterase inhibitor ibudilast induces autophagy by promoting TFEB activity, which enhances clearance of TARDBP aggregates and reduces cytotoxicity [360].

Although stimulating autophagy improves outcomes in TARDBP ALS models, autophagy induction has complex effects in ALS due to *SOD1* mutations. Inducing autophagy with lithium [361] rescues SOD1^{G93A}-related pathology and cell death, and overexpression of TFEB enhances LC3 expression and prevents cytotoxicity in SOD1^{G93A}-expressing NSC-34 cells [362]. Furthermore, antagonizing the unfolded protein response (UPR) through deletion of *XBP1* [335] triggers autophagy and the clearance of SOD1 inclusions, in the process delaying disease onset and improving survival. Although these studies suggest that therapies capable of stimulating autophagy hold great promise for SOD1-related ALS, many caveats still remain. For instance, neurodegeneration may itself stimulate autophagy, and it is unclear whether this response is beneficial or maladaptive [78]. In a SOD1^{G93A} transgenic mouse model of ALS, motor neurons of symptomatic animals show increased levels of LC3-II and autophagic vacuoles compared to controls or pre-symptomatic mice. Although elevated LC3-II in this context could represent compromised autophagy flux, reduced levels of active/phosphorylated markers of the MTOR pathway [337] suggest autophagy induction. In addition, rapamycin facilitates motor neuron degeneration and rapid disease progression in SOD1^{G93A} ALS mice [338], and excess mitophagy accelerates neurotoxicity and metabolic compromise in ALS models

[363]. It is possible that overactive autophagy in SOD1-related ALS may generate toxic fragments of SOD1 or accelerate the atrophy of denervated muscle [71,364]. Regardless, the nature of autophagy induction in these biologic settings, whether representing a coping response to antecedent proteotoxicity or an active component of pathogenesis at disease onset (or both), remains incompletely defined.

Interestingly, mutant SOD1 may act in combination with autophagy dysfunction to promote motor neuron loss in ALS. Ubiquitous expression of ALS-related *TBK1* mutations, heterozygous *TBK1* deletion, or motor neuron-specific *TBK1* deletion result in loss of function phenotypes but are insufficient to produce ALS-related neurodegeneration in mice [214,215]. However, in the setting of the SOD1^{G93A} mutation, loss of *TBK1* or pathogenic *TBK1* mutations accentuate several pathologic features of ALS (disease onset, muscle denervation) while ameliorating others (inflammation, early death) [214]. Similarly, conditional deletion of *Atg7* in mouse motor neurons accentuates early ALS pathology but mitigates glial inflammation and early death [336]. These findings not only suggest that impaired autophagy sensitizes the nervous system to key pathogenic events occurring early in ALS pathogenesis, but also highlight the importance of neuroinflammation in disease progression [215].

The interplay between autophagy and *TARDBP* or *SOD1* mutations is complex and influences ALS pathogenesis within and between different cell types of the nervous system. Identifying whether and how specific autophagy factors are differentially affected by *TARDBP* and *SOD1* mutations, and to what extent these alterations are salient for neurons, glia, and/or muscle, is critical for refining therapeutic strategies and targeting cell type-specific regulators. In so doing, future ALS therapies may be able to effectively induce autophagy in the relevant cell types without adversely affecting others, maximizing on-target benefits while reducing off-target side effects that would be unavoidable with indiscriminate autophagy inducers.

Cell type-specific regulation of autophagy

Genetic disruption of key autophagy effectors is sufficient to cause ALS, highlighting the integral relationship between autophagy and neuronal survival. Moreover, individual ALS mutations affect all phases of autophagy, from early (*C9orf72*), intermediate (*SQSTM1, CHMP2B*), and late (*VCP*) stages of the pathway. Since neuronal death and dysfunction occur regardless of the point at which autophagy is impaired by disease-causing mutations, enhancing flux through the pathway as a whole is indispensable for preventing neurodegeneration. Notably, with global expression of germline mutations associated with ALS, neural and non-neural tissues manifest the detrimental effects of compromised autophagy [11], but to varying extents [365,366]: *SQSTM1* mutations affect muscle, bone, and liver [125,367,368]; *C9orf72* deficiency leads to lymphocytic inflammation, lymphadenopathy, and splenomegaly [149]; *CHMP2B* mutations induce oligodendroglial pathology [267]; *OPTN* mutations produce microglial activation and glaucoma [288]; and *VCP* mutations cause extensive cardiac

and skeletal muscle pathology [248]. Since a given autophagy-related mutation is identical in all tissues, the heterogeneous manifestations of autophagy dysfunction between tissues may relate to differences in autophagy regulation specific to each cell type. Below we describe cell type-specific mechanisms regulating autophagy, emphasizing the distinct phenotypes observed in each tissue upon autophagy dysfunction, and outlining therapeutic strategies for selectively modulating autophagy within predetermined cell types. A summary of key cell type-specific regulators is included in Figure 2 and Table 1.

Neurons

Neurons exhibit specialized topology that includes not only dendrites capable of extensive arborization, but also axons that extend at times over a meter in length [369]. As a result, autophagy must operate within the contexts of these localized compartments since the autophagy-lysosomal system of the soma is metabolically and temporally incapable

of meeting all dendritic, axonal and synaptic demands [370]. Another critical factor that uniquely impacts neuronal autophagy is the requirement of neurons to maintain protein homeostasis for the entire lifespan of an organism; this arises from the extended post-mitotic state of neurons, and the lack of their renewal and replacement [371]. Neuronal autophagy also exhibits physiologic characteristics that are fundamentally distinct from those in other cellular types, as detailed further below.

Autophagic activity varies significantly within each subcellular compartment of the neuron. Autophagy initiation typically begins outside of the soma, in the distal axon tip [370,372,373]. Newly-formed autophagosomes in neuronal processes undergo retrograde transport to the soma and proximal segments of neurites to fuse with somatodendritic lysosomes for cargo degradation, with maturation and acidification of the autophagosomal lumen developing *en passant* [370,372,374]. Axonal autophagy is dependent on specialized enzymatic apparatuses, including the PINK1-PRKN complex for localized mitophagy [24], KIF1A/ UNC-104 for autophagosome formation at the synapse [22,25],
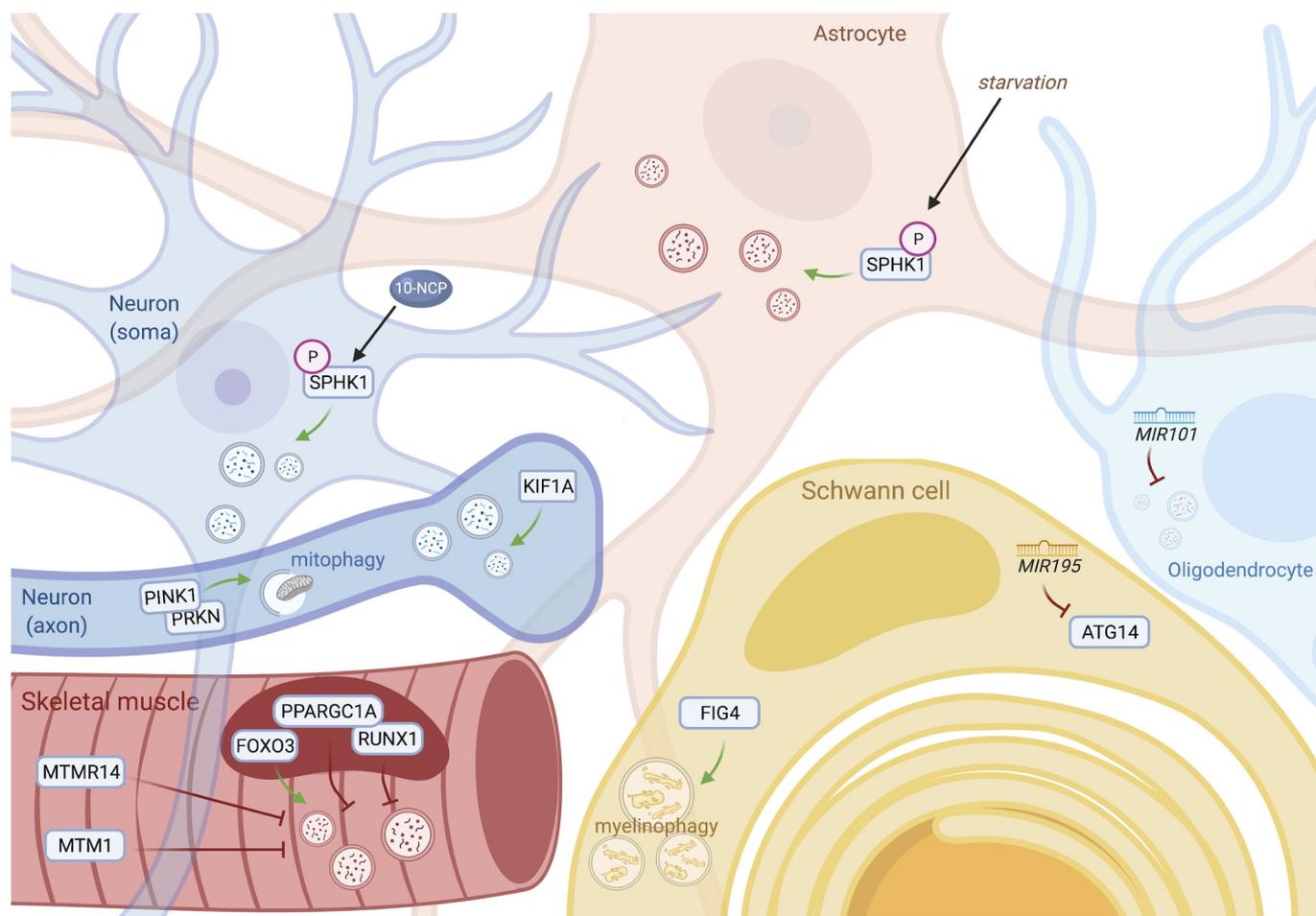

Figure 2. Distinct factors regulate autophagy among different cell types of the nervous system. In each of the cells which comprise the central and peripheral nervous systems, autophagy is differentially regulated by cell type-specific effectors. In neurons (*top left*), modulation of SPHK1 by phenoxazine compounds such as 10-NCP, but not starvation, potently induces autophagy. In contrast, nutrient deprivation is sufficient to promote SPHK1 signaling and autophagy induction in astrocytes (*top center*). In the axonal compartment of neurons, KIF1A and PINK1-PRKN are critical for facilitating local autophagic activity (*left, middle*). MicroRNAs such as *MIR101* and *MIR195* suppress oligodendrocytic and Schwann cell autophagy, respectively (*right*). Schwann cells also clear myelin debris through myelinophagy, a unique form of selective autophagy that is dependent on FIG4 (*bottom*). In muscle, specific transcription factors can exert activating (FOXO3) or suppressive (PPARGC1A, RUNX1) effects on autophagy, and phosphatases such as MTM1 and MTMR14 inhibit autophagy by recycling phosphoinositides needed for autophagy induction (*bottom left*).

Table 1. Role of starvation-induced MTOR inhibition in various cell types of the nervous system.

cell type		Starvation	MTOR inhibition	common factors	cell type-specific factors
Neurons		Insensitive or mild activation [385]	Insensitive [370]	AMPK	PINK1, PRKN (axonal mitophagy) [24]
				TFEB	KIF1A (synaptic autophagy) [22,25]
				ULK1	HAP1, MAPK8IP1 (axonal transport) [23,26]
				PIK3C3/VPS34	SPHK1 (benzoxazine treatment) [11]
Glia	Astrocytes	Activates [404]	Activates [404]	BECN1	SPHK1 (starvation induction) [11]
	Oligodendrocytes	Activates [450]	Activates [530]	ATG5	MIR101 [12]
	Schwann cells	Activates [465]	Activates [467,468]	ATG7	FIG4 [13] MIR195 [14,15]
Skeletal muscle		Activates [475]	Activates [482]	LC3	RUNX1 [474]
				ATG12	MTM1 [17]
				ATG14	MTMR14 [20]
				SQSTM1	PPARGC1A [19]
				VCP	AKT, FOXO3 [16,18]
				LAMP2 OPTN	

and the HTT-HAP1 complex and MAPK8IP1/JIP1 for retrograde transport of autophagosomes [23,26]. Autophagy is also necessary for maintaining the structural integrity of the axon [375], as Purkinje-specific deletion of *Atg7* (encoding an essential E1-like enzyme required for activating ATG12, ATG8, and LC3-I) results in Purkinje cell-autonomous axonal dystrophy and degeneration in mice [376]. In addition, autophagy helps eliminate synapses during development [377,378], but can also accentuate axon degeneration during excitotoxic stress [375].

Baseline autophagy in neurons constitutively operates at a highly processive level, such that maturing autophagosomes are rapidly incorporated into autolysosomes, which represent the majority of the autophagic vesicle population after autophagy induction or flux inhibition [379]. Basal autophagy in neurons is essential for maintaining long-term neuronal health, since disruption of baseline autophagic activity *in vivo* causes neurodegeneration and the accumulation of inclusions containing polyubiquitinated proteins [380,381]. With increasing age, basal autophagy and the capacity for efficient autophagosome maturation progressively deteriorate in neurons, leading to accumulation of malformed and immature autophagic vesicles, implying reduced proteostatic reserve over time [382].

Neuronal autophagy is distinct in several additional physiologic respects. For example, despite serving as a potent autophagy inducer in most cell types [383], starvation is an ineffective means of stimulating autophagy in neurons, even in the face of starvation-related downregulation of neuronal MTOR signaling [370]. Similarly, rapamycin and related MTOR inhibitors, as well as AKT inhibitors, are ineffective inducers of neuronal autophagy [338,384]. Alternative methods have proven more successful in neurons, however, including selective MTORC1 inhibition via everolimus [385–387], or modulation of MTOR- and AKT-independent pathways with 10-NCP, an N^{10}-substituted derivative of the heterocyclic dye compound and antipsychotic analog phenoxazine [384]. These observations imply the presence of unknown, cell type-specific pathways regulating autophagy in neurons [388].

Glia

In addition to neurons, the central and peripheral nervous systems (CNS and PNS) are populated with glial cells in approximately equal proportions to neurons [389] and are comprised of several subtypes: astrocytes, oligodendrocytes and Schwann cells (which myelinate neurons in the CNS and PNS, respectively), ependymal cells, microglia, enteric glia, and satellite cells [390,391]. For the purposes of this review, we will examine the current state of knowledge regarding glial autophagy in astrocytes, microglia, oligodendrocytes and Schwann cells.

Astrocytes. Astrocytes are the most abundant subtype of glial cells and provide structural and metabolic support for neurons through several mechanisms, including neurotransmitter clearance, free radical scavenging, modulating neuronal excitability, maintaining ion homeostasis, regulating synaptic maintenance and plasticity, paracrine and autocrine signaling, blood-brain barrier formation, modulating inflammation and injury with reactive astrogliosis, among other functions [391]. Astrocytes are enriched in intermediate filaments (including glial fibrillary acidic protein, GFAP) and exhibit morphologic heterogeneity ranging from intricately branching processes of protoplasmic astrocytes in gray matter to longer but less elaborate processes of fibrous astrocytes in white matter [392–396]. Not only do astrocytes and neurons harbor extensive morphologic and physiologic differences, but the regulatory pathways coordinating autophagy and the physiologic consequences of autophagy activation in each cell type may also differ considerably, depending on the pharmacologic or pathologic context:

- *Adaptive proteostasis*: Similar to neurons [397], autophagy is activated in astrocytes to compensate for inhibition of the UPS [398]. Such compensatory stimulation may underlie the activation of astrocyte autophagy observed in Alexander disease, a pediatric leukodystrophy caused by *GFAP* mutations which lead to accumulation of Rosenthal fibers and, in turn, proteasomal impairment [399].

- *Mitochondrial homeostasis*: Mitochondrial compromise and ATP synthase inhibition with oligomycin A stimulate autophagy to a greater extent in astrocytes than in neurons, although both cell types activate autophagy to similar extents in the face of ischemia [400]. In reactive gliosis, a process unique to astrocytes, intact mitochondrial health is essential and is maintained through autophagy, such that astrocyte-specific deletion of *Atg7* perturbs the balance of mitochondrial dynamics

to favor fission over fusion and thereby reduces oxidative capacity [401]. Unlike neurons, astrocytic loss of *Atg7* or *Atg5* is insufficient to cause cell death or the accumulation of misfolded and ubiquitinated proteins [380,381], implying greater proteostatic reserve in astrocytes compared to neurons.

- *Differential signaling cascades*: In contrast to neurons, MTOR inhibition and starvation robustly activate autophagy in astrocytes [402], whereas MTOR-independent activation of autophagy with benzoxazines (e.g., 10-NCP and fluphenazine) takes place in neurons but not astrocytes [11]. These differences in autophagy may be due to enhanced phosphorylation of astrocytic SPHK1/SK1 (sphingosine kinase 1) with starvation, whereas benzoxazines activate neuronal (but not astrocytic) SPHK1 to induce autophagy [11]. Notably, SPHK1 signaling may promote autophagy in SH-SY5Y cells and fibroblasts, but its role in mature neurons is incompletely elucidated [403–406]. Notably, although benzoxazines induce differential responses in SPHK1 phosphorylation and autophagy activation, these compounds are sufficient to reduce TARDBP^{M337V}-associated toxicity in iPSC-derived astrocytes [357], presumably through autophagy upregulation.

- *Non-cell autonomous effects on neuronal autophagy and survival*: Astrocytes regulate neuronal autophagy and exert non-cell autonomous, neuroprotective effects. In the context of neurodegeneration, astrocytes take up aggregate-prone MAPT/tau, amyloid beta (Aβ) and SNCA (synuclein alpha), and degrade these proteins through autophagy [407–412]. Furthermore, not only is autophagy responsible for degrading HTT in astrocytes, but it also promotes glutamate transporter expression in these cells [413], thereby providing greater capacity for astrocytes to buffer and mitigate glutamate-mediated excitotoxicity in disease. In transgenic mice overexpressing mutant SOD1 associated with ALS, astrocytes display more SOD1-positive inclusions than do neurons, and at an earlier stage [414]. Selective clearance of SOD1-positive inclusions from astrocytes is sufficient to slow disease progression [414], while conditioned media from SOD1-expressing astrocytes is toxic to cultured human motor neurons *in vitro* [181,415],, implying that astrocytes are both necessary and sufficient for neurodegeneration in this ALS model. Notably, reactive astrocytes from SOD1 transgenic mice secrete TGFB1, which stimulates MTOR signaling and impairs autophagy in co-cultured motor neurons [415], suggesting that astrocytes can exacerbate neuronal toxicity by compromising neuronal proteostasis in a non-cell autonomous fashion.

Further characterization of astrocyte-specific pathways will provide critical information for honing the precision of therapies for ALS by targeting neuron-specific autophagy in particular, potentiating the beneficial cell autonomous and non-cell autonomous effects of astrocyte autophagy, or both.

Microglia. Microglia are macrophage-like cells that comprise 10–15% of the CNS, and mediate diverse functions ranging from innate immunity and phagocytosis to synaptic pruning and the regulation of synaptic activity [416,417]. Activation of microglia through genetic, infectious, inflammatory, or physical stimuli, leads to stereotypical changes in their morphology, accompanied by proliferation, migration, and the release of proinflammatory cytokines and chemokines [417–419]. In neurodegenerative diseases, microglial activation may accentuate neuron loss by promoting and propagating regional and local neuroinflammation [420,421]. Consistent with this, ALS patients exhibit elevated macrophage markers in their CSF compared to unaffected controls [422], presumably due to increased CNS microglia or their activation, as suggested by positron emission tomography (PET) imaging using radiotracers specific for activated microglia [423,424]. Moreover, loss of function mutations in *TREM2* (triggering receptor expressed on myeloid cells 2), which affect microglia phagocytosis and inflammation, are risk factors for ALS [425–427], confirming the pivotal role played by microglia in ALS pathogenesis.

- *Phagocytosis*: Autophagy and phagocytosis share key morphological and mechanical features [428]. For instance, both pathways rely on vesicular structures to deliver contents to lysosomes for degradation. Microglia actively clear apoptotic cells, myelin and synaptic debris, axonal fragments and protein deposits from the CNS through phagocytosis [428–430]. In line with these connections between phagocytosis and autophagy, microglial phagocytosis of Aβ deposits in Alzheimer disease models [430,431] requires the autophagy-related factors BECN1, ATG7 and LC3 [432,433].

- *Neuroinflammation*: The activation of CNS microglia and astrocytes, and the subsequent production of pro-inflammatory cytokines and interleukins (neuroinflammation), is intricately connected to ALS pathogenesis. In pre-symptomatic mutant SOD1 transgenic mice, activated microglia initially display neuroprotective properties that slow disease progression; however, with time the continued and unabridged neuroinflammation only enhances neurodegeneration and accelerates disease progression [434–436]. Transplanted wild-type microglia effectively extend the survival of mutant SOD1 transgenic mice [437], confirming the importance of microglial activation in ALS pathogenesis. Supporting this, ALS patients carrying *C9orf72* mutations display microglial activation that correlates with disease progression [438]. The C9orf72 protein – itself closely tied to autophagy, as discussed above – is highly expressed in myeloid cells such as microglia. *C9orf72*-deficient mice display lysosomal accumulation and a hyperactive immune response characterized by enhanced production of IL1B and IL6 [184,185,187,439], a phenotype recapitulated by myeloid-specific *C9orf72* knockout, indicating the significance of C9orf72 in maintaining physiological immune responses [440]. Emphasizing the link between autophagy and inflammation, impaired autophagy in C9orf72-deficient myeloid cells leads to the accumulation of the pro-inflammatory STING (stimulator of interferon genes) protein [440]. Likewise, ALS-

associated mutations in *TBK1, OPTN, SQSTM1*, and *VCP* all affect autophagy as well as microglial function and innate immunity [192,205,206,441–443]. Collectively, these findings reinforce the convergence between inflammation and autophagy in microglia, and their importance for ALS pathogenesis.

Oligodendrocytes. Oligodendrocytes comprise 45–75% of the glial population, depending on the region of cerebrum or brainstem sampled, and are essential for CNS axon myelination [389]. Although much of oligodendrocyte autophagy remains unknown, this process is important for several aspects of development and disease:

- *Developmental CNS myelination*: Autophagic activity increases during progenitor cell differentiation into mature oligodendrocytes [444]. During oligodendroglial precursor (OPC) differentiation, autophagy becomes most active in distal OPC processes, and autophagosomes are trafficked proximally to the soma in a manner similar to neurons [444]. Conditional oligodendrocyte-specific deletion of *Atg5* in mice leads to incomplete differentiation, reduced myelination, abnormal myelin compaction, oligodendrocytic death, and shortened life span [444]. In addition, oligodendrocyte autophagy, OPC differentiation, and myelination during development are all regulated by MTOR [445–447]. Consistent with these findings, autophagy activation enhances oligodendrocyte survival and promotes myelination in a rat model of demyelinating disease [448].
- *Non-cell autonomous trophic support*: Loss of autophagy with oligodendrocyte-specific deletion of *Atg5* impairs motor recovery after spinal cord contusion in mice [449], but rapamycin fails to enhance recovery [449] despite effective stimulation of autophagy in these cells [449–451]. Paradoxically, suppression of autophagy with NTF3 (neurotrophin 3) disinhibits oligodendrocyte proliferation and promotes motor recovery in a separate model of spinal cord contusion [452]. These observations suggest that oligodendrocyte autophagy may play a necessary but insufficient role in post-injury repair of the spinal cord [453], but excessive autophagy in oligodendrocytes may lead to neuronal toxicity in a non-cell autonomous manner.
- *Impaired proteostasis in neurodegeneration*: In multiple system atrophy (MSA), the pathologic hallmark consists of cytoplasmic SNCA inclusions in oligodendrocytes [12], and the accumulation of these inclusions is exacerbated by autophagy inhibition, either pharmacologically with chloroquine or genetically by *MIR101* overexpression [12,450,454]. Separately, oligodendrocytes in ALS patients demonstrate inclusions containing TARDBP and FUS [455–457] and lower levels of SLC16A1/MCT1 (solute carrier family 16 member 1), an essential lactate transporter in the CNS whose expression is regulated by autophagy [458]. These findings constitute indirect evidence for impaired oligodendrocyte autophagy in ALS; however, direct assessment of oligodendrocyte autophagy in ALS models has not been fully explored.

Current knowledge regarding oligodendrocyte-specific mechanisms of regulating autophagy is limited. Additional studies are needed to establish the specific autophagy factors that distinguish autophagy in oligodendrocytes from that in neurons.

Schwann cells. Details regarding Schwann cell-specific autophagy are also limited and are largely devoted to examining Schwann cells in development and after traumatic injury to peripheral nerves:

- *Developmental PNS myelination*: During development, autophagy is required for refining and culling of Schwann cell-mediated myelination, and conditional *Atg7* deletion in mouse Schwann cells leads to cytoplasmic swelling, ribosomal accumulation, dysmorphic ER, and dysmyelination [459]. Furthermore, conditional deletion of *Pik3c3* in murine Schwann cells not only results in hypomyelination, but also in non-cell autonomous disruption of neuronal autophagic flux and dysregulated neuronal trafficking of ERBB2 or ERBB3 receptor tyrosine kinases [460]. Additional non-cell autonomous effects are observed with Schwann cell-specific *PTEN* deletion or EGFR overexpression, which attenuate peripheral nerve autophagy and lead to disorganization of the nascent neuromuscular junction [461].
- *Peripheral nerve trauma*: After nerve injury, Schwann cells oversee the capture of myelin debris in autophagosomes in a process termed "myelinophagy" [459,462,463], and this function is impaired by Schwann cell-specific *Atg7* deletion [459,464]. Remyelination after peripheral nerve injury is hindered by injury-induced upregulation of the microRNA *Mir195a*, which inhibits *Atg14* and, in turn, suppresses Schwann cell autophagy [14,15]. Conversely, stimulating Schwann cell autophagy with rapamycin promotes myelination and improves peripheral nerve regeneration [465,466].
- *Inherited demyelinating neuropathies*: Abnormal Schwann cell autophagy is also observed in dysmyelinating forms of inherited polyneuropathies. For instance, in Charcot-Marie-Tooth (CMT) type 1A caused by duplication of *PMP22* (peripheral myelin protein 22), Schwann cell autophagy is aberrantly elevated and associated with higher levels of JUN/c-Jun, which may be part of a compensatory, neuroprotective response to the pathogenic effects of the *PMP22* duplication [467–469]. In CMT4J, autosomal recessive mutations in the gene encoding FIG4, a phosphatase required for recycling phosphoinositides involved in vesicle trafficking, lead to incompetent myelinophagy [13]. Autosomal dominant *FIG4* mutations are associated with rare forms of familial ALS [470], suggesting that inefficient myelinophagy may result in the loss of motor neurons and muscle atrophy.

Together, these studies indicate that targeting Schwann cell autophagy in development or following traumatic injury has strong therapeutic potential not only for enhancing remyelination, but also for promoting axonal regrowth through non-cell autonomous mechanisms. Even so, treatments for specific peripheral myelination disorders must be individualized based on the specific pathologic context and the perceived need to enhance deficient autophagy, or conversely slow overactive autophagy.

Skeletal muscle

Skeletal muscle comprises another integral component of the peripheral nervous system. Similar to neurons, muscle fibers must contend with life-long metabolic needs due to their post-mitotic state, but unlike neurons these cells must adapt to mechanical stressors arising from weight-bearing activity and locomotion [471]. Structural and oxidative maintenance, adaptive hypertrophy, senescent sarcopenia, and pathogenic atrophy from neuromuscular disease all appear to require regulatory changes in autophagy, and the mechanisms underlying these different physiologic states and their impact on proteostasis will be the focus of this section.

- *Muscle atrophy*: There is conflicting evidence as to whether autophagy contributes to or mitigates muscle atrophy in ALS. On the one hand, skeletal muscle denervation causes neurogenic atrophy and may activate autophagy; such a protective response (mounted by the transcription factor RUNX1) limits muscle wasting after denervation by suppressing autophagy. Conversely, genetic ablation of RUNX1 disinhibits autophagy and worsens atrophy [472]. Starvation-related atrophy is also associated with upregulated autophagy, which in turn is controlled by the transcription factor FOXO3 and is independent of MTOR activity [473]. On the other hand, neurogenic atrophy can be partially mitigated by adrenergic stimulation, which activates autophagy through *Atg7* and counteracts denervation-induced inhibition of autophagic flux [474]. Autophagy appears to be required for muscle regeneration, since autophagy inhibition with 3-methyladenine blunts recovery in mice after myotoxic injury [475]. Similarly, *ulk1* knockout impairs muscle regeneration in zebrafish [476], and conditional *Atg7* deletion in mouse skeletal muscle leads to atrophy, weakness, and accumulation of unhealthy mitochondria and sarcoplasmic reticulum [477]. Analogously, conditional *Atg5* deletion in mouse skeletal muscle leads to accumulation of ubiquitin-positive inclusions and ultrastructural disorganization [478]. The discrepancies suggested by these paradoxical findings may indicate that autophagy is required for muscle maintenance and homeostasis, but there is a limit or threshold beyond which autophagy stimulation may exacerbate fiber atrophy.
  - *Neuromuscular disease*: In ALS, skeletal muscle-specific expression of SOD1[G93A] is associated with atrophy and elevated autophagy [479], but these effects are mitigated by siRNA-mediated *MAP1LC3B*

knockdown and the subsequent reduction of autophagy flux [479]. In addition, inherited myopathies demonstrate muscle pathology that includes markers of elevated autophagy. For instance, centronuclear myopathy is caused by mutations in the genes encoding MTM1 (myotubularin1) or MTMR14/Jumpy (myotubularin related protein 14) [17,20]. These loss-of-function mutations prevent these enzymes from metabolizing phosphoinositides required for autophagy, thereby disinhibiting autophagy flux and muscle atrophy [17,20]. In contrast, enhancing autophagy in mouse models of Duchenne muscular dystrophy (DMD) pharmacologically with rapamycin or 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, an AMP analog which stimulates AMPK) improves muscle strength and contractility [480,481]. Moreover, PPARGC1A/PGC1A (PPARG coactivator 1A) overexpression drives non-MTOR-dependent autophagy by increasing TFEB nuclear localization in dystrophin-deficient muscle fibers [19]; however, the extent to which this manipulation rescues DMD-related pathology, atrophy, or weakness was not assessed. These discrepant results may be due to effects of the genetic background unique to each disease, resulting in excessive autophagy stimulation in centronuclear myopathy, but insufficient autophagy in DMD. In either case, deviation from normal proteostasis results in muscle pathology.

- *Autophagy and exercise*: The positive effect of exercise on autophagy was first noted in 1984, with the observation of autophagosome accumulation after exercise in rat skeletal muscle [482]. Similar effects have been seen in humans and mice after strenuous exercise, interpreted as a response to exercise-related muscle breakdown [482,483]. Exercise triggers autophagy by activating ULK1 and dissociating BCL2 from BECN1, thereby disinhibiting the initiation complex [484,485]. In muscle biopsies collected from human volunteers after endurance exercise, measurements of autophagy markers revealed downregulated AKT and MTOR signaling, elevated ATG5, ATG12, and LC3-II [486]. Similarly, *vastus lateralis* biopsies taken from human runners after a 200-kilometer race showed increased transcription of autophagy factors *ATG4B, ATG12, GABARAPL1, LC3B, CSTL* (cathepsin L), *BNIP3*, and *BNIP3L* [487], which may be an adaptive response to muscle injury related to long-distance running. Exercise also potentiates autophagy activation due to starvation and the resulting reduction in plasma insulin, which in turn downregulates AKT and FOXO3 signaling [16,18]. Autophagy induction is not only seen with endurance exercise, but also acute bouts of exercise, and this short-term increase in autophagy appears to require PPARGC1A [488].
- *Autophagy and aging*: Aging is associated with a decline in muscle mass (termed sarcopenia) and mitochondrial function, both of which are associated with dysregulated autophagy [489]. However, there remains considerable controversy as to whether

these events arise due to impaired or overactive autophagy with age. On the one hand, skeletal muscle autophagy declines with aging in *Drosophila* and can be rescued by FOXO overexpression or constitutive activation of EIF4EBP1 [490]. Mouse muscle also shows age-related decline in autophagy machinery (LC3, BECN1, and LAMP2), and these deficiencies are partially mitigated by 8 weeks of treadmill exercise [491]. Age-related declines in MFN2 (mitofusin 2), which is associated with macroautophagy and mitophagy [492], together with mitochondrial fragmentation and mitochondrial fission machinery in muscle, indicate inefficient mitophagic flux in aging muscle [493]. Supporting this, skeletal muscle from aged rats exhibits lower LC3 expression, suggesting reduced levels of basal autophagy and an overall decreased capacity for autophagy [494].

On the other hand, sarcopenia involves autophagy of muscle structural components, and attenuation of autophagy with PPARGC1A overexpression mitigates sarcopenia [495]. In aged rats, muscle tissues display elevated basal levels of proteolysis, higher expression of autophagy proteins and the accumulation of unfolded protein response elements. These changes are accompanied by reduced adaptive reserve in response to denervation, with impaired autophagy induction and lower levels of mitophagy compared to young rats [496]. Aged rats also show elevated basal mitophagy, higher levels of mitophagy proteins, higher TFEB expression and lysosomal markers than young mice; these changes were reduced with chronic and forced contractile activity induced by implanted stimulators [497]. Taken together, the mixed evidence describing age-related changes in muscle autophagy emphasizes the need for further investigations of the regulatory machinery overseeing autophagy in muscle. This issue is particularly prominent given the potential for nonselective autophagy-inducing therapies to be used for highly prevalent age-related neurodegenerative conditions, and their unknown effects upon sarcopenia in susceptible populations.

Concluding remarks: remaining questions & therapeutic implications

Although autophagy affords much potential for disease-modifying benefit in neurodegenerative disease, additional challenges arise when administering autophagy-modulating drugs on a clinical basis. For instance, cell and animal models may not faithfully reproduce autophagy regulation in healthy subjects or humans with disease. Additionally, treatment with autophagy modulating drugs runs the risk of incurring intolerable side effects and toxicities, especially when given on a long-term basis that may be required to prevent or delay late-onset neurodegenerative diseases.

Autophagy induction using MTOR inhibitors is currently being tested in ALS – a phase II trial of rapamycin (sirolimus) for ALS patients began in 2018 [498]. Results from this trial have yet to be announced or published, but the use of rapamycin in ALS is complicated not only by its potential for insufficient induction of neuronal autophagy *per se*, but also its capacity for modulating multiple MTOR-dependent downstream pathways (Figure 3). Rapamycin has been extensively utilized for transplant rejection prophylaxis and graft-versus-host disease, as well as seizure control and reduction of tumors in tuberous

sclerosis complex patients [499–501]. As a result, the drawbacks of chronic rapamcyin therapy – including cytopenias, nephrotoxicity, metabolic syndrome, dermatitis, and pneumonitis [502,503] – are well known. Clinicians may therefore be required to implement regular monitoring of serum drug levels, blood pressure, skin exams, complete blood counts, liver and renal function, cholesterol, and triglycerides, as is commonly performed in those taking rapamycin for FDA-approved indications [504].

Additional methods for bypassing MTOR to modulate autophagy exist, however, and should be explored in the context of ALS. Despite early enthusiasm over lithium in patients and mouse models [505,506], five subsequent trials showed no clinical benefit in survival or neuromuscular outcomes for ALS patients [507–511]. These negative trials, together with lithium's narrow therapeutic index, severely limit the therapeutic utility of lithium for ALS (Figure 3). Modulation of amino acids represents an alternative strategy for regulating autophagy in an MTOR-independent fashion: amino acid starvation induces autophagy, whereas amino acid provision results in RPS6KB/p70S6K phosphorylation and autophagy suppression [512]. Neither event is affected by rapamycin, suggesting that MTOR is dispensable for this pathway [512]. 10-NCP and related compounds are also capable of inducing neuronal autophagy through an MTOR- and AKT-independent signaling cascade [357,384]. The array of MTOR-independent methods for stimulating autophagy also includes direct stimulation of AKT and BECN1, activating MAPK8/JNK1 to dissociate BCL2 from (and thereby disinhibit) BECN1, applying the synthetic peptide Tat-Beclin 1, SIRT1-associated starvation, and disrupting nonsense-mediated RNA decay [513–517] (Figure 3). Notably, with the exception of 10-NCP, the above strategies have been tested almost exclusively in non-neuronal model systems. Therefore, the applicability of these biologic phenomena to neurons, and whether manipulating MTOR-independent effectors and pathways are sufficient to modulate neuronal autophagy, must be investigated in relevant neuronal model systems to inform their suitability for therapy development.

Given the limitations demonstrated by therapeutic strategies empirically trialed to date, the rational design of autophagy inducers for neurodegenerative disease will require several key advances. First, cell type-specific regulatory mechanisms controlling autophagy flux must be elucidated in neurons, astrocytes, microglia and muscle. Second, the means to accurately measure autophagy induction in each cell type must be established. Traditional measures relying upon the accumulation of pathway intermediates such as LC3-II cannot discriminate between true induction and late-stage inhibition of autophagosome maturation and should therefore be utilized cautiously in estimating the ability of compounds to stimulate autophagy. Third, these approaches must be adapted for high-throughput screening, enabling the unbiased identification of effective autophagy inducers in multiple cell types [518–520]. Fourth, the effectiveness of these compounds must be tested rigorously in model systems that do not depend upon the overexpression of disease-associated proteins, since this approach may inappropriately emphasize strategies that reduce the non-physiological accumulation of exogenous proteins. In this light, the use of alternative model systems, such as iPSCs and mature cells
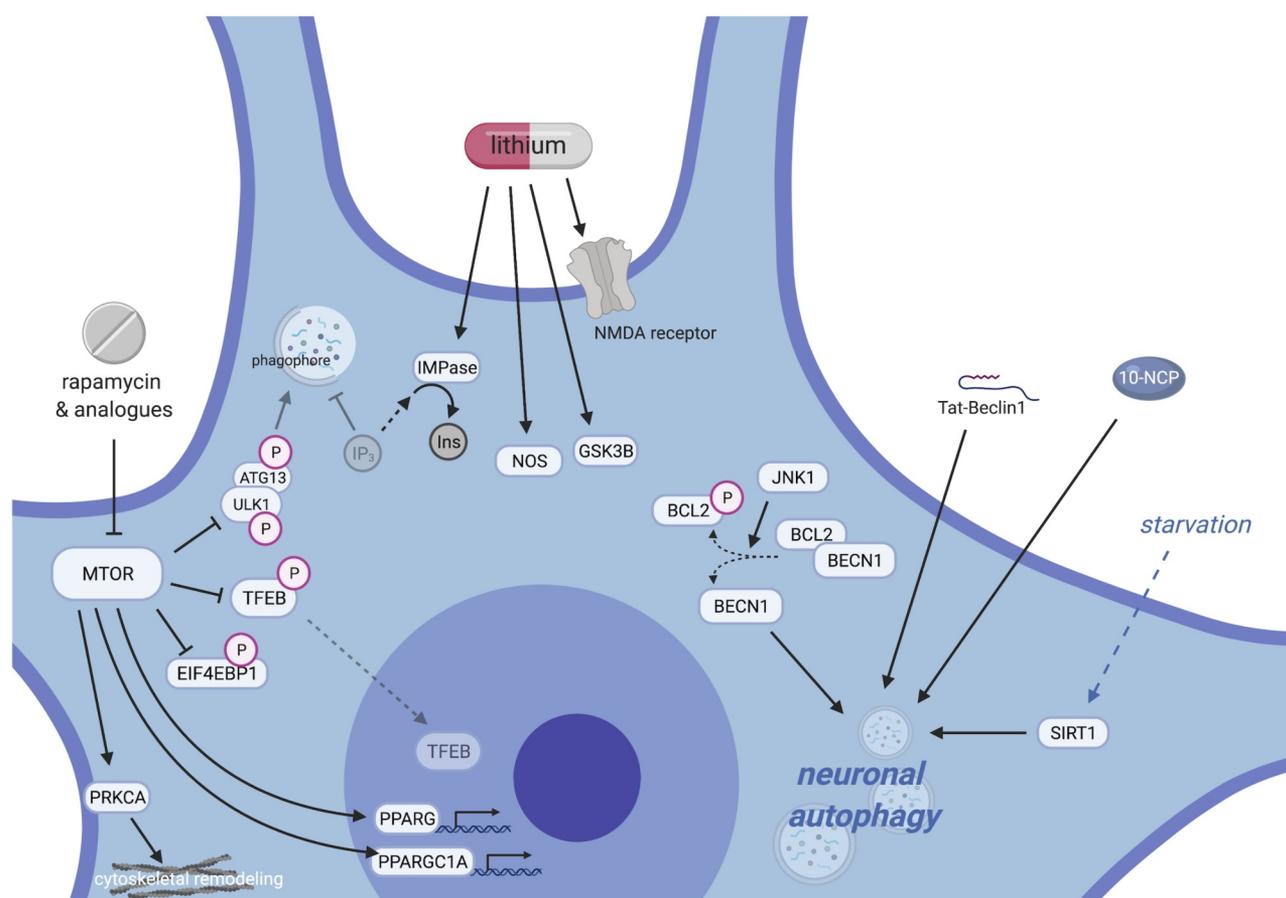
Figure 3. Mechanisms of neuronal autophagy and their impact for therapeutic design in ALS. Pharmacodynamic considerations limit the use of currently available drugs for modulating autophagy. Inhibitors of MTOR inhibitors (*rapalogs*) only weakly activate autophagy in neurons, and their wide-ranging effects target myriad cellular pathways, including growth signaling, translation, stress responses, transcriptional regulation, and cytoskeletal remodeling (*left*). Such pleiotropy leads to well-documented and multi-systemic toxicities, especially with long-term use; however, newer rapalogs may enable more specific targeting and selective autophagy modulation. Similarly, lithium has numerous multi-target effects, including depletion of IP_3 through IMPase, activating nitric oxide synthase, inhibiting GSK3B, stimulating NMDA receptors and enhancing glutamatergic tone, among many others (*middle*). This results in a narrow therapeutic index for lithium, potentially explaining its apparent lack of neuroprotective effects in human trials for neurodegenerative disease to date. To reduce off-target effects, recent efforts have focused on MTOR-independent strategies for stimulating autophagy, including modulation of SIRT1, phenoxazine compounds such as 10-NCP, or synthetic peptides such as Tat-Beclin 1 (*right*).

differentiated from iPSCs, provides a unique advantage, enabling investigations of heretofore untestable aspects of neuronal biology and cell type-specific autophagy regulation.

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