REVIEW

Autophagy–lysosomal-associated neuronal death in neurodegenerative disease

Ralph A. Nixon[1,2,3,4]

Abstract

Autophagy, the major lysosomal pathway for degrading damaged or obsolete constituents, protects neurons by eliminating toxic organelles and peptides, restoring nutrient and energy homeostasis, and inhibiting apoptosis. These functions are especially vital in neurons, which are postmitotic and must survive for many decades while confronting mounting challenges of cell aging. Autophagy failure, especially related to the declining lysosomal ("phagy") functions, heightens the neuron's vulnerability to genetic and environmental factors underlying Alzheimer's disease (AD) and other late-age onset neurodegenerative diseases. Components of the global autophagy–lysosomal pathway and the closely integrated endolysosomal system are increasingly implicated as primary targets of these disorders. In AD, an imbalance between heightened autophagy induction and diminished lysosomal function in highly vulnerable pyramidal neuron populations yields an intracellular lysosomal build-up of undegraded substrates, including APP-βCTF, an inhibitor of lysosomal acidification, and membrane-damaging Aβ peptide. In the most compromised of these neurons, β-amyloid accumulates intraneuronally in plaque-like aggregates that become extracellular senile plaques when these neurons die, reflecting an "inside-out" origin of amyloid plaques seen in human AD brain and in mouse models of AD pathology. In this review, the author describes the importance of lysosomal-dependent neuronal cell death in AD associated with uniquely extreme autophagy pathology (PANTHOS) which is described as triggered by lysosomal membrane permeability during the earliest "*intraneuronal*" stage of AD. Effectors of other cell death cascades, notably calcium-activated calpains and protein kinases, contribute to lysosomal injury that induces leakage of cathepsins and activation of additional death cascades. Subsequent events in AD, such as microglial invasion and neuroinflammation, induce further cytotoxicity. In major neurodegenerative disease models, neuronal death and ensuing neuropathologies are substantially remediable by reversing underlying primary lysosomal deficits, thus implicating lysosomal failure and autophagy dysfunction as primary triggers of lysosomal-dependent cell death and AD pathogenesis and as promising therapeutic targets.

Keywords Autophagy · Lysosomal disorder · Cell death · Neuronal death · Neurodegenerative disease · Alzheimer's disease · Amyloid · Senile plaque

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AVs	Autophagic Vacuoles
ER-phagy	Autophagy of ER
BMP	Bis (monoacylglycerol) phosphate
CMA	Chaperone-mediated autophagy
ELA	Endosomal-lysosomal-autophagy
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ESCRT	Endosomal sorting complexes required for transport
fAD	Familial AD
Fe^{2+}	Ferrous iron ions
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration

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| HD | Huntington's disease |
LMP	Lysosomal membrane permeabilization
LSDs	Lysosomal storage disorders
LCD	Lysosome-dependent cell death
MPR	Mannose-6-phosphate receptor
MAMs	Mitochondria-associated ER membrane interactions
MMP	Mitochondrial membrane potential
NFTs	Neurofibrillary tangles
PANTHOS	p-anthos or poisonous flower
PD	Parkinson's disease
Ph	Phagophore
PSEN 1	Presenilin 1
ROS	Reactive oxygen species
TFEB, TFE3	Transcription factors
TPC2	Two-pore channel 2
vATPase	Vacuolar ATPase

Introduction

Autophagy comprises a network of cross-regulated pathways that engage and deliver potentially toxic and damaged organelles for degradation in lysosomes (Fig. 1). The cellular systems involved in its regulation offer powerful protection against a premature triggering of apoptosis when cells are stressed. This is a particularly critical function of autophagy for neurons—a cell type that cannot regenerate and must survive in some cases for more than a century in a long-lived individual despite these stresses. This review, part of a series on neuronal cell death, addresses the pathobiology in Alzheimer's disease that overwhelms these survival mechanisms. It focuses especially on the converging disease factors that impede lysosomes from completing the autophagy clearance process and turn damaged lysosomes into triggers of cell death.

Autophagy regulators of protein quality control and metabolic homeostasis have been established as key determinants of species longevity [159], which in turn depends on the long-term survival of neurons [87] and their resilience to brain disorders. Capture and *complete* degradation of an autophagic substrate, termed "autophagy flux", must be optimally maintained over the individual's entire life. Although abnormal accumulation of autophagy-related compartments (autophagic vacuoles or AVs) is often the most striking feature of neurodegenerative disease, a primary failure of lysosomes is more often the basis rather than heightened substrate sequestration and self-digestion. Being the repository of dozens of activated hydrolytic enzymes, lysosomes are more than qualified to have been designated potential "suicide bags" by their discoverer, Christian DeDuve, to underscore a potential to release damaging hydrolases into the cytoplasm [66] while triggering other cell death routines [80,

235]. Either acute lysosome disruption or gradual leakage of enzymes from damaged were each recognized by DeDuve's associates in the 1960s as primary effectors of neuronal cell death [54, 173]. However, the appreciation of lysosomes or autophagy in neuronal cell death adult neurodegenerative disorders escaped the attention of most investigators until this past decade, despite prior knowledge of > 50 congenital lysosomal storage disorders (LSDs), most featuring prominent neurodevelopmental or neurodegenerative phenotypes [172]. It is important to note that investigators often incorrectly equate "autophagy" with just the substrate sequestration steps of the pathway even though autophagy derives its name from its digestive 'phagy' ("eating") lysosomal step. Despite the obvious redundancy, a useful convention is to refer to an "autophagy–lysosomal pathway", or ALP, to underscore the crucial importance of the degradative step in autophagy and its outsized importance within the pathway as a target in neurodegenerative disease [169].

In this review, the author will discuss how ALP failure in AD evolves, leads to neuron death, and serves as a conceptual framework for explaining the emergence of hallmark neuropathological features in the disease. Relevance to other neurodegenerative disorders is briefly mentioned here and more broadly reviewed recently in Ref. [169]. Adult-onset neurodegenerative diseases have been commonly referred to as proteinopathies, emphasizing the toxic action(s) of a particular aggregation-prone pathogenic protein. Their toxicity, however, is often manifest as clinical disease only after these substrates accumulate in the failing lysosomes of neurons in the aging brain, underscoring the critical roles of lysosomes in precipitating disease. This review also describes a unique morphological pattern of extreme autophagy dysfunction recently identified in select neurons within the broader autophagy-compromised populations of highly vulnerable pyramidal neurons at early stages of AD. This select subpopulation of neurons undergoes a massive build-up of autophagic vacuoles laden with undegraded substrates, including Aβ, and form plaque-like β-amyloid fibrillar aggregates intraneuronally. Although it is only one of multiple possible cell death cascades operating in AD brain [224], autophagy-associated lysosomal-dependent neuron cell death is directly driven by the genes and risk factors responsible for AD. The unique pattern of extreme pathological autophagy offers a rare opportunity to characterize cell-autonomous neuronal death evolving during a disease stage preceding hallmark AD lesions and inflammation, which complicate distinguishing primary from secondary neurodegeneration events [130]. Emergence of the pattern at a "pre-pathology" stage of AD and its role in β-amyloid plaque formation highlights an exceptionally early *intraneuronal* phase of Alzheimer's disease that has been largely unexplored, especially in relation to cell death (Fig. 2).
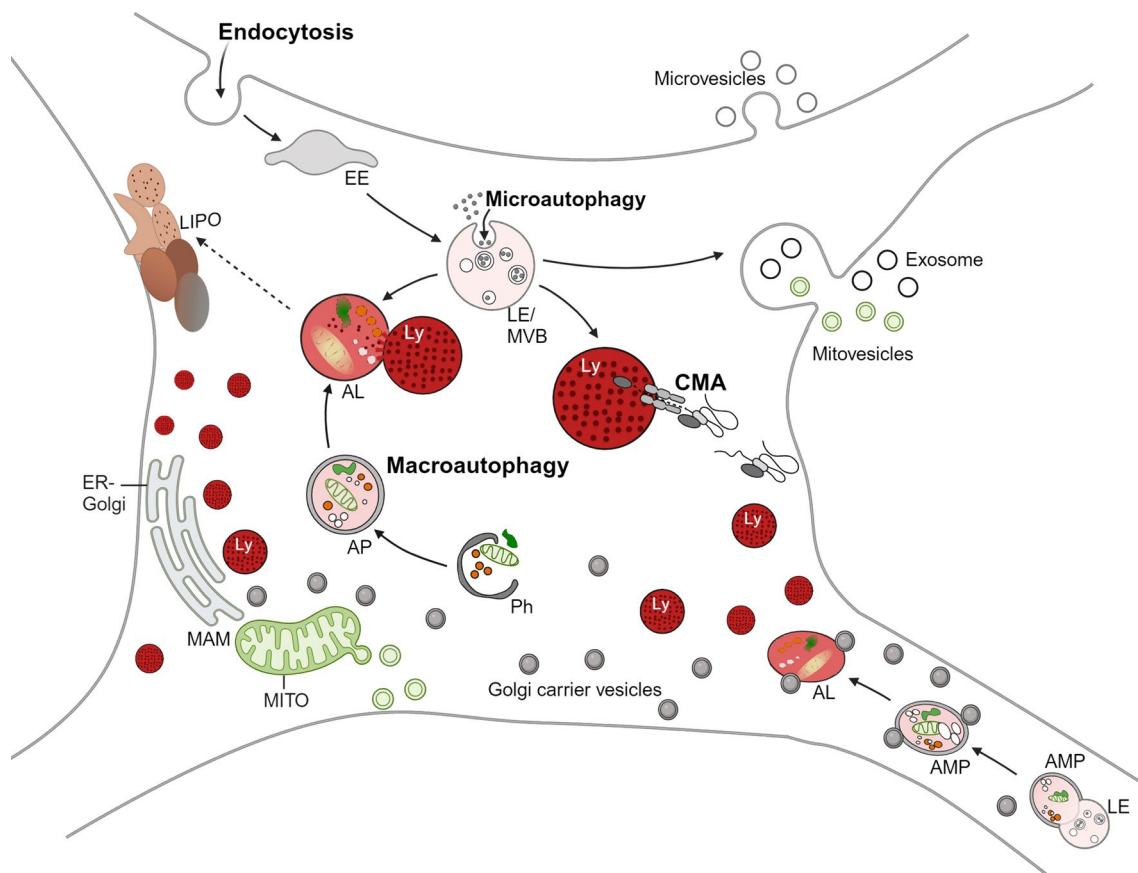
Fig. 1 Major routes of substrate delivery to lysosomes. Macroautophagy is characterized by the formation of a double-membrane enveloping structure, the phagophore (Ph) and the sequestration of cytoplasmic constituents, including organelles, targeted for degradation into double-membrane vesicles called autophagosomes. Fusion with lysosomes introduces acid hydrolases and a proton pump (vATPase) that acidifies the lumen and activates an array of hydrolases that can fully digest most substrates to unit metabolites, which are recycled for energy or new synthesis. Import of chloride and fluxes of other ions balance the electrogenic gradient during proton import to facilitate acidification. An intermediate step particularly active in axons is autophagosome fusion with a rab7-positive late endosome to form an amphisome (AMP), which amplifies its retrograde motility [31, 133]. In chaperone-mediated autophagy (CMA), proteins carrying pentapeptide KFERQ-like sequences are recognized by Hsp70, which associates with the integral lysosome membrane protein LAMP-2A, triggering translocation of the bound protein into the lysosome interior. In microautophagy, cytoplasmic substrates are internalized into late endosomes/MVB by membrane invagination followed by release of the cargo by membrane scission into the lumen for degradation in lysosomes. Heterophagy involves the lysosomal degradation of plasma membrane components and exogenous substrates after they are internalized by receptor-mediated or bulk endocytosis. Selected proteins are sorted to different cellular destinations or recycled to the plasma membrane. Proteins targeted for degradation are trafficked to late endosomes/ multivesicular bodies (MVB), which mature to lysosomes to effect complete degradation [162]. Buildup of lipofuscin reflects declining clearance inefficiency as neurons age. Compaction of ineffective autolysosomes containing hydrolysis-resistant substrates reduces but does not eliminate the damaging impact on cell function. Lysosomes fully mature and concentrate within the soma of neurons and are scarce or absent in axons. Instead, anterogradely moving Golgi carrier vesicles deliver lysosomal components to the amphisomes and late endosomes moving toward the soma to facilitate their maturation

Autophagy in healthy and aging neurons

Brief overview of the autophagy–lysosomal network

In macroautophagy, the pathway of this network most crucial for neuron survival [118], cytoplasmic constituents including damaged or obsolete organelles are captured in double-membraned autophagosomes either constitutively at a bulk level or selectively by engaging members of a family of adaptor proteins [64, 166, 194] (Fig. 1). The same processes are induced further under stress conditions as a neuroprotective response [39, 164]. Substrate-laden autophagosomes mature to autolysosomes via direct fusion with lysosomes which introduce dozens of hydrolases capable of completely digesting most normal substrates completely to their unit components (amino acids, lipids, etc.) for reutilization in new synthesis or to generate energy (detailed reviews [64, 194]). A proton (H$^+$) pump, the vacuolar ATPase (vATPase), is also introduced enabling intra-lysosomal acidification
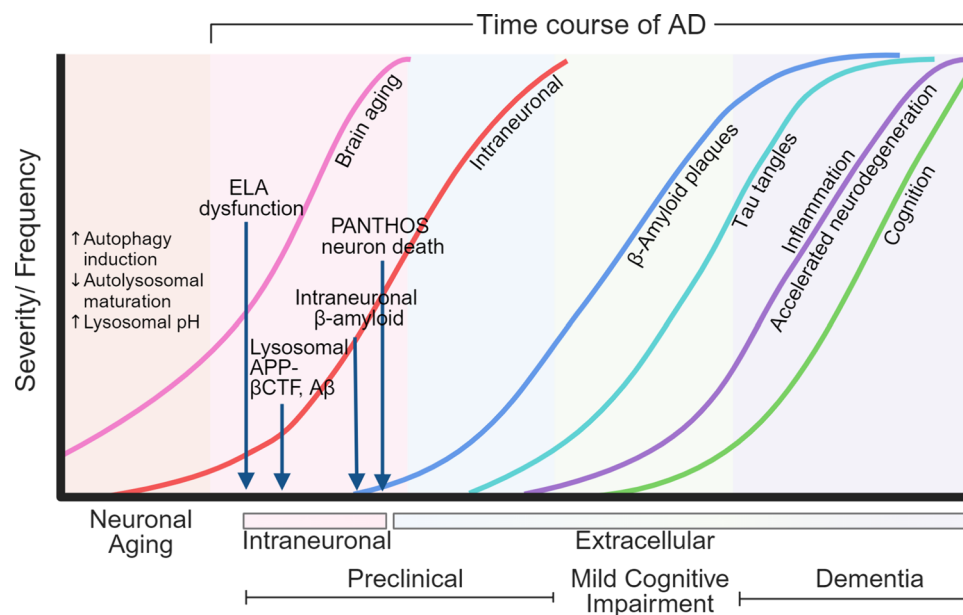
Fig. 2 Autophagy–lysosomal pathway (ALP) abnormalities progress during an exceptionally early "intraneuronal" stage of AD. A hypothetical timeline of pathological changes in Alzheimer's disease (AD) is depicted. Beginning during the preclinical ("intraneuronal") stage and continuing in later stages, primary lysosomal dysfunction initiates a cascade of autophagy failure, lysosomal membrane permeability, intraneuronal amyloid plaque formation, and death of select neurons, which instigates and propels extracellular AD neuropathology, as discussed further in the review. This intraneuronal disease stage is followed by the earliest detection by PET (or other imaging modalities) of sequential emergence of extracellular β-amyloid plaques, tau tangles, inflammation and accelerated neurodegeneration associated with more rapid cognitive decline. It should be noted, based on neu-ropathological studies [88], that the first tau lesions precede by age of occurrence the first amyloid-β plaques. A caveat to ordering definitively the sequence of appearance of different anomalies is the relative sensitivity of the detection methods applied. Biomarkers for soluble amyloid-β peptide and modified tau species in cerebrospinal fluid (not shown) become abnormal before amyloid-β and tau aggregates are detectable by PET or histologically. Elevated levels of these soluble biomarkers in sporadic AD may precede detectable extracellular lesions by 1–2 decades or more before symptoms [139] which could possibly overlap temporally with changes during the "intraneuronal" stage, although this has not been studied. Adapted from Jack et al., 2010 [98] and 2013 [97], Leuzy et al., 2019 [134], and McDade and Bateman, 2018 [150]

down to the pH range of 4.5–5.0 needed to optimally activate the "acid" hydrolases with varying acidic pH optima. Cross-dependencies also exist between the autophagy–lysosome pathway and the endolysosomal–lysosomal pathway [72, 198]. For example, an amphisome is created when an autophagosome fuses with an endosome [75]—a process especially important in neurons to enhance motility during delivery of sequestered cargoes from long neuronal processes to the soma where lysosomes are concentrated (Fig. 1).

In addition to macroautophagy, proteins containing a KFERQ targeting motif are delivered by chaperone-mediated autophagy directly to lysosomes after binding to the chaperone HSC70 and a LAMP2a complex that delivers the protein inside the lysosome [110]. By microautophagy, cytoplasmic substrates can also be introduced through invaginations of late endosomal membranes and delivered to lysosomes [18]. While not an autophagy route per se, the endocytic pathway delivers certain internalized extracellular materials and plasma membrane components to lysosomes if they are not sorted to other cellular destinations or recycled to the cell surface (Fig. 1). Within this network of systems for capturing substrates, lysosomes deserve special emphasis as the only degradative compartment shared by all autophagy and endocytosis-related substrate delivery routes.

Autophagy in healthy neurons confers resilience to aging and disease

Healthy neurons efficiently eliminate newly formed autophagosomes or amphisomes by rapidly fusing with lysosomes and degrading the content within autolysosomes. Even very high levels of autophagy induction via mTOR or AMPkinase and substrate sequestration do not cause autophagosomes in most neurons to build up. In fact, the ultrastructural detection of more than occasional autophagosomes with undegraded material in a cortical pyramidal cell body is rare [171] and likely a harbinger of a declining lysosomal efficiency rather than an over-active induction or autophagosome over-production. By contrast, even brief exposure of neurons to inhibitors of cathepsins or lysosome acidification prompts the rapid accretion of autophagy

intermediates [13]. These observations imply that healthy neurons can have a relatively high rate of constitutive autophagy induction but also normally have a lysosomal clearance system with enough reserve capacity to prevent temporary surges of induction from causing dangerous substrate build-up [13].

Additional responses in healthy neurons confer resilience to an increase of damaged proteins and organelles that can threaten survival. Upregulation of transcription factors (e.g., TFEB, TFE3) controlling the "CLEAR Network" of genes encoding autophagy and especially lysosomal biogenesis constitutes a successful strategy to delay effects of cell aging or extend longevity in vivo in aging models [179] and to slow or prevent neurodegenerative disease progression in mouse models [149]. In addition, in some neural cell types, failing lysosomes can jettison accumulated lysosomal cargoes by exocytosis [93, 178, 243]. Over-burdening lysosomes with cargoes from the endocytic pathway can also be attenuated by a default release via exosomes that are formed via late endosome membrane invaginations that capture cytoplasmic materials into vesicles [135]. These vesicles are released when an endosome fuses with the plasma membrane. In addition, endosome cargoes can enter the autophagy pathway by fusing with autophagosomes that can expel their contents by exocytosis—a process so far documented mainly in non-neuronal cells [153]. These various "unconventional secretory" pathways may be constitutive but are upregulated as default pathways when lysosomal efficiency declines in aging and disease [3]. In a final adaptation to cope with poorly degraded substrates in autolysosomes, ineffective congested autolysosomes can fuse and undergo further compaction via residual hydrolysis and chemical modification, which yields the aging-related lipopigment, lipofuscin, a relatively but not completely inert family of lipo-proteinaceous granules [162].

## Neuronal aging: the gateway to lysosomal failure and neuron death in AD

Disease emergence in aged adults coincides temporally with waning neuronal proteostasis, especially in autophagy involving declines in autophagosome biogenesis [183, 230], waste trafficking and degradation [230], and translocation of CMA substrates into lysosomes [49, 50]. Particularly influential to the autophagy pathway decline in aging cells of lower species [30] and likely also mammals [24] is progressive lysosomal dysfunction tied to failing lumenal acidification [230]. This is shown, at least in part, to be due to ROS and aldehydes (e.g., 4-hydroxynonenal) from oxidized lipids [190] causing oxidative damage to vATPase complex subunits and lysosomal enzymes [189, 192, 253].

Proteostasis protects against pathogenic protein accumulation for decades until aging-related autophagy impairments [26, 60, 188, 191] trigger a rise in levels of toxic substrates, boosting oxidative stress [199] and mitochondrial damage that yield calcium dyshomeostasis and calpain activation, known to mediate varied effects of cell aging [197, 228]. The long-term protection against these challenges until late age explains why even individuals with autosomal dominant mutations in a pathogenic protein are functionally normal until sufficient lysosomal dysfunction emerges. Aging's role in precipitating neurodegenerative disease involving lysosomal mechanisms can be appreciated from genes that, in homozygote mutant form, cause childhood lysosomal storage diseases but, even in heterozygote form, increase risk for a late-age onset neurodegenerative disorder, such as Parkinson's disease (GBA) or frontotemporal dementia (CLN11) [233].

Lysosomal membrane permeabilization (LMP) to a variable extent (Fig. 3) is the inevitable outcome of these cumulative aging-related insults to lysosomes and is often the harbinger of neurodegeneration [79]. LMP is defined as the selective destabilization of the lysosomal membrane, which allows certain lysosomal contents to be released into the cytoplasm. Instigating factors include free radicals, membrane incorporation of damaged proteins and oxidized lipids, osmotic shifts due to ion flux changes [121, 176], and changes in membrane lipid composition, such as increased lyso-phosphatidylcholine and ceramide [12]. Calpain activities actually increase in aging [147, 170] and can potentially act upstream of, or coincident with, pH dysregulation [151, 158] to damage organelles and cytoskeleton, adding to LMP and necrotic damage in AD [197, 228]. Aging-related declines in the endogenous inhibitor of calpains, calpastatin [170, 197], also lie upstream of cathepsin release during lysosomal-associated cell death [234].

These effects of aging are countered by the chaperone Hsp70, which binds to the endolysosomal anionic phospholipid BMP [116], a co-factor that enhances acid sphingomyelinase activity. Under the vATPase deficient conditions that develop during aging [46, 127], heightened lysosomal calcium release into the cytosol activates calpains and protein kinases that have significant pathogenic consequences discussed below. Mitochondrial deterioration, a major contributor to cell aging activates neuronal mitophagy [22, 188] represents another LMP instigator [107, 231]. A strong surge of cytosolic calcium and calpain activation is sufficient to induce necrotic cell death [170]. Collectively, aging-related changes precede and add to lysosomal insults imposed by causal and risk disease genes discussed below.
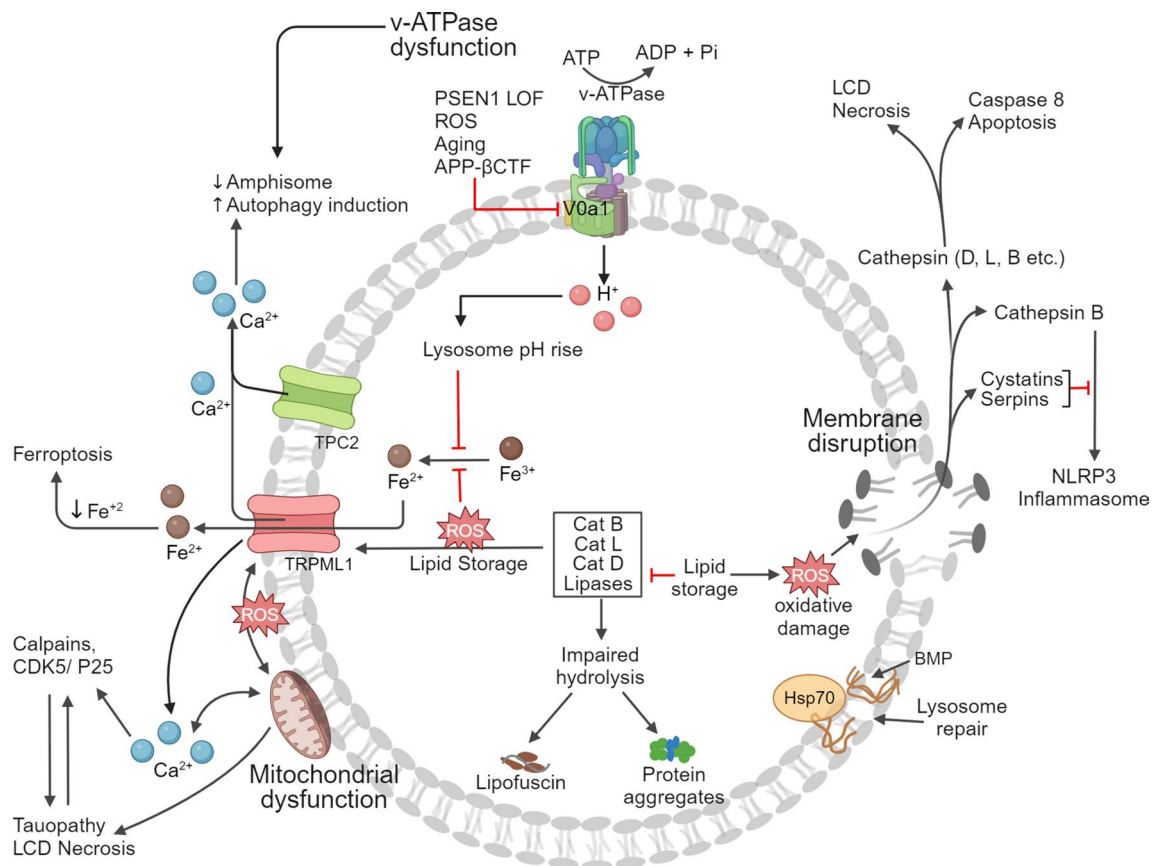
Fig. 3 Lysosomal membrane permeability (LMP) and lysosomal neuronal death cascade in AD. This diagram of the lysosome illustrates a crucial inciting decline in vATPase activity and lysosome acidification, which is a primary target of AD causative genes/gene products (PSEN1 mutations, APP, and genetic and environmental risk factors (e.g., ROS, neuronal aging, and high cholesterol), as discussed further in the text. Counter-productive induction of autophagy when lysosomal dysfunction is advanced may also exacerbate waste storage, ROS, and deacidification, thus promoting LMP. Also depicted is the cascade of further lysosome disruptions and lysosome- associated processes affecting mitochondria and innate immune function. The diagram further illustrates how LMP is connected to other pathways of cell death that can be upstream contributors (e.g., calpain activation and Hsp70), coincident exacerbating factors (e.g., ferroptosis), or downstream consequences of the release of cathepsins during LMP or lysosomal cell death (cathepsins, caspases, calpains, and Cdk5/ p25), which facilitate end stages of cell death. Collective injury to the lysosomal limiting membrane from the depicted sources induces lysosomal membrane permeability (LMP)—a state of injured membranes that allows relatively small proteins like cathepsins to pass through into the cytoplasm from an otherwise intact lysosome. Some hydrolases commonly released during LMP, such as cathepsins B and D, remain partially active at neutral pH and their potential cytotoxicity is buffered by cystatin C, an endogenous cysteine protease inhibitor in the cytosol and lysosomes [217]. The release of cathepsin B is linked to IL-1 activation, NLRP3 inflammasome activation, and neuroinflammation and can activate multiple cell death cascades, including caspases that trigger apoptosis. Cathepsin D release by LMP promotes necroptosis [143, 257]. Impaired ferritin degradation invokes features of the cell death pattern seen in ferroptosis [201]. Calcium release via TRPML1 and TPC2 channels activates calpains and calcium-dependent protein kinases promoting hyperphosphorylation of pathogenic proteins like tau and activation of RIPK1, which initiates necrosis-associated neurodegeneration. TRPML1- and TPC2-mediated calcium release is linked to mitochondrial dysfunction, and mTORC1 activation

How cell death evolves in the first neurons to die in AD

Faulty lysosomal acidification and autophagy flux failure arise exceptionally early

As early as Braak stage II, pyramidal neurons in prefrontal cortex of the late-onset AD (LOAD) brain exhibit increased numbers of pro-cathepsin D and cathepsin D-positive lysosomes, elevated cathepsin D mRNA transcription, and mannose-6-phosphate receptor (MPR) trafficking of lysosomal enzymes to endosomes [32, 33], all implying upregulated lysosomal biogenesis. Microarray analysis of laser-captured CA1 hippocampal neurons in LOAD (Braak III and V) further demonstrate upregulated lysosomal gene transcripts [16] and proteomic analysis of the large ROSMAP

[106, 129], and Banner [239] AD cohorts indicate early and sustained inhibition of mTOR [129]. These data suggest that, surprisingly, elevated autophagy induction persists [16, 141] even as rising lysosome levels of LC3 and inactive catD document a decline of lysosomal clearance [129]. Dual-immunolabeling with antibodies to catD and LC3 by Braak III stage confirm build-up of grossly enlarged catD/LC3-positive autolysosomes and depletion of CatD-positive lysosomes [129]. Collectively, these analyses indicate that autolysosome maturation to lysosomes (i.e., substrate digestion) stalls early in AD and worsens with disease progression. Proteomic analyses revealing declines in levels of most vATPase subunits in AD brain [106, 129], which together with complementary evidence in mouse AD models discussed below, strongly suggest that a lysosomal acidification deficit underlies impaired autolysosome maturation. Sustained autophagy induction compounds autophagic stress under these conditions—a seemingly maladaptive neuroprotective response that over-burdens failing lysosomes by delivering even more substrates [16, 129, 225].

An autophagy–lysosomal pattern similar to that in late-onset AD brain has been extensively detailed in neurons of different mouse AD models ranging from late disease onset in mice expressing human wt APP or mutant APP, to early onset in mice expressing mutant forms of both APP and Presenilin 1 (PSEN 1) [130, 140]. To track autophagy changes in neurons in vivo, a probe of autophagy and vesicle pH, the mRFP-eGFP-LC3 ("TRGL") construct, was stably expressed selectively in neurons (Fig. 4). As in AD brain, autophagy becomes dysregulated broadly in vulnerable populations of cortical and hippocampal neurons well before β-amyloid or glial reactive responses are detectable. Most of these neurons accumulate enlarged poorly acidified autolysosomes filled with undegraded substrates. Lysosomes isolated from these brains have significantly lowered vATPase activity (Fig. 5). The basis for the defect is impaired autolysosome acidification stemming from deficient lysosomal vATPase activity related to APP-βCTF [95] which accumulates together with Aβ selectively in these poorly acidified autolysosomes [95] (Figs. 3, 5). More generally, lysosomal pH dysregulation originating from causative mutations of one of multiple ion channels is an emerging common theme in adult neurodegenerative diseases, which may potentially disrupt pH in either direction [169].

In a select small subpopulation neurons that are most affected, poorly acidified autolysosomes build-up so massively that these fluorescent autophagic vacuoles bulge the plasma membrane outward, forming rosettes of large petal-shaped blebs—a flower-like pattern referred to as PANTHOS (p-ANTHOS or poisonous flower), which is seen in mouse and human AD brain [129] (Fig. 6). Within the soma, proliferated autophagosomes are frequently seen fused with endoplasmic reticulum (ER) tubules, suggesting that they

have stalled in completing the normally highly active turnover of ER by autophagy ("ER-phagy") (Fig. 7b). Proteins, including amyloidogenic metabolites of APP, build up in the ER [130], as they can in other abnormal states [85, 174], and, in a mechanism still not fully understood, form fibrillar aggregates of β-amyloid intracellularly within the ER membrane tubular network that rings the nucleus [186]. A neuron at this stage of PANTHOS, which is still fully intact by confocal and 3D ultrastructural analysis, is likely to be misclassified as an *extracellular* amyloid plaque when examined only using β-amyloid immunocytochemistry or silver staining (Figs. 6, 7).

Balancing lysosome membrane permeability (LMP) against lysosomal repair, lysophagy, and other defenses

In the mouse AD models studies above, LMP detected at the initial stage of AD using the classical assay devised by Christian DeDuve, the discover of lysosomes, coincides with impaired lysosomal acidification, an established cause of LMP [21–23]. Other likely routes contributing to LMP [176] in AD are damage from free radicals, including especially oxidative stress-induced Hsp70.1 carbonylation and calcium- and calpain-mediated cleavage [244]. Lysosomal repair is a critical physiological/homeostatic defense against worsening LMP that leads to catastrophic disruption and irreversible LCD via release of hydrolases and calcium. Repair is assisted by Hsp70, which binds to bis(monoacylglycerol) phosphate (BMP) and increases acid sphingomyelinase activity, which helps to rebalance lysosomal membrane lipid composition [180] (Fig. 3). Recruitment of the endosomal sorting complexes required for transport (ESCRT) machinery plays a key role [195, 211, 256], leading to restoration of lysosomal acidity. The upregulation and acetylation of Hsp70 also induces an autophagic induction response [184] and attenuates the likelihood of caspase-independent and caspase-dependent cell death by inhibiting Apaf1 and AIF [156]. Autophagy upregulation is anti-apoptotic in several additional ways. Beclin1, a key component and regulator of autophagy, binds and suppresses the pro-apoptotic molecule Bcl2 [11, 57]. Pro- versus anti-apoptotic balance plays out differently in a given neuron population. Although Beclin 1 levels were reported to be lowered in AD brain, subsequent analyses have measured normal levels [106, 129]. In PS1/APP mouse neocortex, rare neurons undergoing classical apoptosis are seen within the larger population exhibiting various degrees of PANTHOS-pattern autophagy–lysosomal dysfunction. Notably, a third population exhibits clusters of autophagic vacuoles containing activated caspase 3, suggesting another way by which autophagy can neutralize an apoptosis threat [247]. As a second line of defense when LMP in a lysosome becomes overwhelming, lysophagy
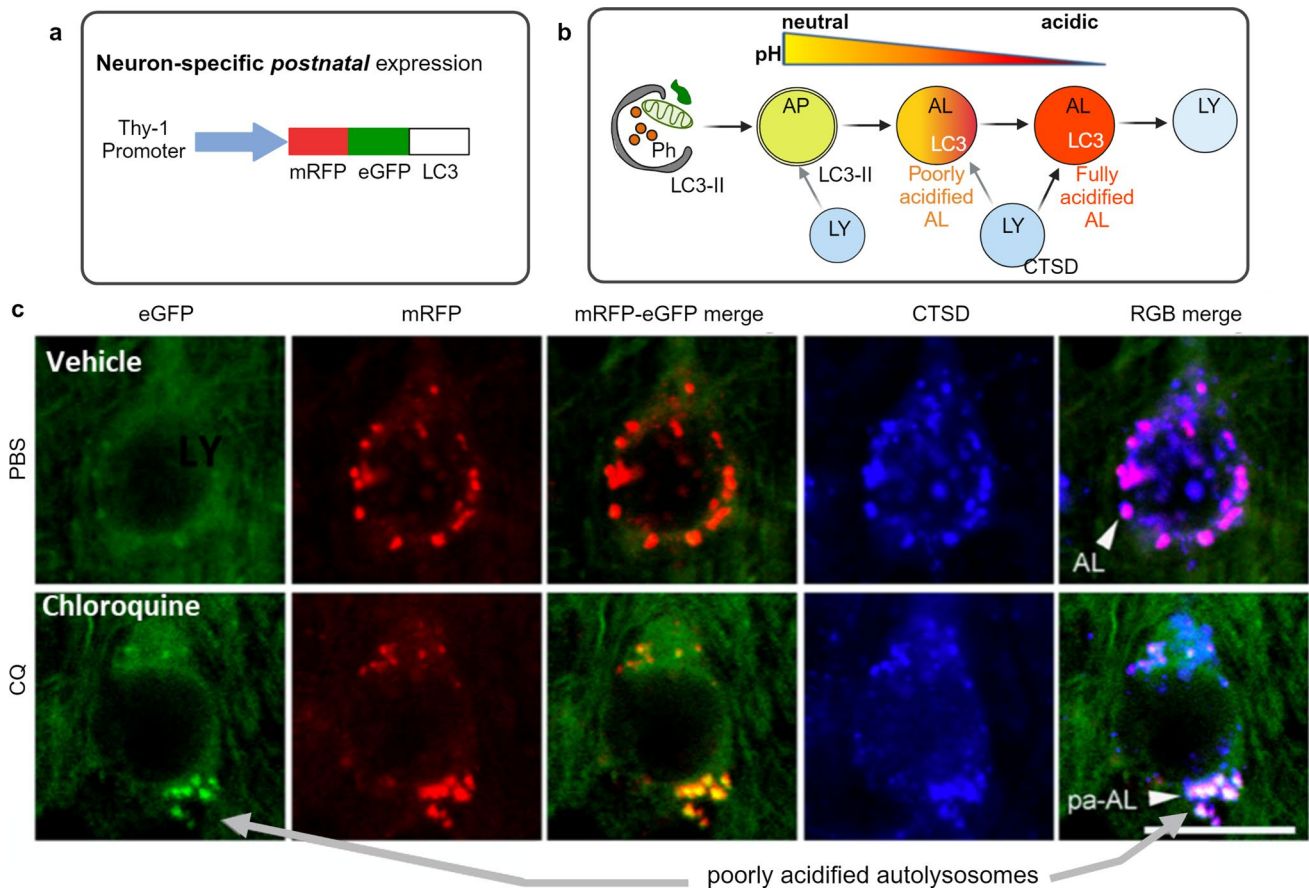
Fig. 4 A transgenic ratiometric autophagy probe in neurons enables interrogation of brain autophagy in vivo. **a** Schematic representation of the dual fluorescence autophagy sensor, mRFPeGFP-LC3 (tfLC3) with a Thy1 promoter ("TRGL mice"). Transgene founders were identified with a forward primer in Thy1 and a reverse primer in the RFP gene. **b** The schematic illustrates changes in fluorescence signals during the progression of autophagy in TRGL mouse neurons. Yellow puncta indicate autophagosomes, orange puncta indicate incompletely acidified autolysosomes, which may in the process of maturing or are autolysosomes pathologically deficient in acidification. Red (mRFP only) puncta identifies autolysosomes that are normally acidified to a pH level that quenches GFP fluorescence after AP-LY fusion, indicating fully acidified ALs with detectable ongoing LC3

digestion. The distinction between a normal maturing autolysosome and an abnormal poorly acidified autolysosome, both appearing yellow/orange, is achieved by IHC co-labeling with a lysosomal marker (CTSD). In the triple fluorescence condition, acidified autolysosomes are purple, white puncta are autolysosomes that are poorly acidified (eGFP not quenched), and lysosomes are blue. Net fluorescence "color" is objectively quantified ratiometrically as hue angle yielding a measure of relative pH. **c** Changes of morphology and pH of autophagy pathway organelles are illustrated in the somas of neocortical neurons in TRGL mice. A vehicle control condition is compared with conditions where lysosomal pH is abnormally elevated (see Ref. [130] for further details). Scale bar: 20 μm

eliminates the lysosome. In this process, galectins enter the lysosome, bind to intralumenal β-galactosides resulting in their surface exposure and LC3-mediated engulfment into autophagosomes for clearance by intact lysosomes [180].

Neurons exhibiting PANTHOS form intracellular β-amyloid plaques and transform into extracellular senile plaques when they die

Despite the massive autophagic pathology in intact PANTHOS neurons, they are not significantly invaded by microglia for multiple weeks in the mouse models of amyloidosis and not until they appear to have lost structural integrity.

The prolonged "agonal" state of PANTHOS neurons and their significantly delayed recognition by microglia as being irreversibly compromised confirms their persistent viability even in this compromised state. The absence of caspase 3 activation [130] and the slow removal of PANTHOS neuron corpses mediated by invading glial cells [130] further distinguishes this form of cell death from apoptosis. It further suggests that even the clearly maladaptive sustained induction of autophagy in the face of lysosome failure is a lasting albeit futile attempt to maintain viability rather than it being a stimulus to enter an autophagic or apoptotic cell death program. PANTHOS is ultimately accompanied by worsening LMP and autophagic-lysosomal death followed by
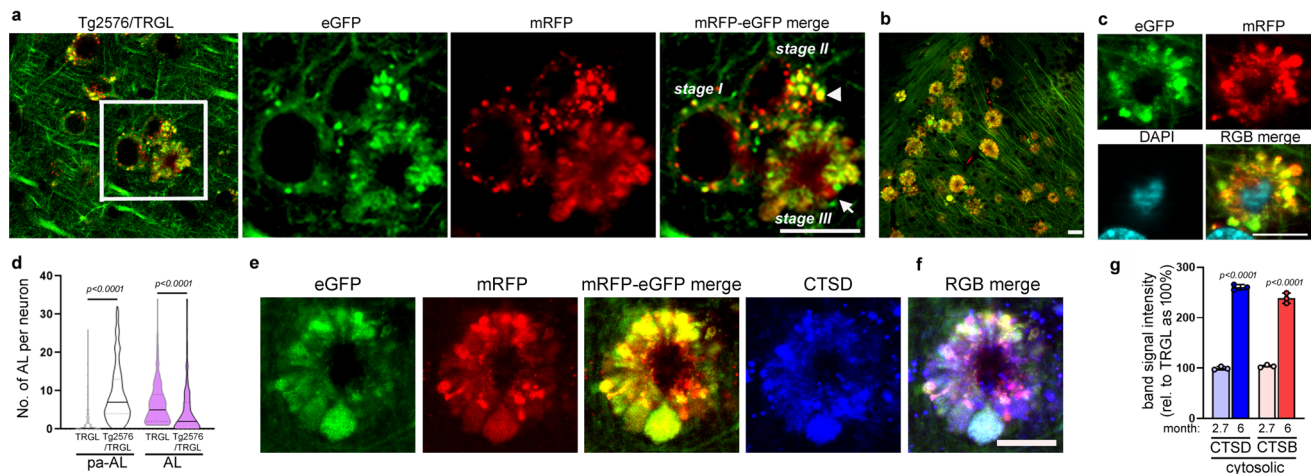
Fig. 5 Extreme autophagy–lysosomal dysfunction ("PANTHOS"), LMP, and loss of highly vulnerable pyramidal neurons in AD: an exceptionally early unique pattern of progressive autophagic stress, autolysosome pH deficits and plasma membrane blebbing ("PANTHOS") is detected in varied mouse models of AD pathology (only Tg2576 model shown here) expressing a dual-fluorescent RFP—eGFP—LC3 autophagy reporter in neurons. Especially when the nucleus is DAPI-stained, the pattern, termed PANTHOS—p-anthos or "poisonous flower", resembles brightly colored flower blossoms and was referring to the slow but inevitable death of these neurons (see also Fig. 6). Appropriate fluorescent antibody markers have recently detected a similar autophagy dysfunction and PANTHOS lesions in human late-onset (LOAD) brain [68] (Fig. 7). **a** Representative tfLC3 fluorescence images of 10-month-old Tg2576/TRGL mouse brain depicting neurons at three different stages toward PANTHOS state: i: early pH change in autolysosomes (CSTD-positive in c.); ii: focal plasma membrane bulges as poorly acidified (yellow) autolysosomes (pa-ALs) enlarge and proliferate (arrowhead); iii: full PANTHOS pattern (arrow). **b** Lower magnification image of cortex highlights the flower-like pattern of PANTHOS, its frequency at a mild/moderate stage of disease, and the preponderance of yellow (poorly acidified autolysosomes in the blebs—see also **e**). **c** Staining of PANTHOS neurons using nuclear marker (DAPI) in 10-month-old Tg2576/TRGL mouse brain. Scale bar, 10 μm. Over 90% of amyloid lesions are PANTHOS (detectable central DAPI nucleus) at early-stage disease and > 65% at later stage [130]. **d** Autolysosome acidification deficits develop early in AD model mice and progress with age. Poorly acidified autolysosome number in 5-month-old Tg2576/TRGL is elevated and acidified autolysosome number is lowered compared to neurons in TRGL littermates. Scale bar, 20 μm. Lysosomal vATPase activity is also decreased at 6-months [130]. **e** A third fluorophore (cathepsin D IHC) reveals that most autophagic vacuoles in the blebs are autolysosomes that have fused with lysosomes but fail to acidify (appearing as white puncta reflecting presence of all 3 fluorescence labels). **f** Lysosomal membrane permeability develops early in mouse models. Lysosomal enzyme distribution (ratio in cytosol vs membrane/vesicle fraction) is normal at 2.7 months of age but significantly abnormal by 6-months in 5xFAD versus WT mice. (Panels reproduced from Nature Neuroscience [130] with permission)

microglial and astrocytic invasion of the PANTHOS corpse which further matures the *extracellular* senile ("amyloid") plaque into lesions of diverse morphologies [8, 14, 52, 73, 94, 102, 124, 126, 130, 163].

The PANTHOS morphology of a still intact neuron in an FAD mouse model of ß-amyloidosis (Fig. 7a) closely matches a corresponding PANTHOS profile in human AD brain at Braak III stage (PFC) (Fig. 7b). Aβ immunoreactivity is present in the Aβ-positive autolysosomes contained within plasma membrane blebs, similar to that described in mouse models [130]. An advanced stage of PANTHOS may also exhibit perinuclear amyloid accumulation or can remain more dispersed and assume a coarse-grained plaque morphology at the *early* Braak stages. As AD pathology progresses in neurons expressing the neuron-specific autophagy reporter, there is greater diversity of plaque morphologies [15, 61, 229] reflecting the evolution of morphological changes that result from the progressive proteolytic clearance and bystander neurodegeneration [169]. The complexity of pathology in later-stage AD, coupled with the fact that

glia strongly immunolabel with lysosomal markers, impedes an unequivocal characterization of plaque origins. The ability in TRGL-expressing mice to track plaque development from a single pyramidal neuron population as it transforms into a senile plaque and further mature provides important new insight into the possibility that a sizeable proportion of the plaque diversity derives from the sequence of neuronal death, neighbor recruitment, and slow progressive clearance by glia.

The evolution of PANTHOS in some highly vulnerable neuron populations supports an "inside-out" origin of neuron cell death and plaque development in AD [51, 82], emphasizing that significantly deleterious Aβ actions are exacted *intraneuronally* on lysosomal compartments and ultimately initiate death of the neuron, yielding a senile plaque [130], which then can trigger additional secondary responses that accelerate disease. In these later phases of disease, it is possible that (possibly defective) microglia dying after taking up amyloid and related plaque debris can also develop PANTHOS-like states that transform into a classic plaque
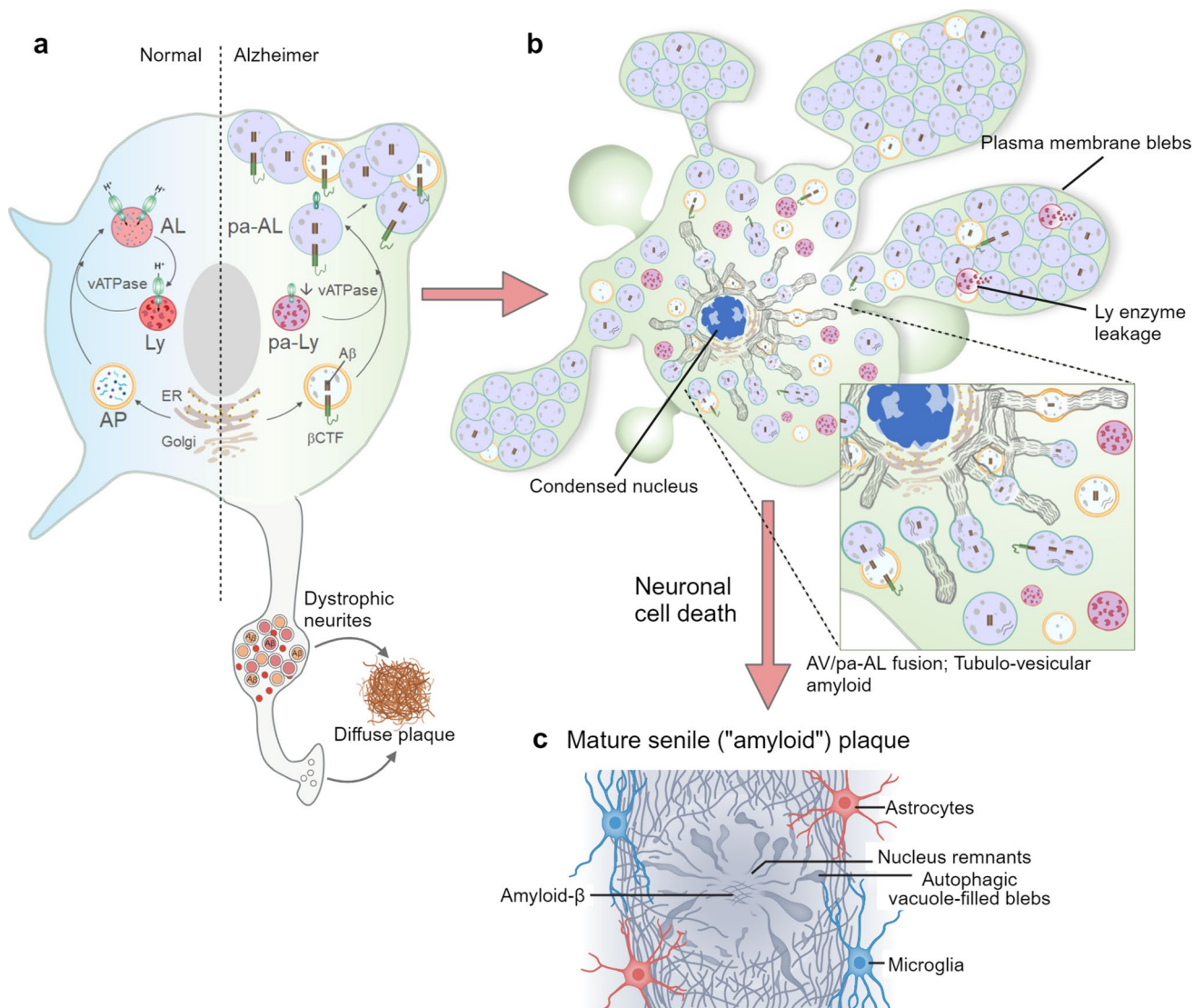
Fig. 6 Evolution of autophagy–lysosome dysfunction in Alzheimer's disease leading to neurodegeneration. **a** A primary effect of APP-βCTF elevation in AD brain [2, 74, 91, 101, 104, 187, 193, 219, 227, 231] is the inhibition of vATPase activity, which disrupts lysosomal acidification [95, 102, 130]. Coupled with multiple genetic, environmental, and cell aging factors [19, 37, 113, 122, 125, 126], APP-βCTF begins to corrupt lysosomal function at the earliest stage of the disease and before hallmark neuropathology appears. **b** Increased autophagy induction, an early neuroprotective cellular stress response, becomes counter-productive as degradative compartments progressively fail to clear the growing waste burden. Ensuing build-up of autophagic vacuoles (AVs), mainly poorly acidified autolysosomes, causes a unique pattern of extreme perikaryal membrane blebbing and trafficking deficits of retrograde moving amphisomes and late endosomes that produce swellings along axons (dystrophic neurites-DN. Within the perikaryon, Aβ accumulation in autolysosomes accelerates, and aggregates of fibrillar β-amyloid form within endoplasmic reticulum (ER) tubules. Reflecting an apparent stalling of ER-phagy—a normally highly active constitutive process of ER turnover by autophagy. **c** This intracellular pathobiology evolving within still intact neurons, precedes lysosomal membrane permeability and the lysosomal-associated neuronal cell death and replacement of each dying neuron with a senile ("amyloid") plaque (SP). An inflammatory response to the extracellular β-amyloid involves the recruitment of reactive astrocytes (A) and phagocytic microglia (M) to the disintegrating neuron and release of damaging cytokines and hydrolases that gradually clear the extracellular debris. Bystander neurotoxicity in nearby neurons and further glial invasion expands some senile plaques (not shown in the diagram). PANTHOS-like centrifugal blebs projecting from the central area of a senile plaque represent the important contribution of somal AV-filled blebs to the neuritic appearance of senile plaques in human AD brain drawn by Oskar Fischer [71] interpreted mainly as dystrophic neurites by later investigators. *Diagrams are adapted from* [130, 169]

via a similar inside-out mechanism [8]. The additional possibilities of neuritic dystrophy [115] or synaptic degeneration seeding diffuse plaques or the expansion of plaques from extracellular amyloid cannot be excluded. However, in mouse AD models, amyloid plaques grow mainly by corrupting neighboring neurons to accelerate PANTHOS and
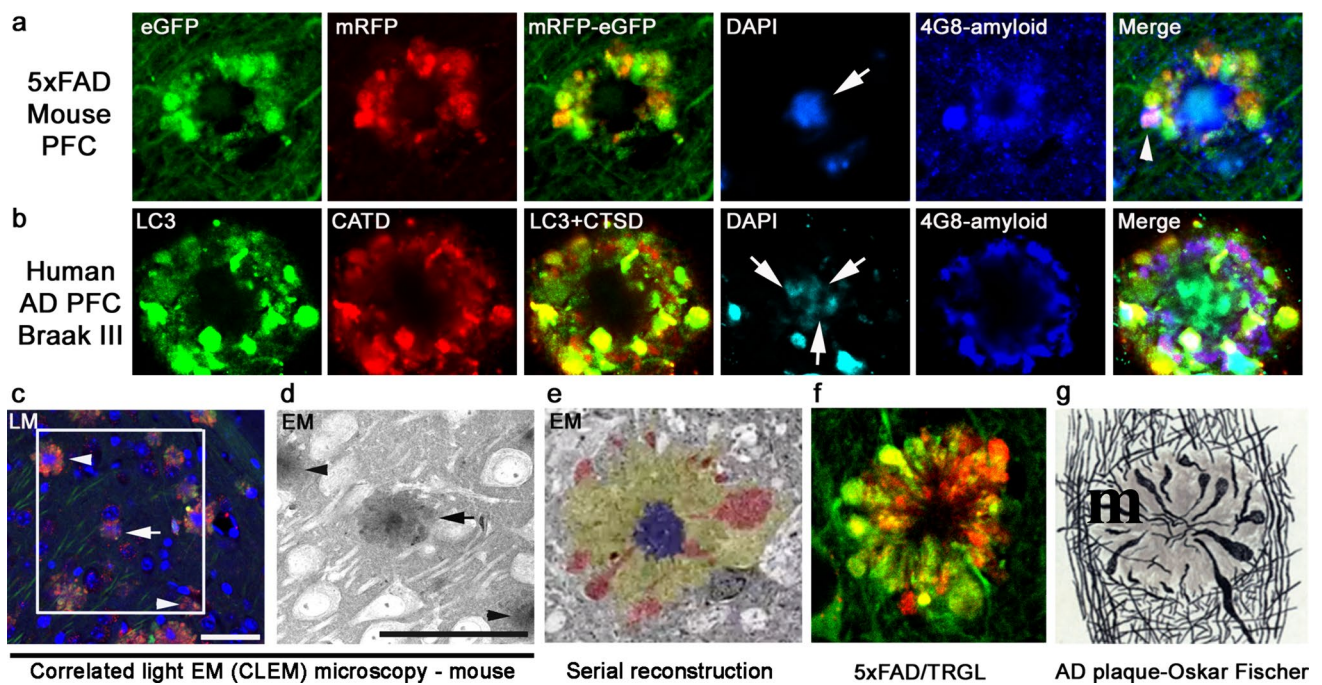
Fig. 7 Comparable PANTHOS-pattern autophagic pathology and intraneuronal β-amyloid in **a** mouse AD models and **b** early-stage human AD brain. IHF labeling using 4 fluorescence labels (TRGL 5xFAD mouse model, DAPI nuclear stain, and amyloid immunolabeling (4G8) demonstrate similar intraneuronal Aβ accumulation surrounding a visible DAPI-positive nucleus within a PANTHOS neuron in both human and mouse brains. Scale bar, 10 μm. **c** Immunohistochemistry image of the ROI (box) used for serial SEM imaging of a 2.7-month-old 5xFAD/TRGL mouse brain. Scale bar, 40 μm. **d** z-stacked serial SEM image of the ROI area, serial sections 370–430 through the PANTHOS depicted in the ROI. Scale bar, 40 μm. Arrow indicates the PANTHOS of interest. **e** 3D reconstruction of 573 serial sections through the indicated PANTHOS neuron demonstrating its integrity as a neuron and the abundance of AVs in blebs with narrow necks (colored pink) projecting from the somal PM. The central nucleus is colorized blue. **f** Confocal image of a PANTHOS state of a neuron from a 5xFAD mouse model highlighting the protrusions of AV-filled blebs with narrow necks originating from the somal plasma membrane and resembling similar plaque lesions in human AD brain depicted initially by Oskar Fischer [71] in panel **g** (panels a, c-f adapted from Lee et al. 2022 [130] with permission)

forming the larger plaques [130]. The foregoing sequence of events is consistent with data showing that neuronal cell death is prominent in mouse models of AD pathology that exhibit greatest intraneuronal accumulation of Aβ and/or APP-βCTF [29, 67, 81, 109, 130, 160], whether or not they develop extracellular plaques [240] or whether ß-amyloid experimentally is redistributed from extracellular to intracellular locations [160]. Neuron loss is a robust feature in a variety of single and multiple transgenic lines: pyramidal neuron loss especially appears related to intraneuronal Aβ accumulation [41].

The genetic basis of primary lysosomal pH dysregulation in AD

That lysosomal acidification impairment is among the earliest pathogenic deficits known in AD brain is consistent with evidence that lysosomal APP-βCTF is a major triggering factor [95], acting together with possible further lysosome damage from other accumulating lysosomal oxidized substrates, including Aβ. Acidification is mediated mainly by the ATP-dependent proton pump, vacuolar H+-ATPase (vATPase) [45, 47, 161], a 14 subunit complex regulated mainly by the extent of reversible association of a cytoplasmic subcomplex (V1) and a membrane associated V0 subcomplex (Fig. 3, [169]). Crucial to pathogenesis of AD and likely additional neurodegenerative diseases [46], the a1 subunit of the V0 subcomplex (V0a1) is responsible for mediating the association between the V1 and V0 subcomplexes that regulates vATPase activity [48]. APP-βCTF binds selectively to the V0a1 subunit and inhibits this association [94], thus linking its elevation in the lysosomes of AD neurons to the early decline in acidification [102, 130] and extreme autolysosomal and lysosomal pathology [94, 102, 124, 126, 130, 163]. The negative impact of APP-βCTF on lysosomes adds to its other pathogenic actions in triggering endosome dysfunction linked to cholinergic neurodegeneration at early stages of AD [100–102, 123, 220] and in dysregulating mitochondria-associated ER membrane interactions (MAMs) that disturb calcium and lipid homeostasis [5] and elevate reactive oxygen species (ROS) levels [5] (Figs. 1, 3).

Evidence tying causative AD genes to lysosomal pH dysregulation first arose from studies of Presenilin 1 (PSEN1) mutations, the most common cause of early-onset familial AD [131]. Most notably as the catalytic subunit of the γ-secretase complex [55, 84], an intra-membrane endoproteinase acting on many endolysosomal substrates, PSEN1 cleaves APP-βCTF to yield amyloid-β (Aβ) peptide. As importantly, PSEN1 by itself in its uncleaved holoprotein form has varied functions [92, 99, 103, 216] including acting as a chaperone in the ER and Golgi to facilitate the folding and glycosylation of the crucial V0a1 subunit [131], thereby stabilizing it against premature ERAD proteolysis before it is delivered to lysosomes for assembly of the vATPase complex [7, 95, 127, 131, 209, 241]. PSEN1 is, therefore, *required* for adequate lysosomal acidification. Its deletion sharply reduces lysosomal vATPase activity and clearance of substrates, including APP-βCTF and Aβ [95, 130].

Restoration or enhancement of lysosomal function attenuates autophagic stress, neuronal cell death, and amyloid plaque formation in AD models

An essential criterion for lysosomal cell death is its inhibition or significant delay by restoring lysosomal functionality. The primary or central role of the autophagy–lysosomal pathway and its closely networked endosomal-lysosomal pathway in driving diverse pathological and pathophysiological features of AD is strongly supported by the broad disease amelioration achieved in AD models by enhancing lysosomal function and autophagy flux [68, 148, 214, 227, 249]. Rescue of deficits ranging from failed waste clearance [17, 45, 127, 236], amyloid and tau pathologies and synaptic and cognitive deficits [89, 117], has been repeatedly confirmed using widely varying approaches sharing the property of enhancing flux through the *entire* autophagy pathway [28, 148, 214, 250]. Notably, similar levels of rescue are achieved when lysosomal proteolytic efficiency is specifically enhanced including elevating cathepsin activities [25, 157] by genetic manipulation of lysosomal protease inhibitors [217, 250], increasing lysosomal biogenesis and transcription of vATPase subunits [56, 148, 213], or pharmacologically re-acidifying lysosome, including using lysosomal-targeted acidic nanoparticles [17, 45, 127]. Pharmacological restoration of lysosomal acidity has been shown in preliminary studies to substantially block death of neurons exhibiting PANTHOS and commensurately lowering senile plaque number.

Autophagy and the concept of lysosomal-dependent cell death in AD

"Heterogeneity" of neuron cell death patterns in AD

The modes of death reported for different cell types in the AD brain vary widely in different pathological contexts. Scattered cortical neurons of the PS1/APP mouse AD model undergo unequivocal apoptosis without the substantial antecedent autophagy pathology evident in most other neocortical cells [247]. In the prolonged neuronal survival battle that is ongoing in neurodegenerative diseases like AD, multiple cascades are activated as disease advances. When a biomarker selective for a single cell death pathway detects its activation at one stage of the demise, other participating modes of cell death may frequently be overlooked. The complexity of cell death analysis in AD brain is compounded as phagocytic, neuroinflammatory, circuit disconnections, and neurotrophic failures superimpose triggers of additional death cascades over the primary initiator. One may ask, for example, whether the demonstrated activation of an apoptotic program should be considered the defining mode of cell death in a disease if lysosomal or necrosis-related triggers have already rendered the neuron irreversibly on a path to death? These are key considerations in deciding whether events leading to death are triggers or executioners or both. PANTHOS associated lysosomal-dependent neuron death evolves during a disease stage preceding superimposition of many complicating disease accelerants (e.g., inflammation and glial proteases).

Roles of lysosomes in the execution of neuronal cell death in AD

An acute massive disruption of lysosomes is sufficient to induce and partially execute death as was classically illustrated by the rapid cell death induced by the uptake of large particles, such as silica, into lysosomes which can be delayed or substantially slowed by cathepsin inhibitors [181]. Severe LMP that overwhelms repair mechanisms and lysophagy can be driven by converging AD-related factors, including inhibition of vATPase [105], APP-βCTF and cholesterol, calcium-mediated activation of calpains, and ROS-generation from oxidized lipids, proteins, and Aβ, which are elevated early in vulnerable neurons in AD brain [20]. Even under extreme conditions of lysosomal disruption, however, other cell death cascades are inevitably activated, resulting in end-stage necrosis and varying levels of caspase activation. Such a chain reaction of cell death executioners is documented from studies of

acute and gradually progressive LMP [79, 23]. Various cathepsins such as aspartic cathepsin D and cysteine cathepsins B, C, F, H, K, L, O, S, V, W, and X are considered effectors in apoptotic cell death [156]. The contribution of cathepsins, however, should not be underestimated, given that restoring acidification of autolysosomes substantially blocks neuron loss and amyloid plaque production.

In addition to promoting LMP and the release of cytotoxic hydrolases into the cytosol, declining lysosomal acidification broadly inhibits intraluminal hydrolase activity and especially the most acidic cathepsins like cathepsin D, which is optimally active at pH 4.0–4.5. Lowered neuronal cathepsin D activity is an outcome of lysosomal pH rise in essentially all mouse models of AD pathology as well as in late-onset human AD [129, 223, 233, 257]. Cat D deficiency alone, causing an autophagy–lysosomal phenotype reminiscent of PANTHOS in mice, induces cell death without a dominant involvement of apoptosis [208].

The foregoing discussion implies that even at the terminal stages of neuronal compromise, autophagy induction is playing a neuroprotective role in prolonging neuron survival by suppressing apoptosis and attempting waste clear despite extreme lysosomal inefficiency that impedes autophagy flux. This pattern is distinct from classical autophagic cell death [44], a death process associated with over-exuberant autophagy induction and preserved or possibly enhanced lysosomal function, enabling the cell to be consumed from within, ultimately killing it by eliminating cellular constituents essential for survival. Autophagy-dependent cell death is executed by the autophagy machinery [58] in the absence of apoptosis [4, 11] and requires evidence that death can be blocked or substantially delayed by selectively inhibiting autophagy. Programmed autophagic death of an entire cell population is seen in some lower invertebrates to achieve organ/tissue reorganization [164]. Over-activated autophagy may also contribute to the death of neurons injured by acute injuries, such as hypoxia/ischemia and trauma [164] and involves enzymes from lysosomes [58]. Beyond these situations, however, lysosome failure is a far more common cause of neuron death than is a defect in upstream autophagy. In AD models, enhancing induction of autophagy experimentally (e.g., rapamycin, TFEB activation) is somewhat effective in attenuating pathology because it can upregulate lysosomal efficiency and autophagy flux by stimulating lysosomal biogenesis and the synthesis of subunits of the vATPase proton pump [114, 185].

Autophagy-associated lysosomal-dependent neuron death in AD in the context of other late-onset neurodegenerative diseases

The lysosome or its related degradative compartments, the autolysosome and endolysosome, are the primary or secondary targets of an expanding list of genes causing late-age onset neurodegenerative diseases besides AD [169]. The point of mechanistic convergence in many cases is dysregulation of ion balance and pH, resulting in signaling and hydrolytic failure. Lysosome dysfunction, and particularly acidification, are being increasingly appreciated as targets of pathogenic proteins causing other neurodegenerative diseases [46]. Channels on the lysosomal membrane regulating the import of chloride (ClC7, CLC5), potassium/H$^+$ balance (TMEM175), calcium efflux/influx/proton leak (TRPML1 and TPC2) among other ions, greatly influence physiological proton content and pH but can also mitigate pathological changes in pH (detailed review: [169]). These ion fluxes influence baseline proton content and pH and can rebalance lysosomal pH under certain pathological conditions. The modulators of lysosomal ion balance and signaling have themselves been implicated as causative for diseases, such as Parkinson's disease, e.g., SCNA and LRRK2), frontotemporal lobar dementia, and several less common diseases [169]. In particular, lysosomal calcium exchanges with the ER and mitochondria maintain the large lysosomal calcium store that influences pH balance [42, 43, 83, 127] as well as the local calcium signaling that regulates varied steps in autophagy endolysosome trafficking and fusion events. *Regulated* calcium efflux from lysosomes mediated by phosphoinositides and other cell signals modulates lysosome motility, fusion with other organelles, exocytosis, and local signaling controlling autophagy flux [70, 144, 221], including transcription of genes encoding autophagy and lysosomal biogenesis [152]. By contrast, uncontrolled calcium efflux caused by a sustained rise of lysosomal pH in AD models dangerously elevates cytosolic calcium levels, which activates calpains and varied pathogenic kinases that collectively are capable of triggering vesicular trafficking deficits, tauopathy, and cell death. One consequence discussed below is AD-like neuritic dystrophy in aged PSEN1 FAD knock-in mice. Reduced lysosomal calcium levels, likely from excessive efflux, are also seen in LRRK2-related PD [112] and are attributable to enhanced activity or responsivity of Two Pore Channel 2 (TPC2), a suspected target of LRRK2 phosphorylation.

Although primary lysosomal dysfunction is shared among multiple major neurodegenerative diseases, the PANTHOS pattern of autophagy pathology seen in AD is not present *to this magnitude* in other adult neurodegenerative diseases so far reported or studied in vivo with the TRGL autophagy probe (e.g., ALS-SOD1 mutation; HD-HTT mutation [10, 215]. Critical differences between AD and these other diseases are the importance of APP, specifically the direct inhibitory action of APP-βCTF on lysosomal acidification, contributory roles of other genes corrupting lysosomes, such as presenilin mutations and APOE4, and, importantly, the persistence of a high level of autophagy induction throughout the course of AD. APP over-expression per se is not

an explanation for PANTHOS given that late-onset human AD [129] exhibit PANTHOS and accumulate higher levels of APP-βCTF. Potentially instructive in this regard is the childhood lysosomal disorder Niemann–Pick type C, which exhibits APP miss-metabolism, AD-like endosome enlargement, modest β-amyloid deposits, and striking autophagic vacuole build-up. Like AD, autophagy induction is sustained and exacerbates the autophagy phenotype because when autophagy induction is experimentally suppressed, the build-up of storage material is attenuated [69, 146, 248]. Moreover, in contrast to AD and NPC, autophagy induction is not increased and may, in fact, diminish during the course of PD and FTLD [108, 182], which is expected to reduce the burden on lysosomes.

Further consequences of lysosomal dysfunction for AD pathological development

Tauopathy

Hallmark neurofibrillary tangles of AD, composed mainly of tau protein, are abundant in AD brain and in a few uncommon aging-related degenerative diseases caused by rare tau mutations, including one form of frontotemporal dementia. An underlying mechanism involving the lysosome is suggested by observations that NFTs are also present in the lysosomal disorders, NPC1 and mucopolysaccharidosis type IIB [175, 203, 204]. Despite its notoriety as a neuropathological AD hallmark, however, NFT accumulation in AD has been proposed to be a marker of resilience [53] compared to the neurons that accumulate abeta [29] and other observations that neurons may live for decades with neurofibrillary tangles [90, 155]. Other work has suggested that accumulation of pTau may not impair neuronal function, at least initially [120] and may instead facilitate neurons escape from apoptosis [136, 242]. Collectively, these studies raise the possibility that NFT formation may not be a primary driver of neuronal death in AD but rather may be a marker of resilience [29].

Abnormal hyperphosphorylation [63, 132], truncation of tau [38, 177], and cathepsin D suppression [223] are each considered to promote tauopathy-related neuronal dysfunctions in AD, whether or not they are associated with tangle formation [63, 77]. Each is also an outcome of lysosomal deacidification and associated with TRPML1 activation [127]. These findings, together with evidence that tau is an autophagy substrate [9], suggest mechanistic links between tauopathy and lysosomal dysfunction, including calcium release from TRPML1 channels which may be regulated in a combined manner by pH, Ca^{2+}, phosphoinositides, and LAMTOR1 subunit of the Ragulator complex [127, 140,

207, 218, 222]. The cytosolic calcium rise in PSEN1 AD cell models via lysosomal TRPML1 activates calpains [127, 140] which generates truncated tau prone to aggregation [38] and the p25 cleavage product of cdk5 that hyper-phosphorylates tau [132] in AD and in FTD models [196] while promoting cell death [132, 165]. Notably, inhibition of calpains specifically by over-expressing its endogenous inhibitor calpastatin markedly reduces tauopathy and neuron loss in tau P301L and P301S tauopathy mouse models [142, 196].

Tau is metabolized by both the ubiquitin proteasome system and autophagy and contains a motif that targets it for chaperone-mediated autophagy [27]. Incomplete chaperone-mediated autophagy of tau generates fragments that aggregate and are cleared by macroautophagy [237]. Moreover, autophagy preferentially degrades a caspase-cleaved fragment of tau implicated in tau neurotoxicity [62]. Consistent with these findings, autophagy induction reduces tau pathology in the triple transgenic AD mouse model [28]. Conversely, autophagic-lysosomal dysfunction amplifies pathology and neurotoxicity of tau in other AD models [86, 111]. In addition, asparagine endopeptidase, a lysosomal cysteine proteinase upregulated during aging and activated in AD [254], is released in neurons injured by brain ischemia and hypoxia [254]. This enzyme and I2PP2A translocate from neuronal lysosomes and the nucleus, respectively, to the cytoplasm where they interact and lead to tau hyper-phosphorylation [9] rescued by asparagine endopeptidase inhibition [254].

Neuritic dystrophy of AD: a selective autophagy-related trafficking deficit

Another hallmark AD lesion linked to autophagy–lysosomal impairment is neuritic dystrophy, referring to focal axonal swellings filled nearly exclusively with autophagic vacuoles and commonly present within senile plaques (or "neuritic plaques") but more widely distributed. As in PANTHOS, amyloid-β, BACE1 and βCTF are enriched in AVs providing for potential release from affected dystrophic regions and formation of diffuse β-amyloid in the local vicinity. However, AD-like dystrophic neurites also form in the absence of β-amyloid as shown in aged PSEN1 knock-in FAD model mice [140] and GGA3-deleted mice that express high levels of BACE1 [115, 145]. In the former model, local calcium release from late endosome/amphisome compartments via TRPML1 activates JNK phosphorylation of dynein intermediate chains, impedes retrograde motility of autophagic/amphisomal vesicles [140] and induces their *selective* accumulation within focal axon swellings [140] recapitulating AD neuritic dystrophy. Restoring proper lysosome acidity blocks these events [140]. Earlier studies showed that pharmacologically inhibiting vATPase in primary neurons *selectively* slows the retrograde motility of vesicles tagged with

LC3 or LAMP1 (i.e., autophagic vacuoles) and also induces their selective accumulation within focal axon swellings [133]. Involvement of calcium release from compromised lysosomes [138] is suggested by the PSEN1 FAD knock-in mouse model study [140] in vivo and in vitro, where the lysosomal calcium release activates c-jun N-terminal kinase and phosphorylates dynein thereby interfering with retrograde axonal transport of endolysosome compartments.

APP-βCTF is also linked to the lysosomal pH dysregulation associated with impaired AV retrograde transport and accelerated neuritic dystrophy in individuals carrying a polymorphism of the AD risk gene, phospholipase D3 (PLD3) encoding a lysosomal protein abundant in neurons [232, 251]. As discussed above under "Tauopathy", the local release of calcium from poorly acidified endolysosomes likely disrupts dynein-mediated retrograde axonal motility [140].

Ferroptosis

In the face of heightened oxidative stress, genes involved in iron homeostasis are essential for neuron survival along with the genes for superoxide dismutases, endolysosomal function, autophagy, and cholesterol handling [226], all of which are widely implicated in neurodegenerative diseases. Rising oxidative stress in aging brain and neurodegenerative disease especially from oxidized lipid accumulations in lysosomes elevate Fe^{2+} entry into cells [96, 252] through the endolysosomal system [6, 210], which helps to further drive oxidative stress via the Fenton reaction [206, 212] and promotes lysosomal deacidification [245]. Iron release from endolysosomes to cytoplasm requires low acidic pH and is key to maintaining appropriate cytoplasmic iron levels. When lysosomal acidification is defective, Fe^{3+} derived from ferritinophagy, mitophagy or transferrin endocytosis cannot be reduced to Fe^{2+} thereby mpeding iron export and causing a deficiency of iron in the cytoplasm and in mitochondria [122]. Iron deficiency activates a pseudo-hypoxia response, loss of mitochondrial function, and non-apoptotic cell death [245]. Moreover, iron deficiency is sufficient to trigger inflammatory cell-autonomous inflammatory gene expression in cultured neurons and in vivo, triggering non-apoptotic cell death known to accompany sterile inflammatory responses [200, 238]. Importantly, inhibition of lysosomal vATPase is sufficient to initiate this cascade in vivo, which can be ablated by repleting iron through the diet [245]. Furthering the link between ferroptosis, LMP, and lysosomal cell death is evidence that ferroptosis requires cathepsin B expression, which can be mediated by STAT3, a positive regulator of ferroptosis in some cell lines and also a promoter of lysosomal-mediated cell death in mice during mammary gland involution [119, 205]. Inhibiting lysosome-dependent cell death by pharmacological blockade of cathepsin activity or vATPase limits erastin-induced ferroptosis [76].

Consistent with the discussion that lysosome dysfunction catalyzes varied cell death cascades, a significant contribution by ferroptosis in AD has been increasingly suggested to be a secondary consequence of lysosomal dysfunction although the mechanism remains unclear and the relevance to human AD is, therefore, circumstantial [78, 130]. Alterations in the three primary factors in this process—iron export [65], thiols (reverse transporter system xc⁻ and glutathione (GSH)/glutathione peroxidase 4) and lipids, are considered contributors to AD pathogenesis. Collectively, these alterations lead to iron dyshomeostasis [59, 154] and iron-dependent lipid peroxide elevations, which are also seen in the brain in AD [1, 246] and are associated ultimately with cell death [255]. In conditions of high iron, such as AD, APP translation is increased [137], which can contribute to elevations of APP-βCTF and Aβ [40, 122, 202], thus accelerating lysosomal dysfunction.

Selected methods for autophagy–lysosomal evaluation in human brain

Striking neuronal autophagy–lysosomal abnormalities were initially appreciated in early AD brain using cathepsin D immunocytochemistry [34–36, 167, 168]. It was proposed at the time that the presence of active *neuronal* lysosomal cathepsins in extracellular plaques reflected a primary origin of plaques from dying neurons [35], although the integrity of these grossly distorted neurons and their relationship to amyloid plaques was difficult to establish using a single marker with or without markers of amyloid. The neuron-specific LC3 autophagy probe selectively expressed in neurons of mouse AD models, however, enabled a visualization of the true extent of autophagy pathology culminating in the unique PANTHOS morphological pattern to be appreciated together with intraneuronal amyloid lesions and their transformation into extracellular plaques. With the PANTHOS evolution having been characterized, detection of this unique autophagy pattern and antecedent autophagic abnormalities in human AD brain can now also be achieved using appropriate pairs of antibodies and nuclear histochemistry [129]. The additional use of axonal markers and confocal imaging in the z-plane dimension facilitates distinguishing PANTHOS ongoing in neurons from the cells that have already transformed into plaques. It is important to note, however, that studies of neurons using TRGL mice do not exclude the possibility that microglial cells engorging amyloid-containing debris at later stages of AD may also exhibit a variant of inside-out plaque formation when they die. Conducting analyses in cortical regions of brain at early Braak stages (II–III) before the later-stage inflammatory response greatly

simplifies the recognition of individual neurons undergoing lysosomal cell death.

Conclusions

Compromise of vulnerable neurons culminating in their death in AD is frequently an indolent process reflecting continuous mobilization of repair and other mechanisms of neuroprotection, including autophagy, to counter aging-related and disease-promoting obstacles. Evidence suggests that cell death modes may differ for individual types of neurons and other cells in brain. Moreover, depending on the pathological context, neurons even within the same population may activate different death mechanisms at varying stages. The characterization of neuronal cell death in AD is fraught with challenges reflecting the possible multiplicity of participating death subroutines, superimposed secondary toxicities from the brain's responses to an initial insult, and technical difficulties of monitoring changes in the multiple cell death pathways that may be involved as neurons progress toward death.

The opportunity to characterize the entire course of an exceptionally early cell death of vulnerable neocortical pyramidal neurons in AD brain and mouse models of AD pathology by probing neuronal autophagy dysfunction prior to conventional neuropathological lesions is fortuitous. While ELA dysfunction emerges early in most layer III and V neocortical neurons, a select subset of these neurons develops unique states of worsening autophagic stress, LMP, abeta aggregation, and lysosomal-dependent death beginning during this poorly understood "intraneuronal" stage of AD and leaving extracellular amyloid plaques in their wake.

The early emergence of LMP beginning as the brain ages evolves to more fulminant AD-related lysosomal dysfunction driven by genetic and environmental factors many of which converge on a mechanism of disrupted vATPase activity causing intralumenal pH to rise. These events leading to cell death are triggered and partly executed by dysregulated lysosomes given that genetic and pharmacological remediation of lysosomal deficits attenuate neuron cell death and ensuing extracellular amyloid deposition and rescue synaptic and cognitive impairments. These observations do not exclude participation by other cell death subroutines at end stages when the fate of the neuron may possibly have already been determined. The foregoing weight of evidence, however, establishes autophagy-associated lysosome-dependent cell death as one cascade in AD having numerous implications for the further progression of the disease. The findings to date encourage investigation of LMP/LCD modulation and therapeutic strategies targeting lysosome dysfunction to defeat AD at its earliest stage.

Declarations

Conflict of interest None.

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