Promoting the clearance of neurotoxic proteins in neurodegenerative disorders of aging

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Abstract

Neurodegenerative disorders of ageing (NDAs) like Alzheimer's disease, Parkinson's disease, frontotemporal dementia, Huntington's disease and amyotrophic lateral sclerosis represent a major socio-economic challenge in view of their high prevalence yet poor treatment. They are often called proteinopathies in view of the presence of misfolded and aggregated proteins that lose their physiological roles and acquire neurotoxic properties. One reason underlying the accumulation and spread of oligomeric forms of neurotoxic proteins is insufficient clearance by the autophagic-lysosomal network. Several other clearance pathways are likewise compromised in NDAs: chaperone-mediated autophagy, the ubiquitin-proteasome system, extracellular clearance by proteases, and extrusion into the circulation *via* the blood-brain barrier and glymphatic system. The present article focuses on emerging mechanisms for enhancing neurotoxic protein clearance, a strategy that may curtail the onset and slow the progression of NDAs.

Abbreviations

Ca^{2+}: intracellular cytosolic calcium, **Aβ:** amyloid-β-protein, **AD**: Alzheimer's disease, **ALN:** autophagic-lysosomal network, **ALS:** amyotrophic lateral sclerosis, **AMPK**: AMP-kinase, **ApoE4**: apolipoprotein Epsilon 4 allele, **APP**: amyloid precursor protein, **Atg**: autophagy-related gene, **BBB**: blood brain barrier, **Bcl**: B-cell lymphoma, **CMA**: chaperone-mediated autophagy, **CSF**: cerebrospinal Fluid, **ER**: endoplasmic reticulum, **FTD**: frontotemporal dementia, **GFP:** green fluorescent protein, **GPCR:** G-protein coupled receptor, **HD:** Huntington's disease, **Hsc**: heat shock cognate, **HSF**: Heat Shock Factor, **Hsp:** heat shock protein, **Htt**: Huntington protein, **ISF**: interstitial fluid, **LAMP**: lysosome-associated membrane protein **LC3**: microtubule-associated proteins 1A/1B light chain 3B, **LRP1:** low density lipoprotein receptor-related protein 1, **LSD**: lysosomal storage disease, **MMP:** matrix metalloproteinase, **mTORC1**: mammalian target of rapamycin complex 1, **NDA:** neurodegenerative disease associated with ageing, **Nrf2**: nuclear factor erythroid 2–related factor 2, **PINK1**: PTEN-induced putative kinase 1, **PROTAC**: proteolysis-targeting chimeric molecules, **SOD1**: superoxide dismutase, **TDP-43**: Transactive response DNA protein-43, **TFEB**: transcription factor EB, **TREM2**: triggering receptor expressed on myeloid cells 2, **Ulk1**: unc-51-like kinase 1, **UPR**: Unfolded protein response, **UPS:** ubiquitin proteasome system, **v-ATPase**: vacuolar-type H^+-ATPase and **Vps**: vacuolar protein sorting-associated protein

Glossary entries underlined and in bold

Neurodegenerative disorders of ageing, neurotoxic proteins and the importance of their clearance

Neurodegenerative disorders of ageing (NDAs) include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and related tauopathies. They are ultimately fatal, have no disease-modifying therapies and are associated with an increasing socioeconomic burden due to their rising incidence. These "**proteinopathies**" display complex and partly distinctive pathophysiological profiles, yet all share a cardinal feature: accumulation of aberrantly-processed and misfolded proteins like **amyloid-β-protein** (Aβ), **tau**, **α-synuclein**, **TAR DNA-Protein 43** (TDP-43) and the polyglutamine protein, huntingtin (Htt). In NDAs, these proteins lose their physiological roles, aggregate and acquire novel neurotoxic functions[1], with an impairment of elimination implicated in their their buildup and spread[1-5].

As summarized in **Figure 1**, several endogenous mechanisms account for neurotoxic protein clearance. The **glymphatic system** and the **blood-brain-barrier** (BBB) extrude neurotoxic proteins from the extracellular space, interstitial fluid (ISF) and cerebrospinal fluid (CSF), where they may also be degraded by proteases or phagocytosed by microglia and astrocytes. Within neurons and other cell types, intracellular elimination is predominantly effected by the ubiquitin-proteasome system (UPS), chaperone-mediated autophagy (CMA) and the autophagic-lysosomal network (ALN) (**Figure 2)**. Owing to its predilection for aggregated forms of neurotoxic proteins, as well as damaged organelles which likewise build up in NDAs, the ALN is an especially attractive target for disease-modification. However, it is unlikely that modulation of the ALN will prove to be a panacea[1,4,5]. Thus, we likewise discuss opportunities for harnessing non-ALN driven mechanisms of clearance for course-alteration in NDAs[2,3].

The autophagic-lysosomal network

Crucial role in clearing aggregated proteins

Autophagy is a phylogenetically-conserved, cytosolic process essential for cellular homeostasis. Three basic types are recognised (**Figure 2**)[3,4].

Macroautophagy ("autophagy") involves sequestration of cytosolic material into *de novo* synthesized, double-membrane-bound autophagosomes that deliver their contents to **lysosomes** for digestion. Autophagic flux (**Box 1**) describes the process from formation of the autophagosome isolation membrane to cargo digestion in the lysosome (**Figures 2 and 3**). Autophagy is crucial for the intracellular clearance of burdensome proteins in all cell types,

including neurons. Further, astrocytes and several subtypes of microglia play important roles in the phagocytosis and subsequent elimination of *extracellular* pools of neurotoxic proteins[6-8]. In addition to bulk clearance of cytoplasmic contents, dedicated autophagy receptors promote sequestration of specific misfolded and/or aggregated proteins, damaged organelles, **aggresomes, stress granules, peroxisomes,** endoplasmic reticulum (ER)/Golgi components, lipids, ribosomes, polysaccharides and nucleic acids[4,9]. LC3-II and adaptor/scaffold receptor proteins like optineurin and p62 recruit discrete classes of protein to autophagosomes[10]. Other scaffolds include "Nix", "BNIP1" and Prohibitin-2 for dysfunctional mitochondria (**Box 2**)[4,9-11]. Ubiquitin and non-ubiquitin dependent autophagy occurs, with ubiquitination of tau and other neurotoxic proteins enhancing capture by autophagic receptors like p62. Post-translational modifications like acetylation (*e.g.,* of Htt) may favour ALN degradation, but await further evaluation[12].

The other two modes of autophagy are microautophagy, where cytosolic material is directly engulfed by invaginations of lysosomes, and chaperone-mediated autophagy (CMA), further discussed below.

Autophagy can be constitutive or inducible, rapidly adapting to alterations in the internal and external environment of cells. Flexibility is important for mainting normal brain function and for ensuring a constant supply of recycled amino acids, sugars, lipids and other products of ALN-mediated catabolism[3,13]. That autophagy serves an essential housekeeping role is demonstrated by genetic ablation of **autophagy-related genes** (Atg). For example, mice with neuron-specific Atg7 or Atg5 deletions develop early post-natal neurodegeneration[14], while knockdown of Beclin 1 (Atg6) exacerbates the vulnerability of hippocampal neurons to energy deprivation[15]. Moreover, post-mitotic neurons cannot dilute harmful proteins *via* mitosis, so they are uniquely vulnerable to impairment of clearance[1,3,5,16-18].

Maintaining efficient ALN flux requires coordination of a suite of modulatory proteins and phospholipids (Figure 3)[3,10] Changes in their amount, stoichiometry and function are characteristic of NDAs[1-3,5,10,18-20].

Operation and regulation of the ALN

Sensing, initiation and regulation of ALN induction

The heterotrimeric serine/threonine kinase, **AMP-regulated Kinase** (AMPK), and **mammalian target of rapamycin complex (mTORC1)** respectively trigger and repress autophagy and mitophagy (**Figure 3, Box 2**)[3,10,20-23]. Unc-51-like kinase (Ulk1) is primarily an autophagy-initiating protein[3,10,19], and the same holds true for mTORC1-suppressed

Transcription Factor EB (TFEB), which orchestrates the synthesis of lysosomal and other proteins critical for maintaining ALN flux[20-23]. Since the Class III deacetylase, Sirtuin-1, requires **nicotinamide adenine dinucleotide** to sustain its activity, this positive regulator of autophagy may also be considered as a sensor[24].

Intrinsic sensors detect changes in intracellular levels of glucose, amino acids, fatty acids, AMP, inositol triphosphate (IP_3), cytosolic Ca^{2+}, reactive oxygen species and metabolic intermediates such as **acetyl coenzyme A (Box 2)**[5,13,19,21,23,25]. For example, decreased glucose availability and impaired mitochondrial respiration compromise ATP production, leading to elevated levels of AMP and ADP which allosterically activate the γ-subunit of AMPK[21]. Extrinsic sensing occurs *via* drug-targetable mechanisms at the plasma membrane. *First*, receptor tyrosine kinases converge onto mTOR1, AMPK or the Beclin 1-Vps 34 complex (Figure 3) to modulate autophagy following stimulation by growth factors[10,26]. *Second*, G-protein coupled receptors (GPCRs) and ion-channel coupled receptors control autophagy *via* signalling pathways that likewise modulate AMPK and mTORC1[27-29]. GPCR-mediated generation of cAMP can negatively regulate autophagy *via*, for example, protein kinase A (PKA)-mediated phosphorylation of Atg proteins[27,29,30]. *Third*, specific classes of cytokine and cytokine receptor also modulate autophagy, although events in the brain remain poorly defined[23].

AMPK exerts several mechanisms that trigger autophagy. Most importantly, phosphorylation-activation of of Ulk1/2 (Ser317 and Ser777) and phosphorylation-inhibition of mTORC1[21,31]. Conversely, mTORC1 inhibits Ulk1/2 by Ser757 phosphorylation[3,4,31]. MTORC1 also restrains autophagy by preventing nuclear translocation of TFEB[20]. Other transcription factors that positively regulate autophagy include Forkhead-Box O1 and O3[22]**.** Conversely, repression is effected by STAT3 (Signal Transducer and Activator of Transcription 3) and, possibly, "ZKSCAN3," although its role has been disputed[22,32]**.** Sirtuin-1 is activated by AMPK-mediated increases in nicotinamide: it drives the ALN by inhibition of mTORC1, induction of Forkhead-O1/O3, and activation of key regulatory proteins like Atg5, Atg7 and LC3. These actions comprise part of a broad palette of Sirtuin-1 mediated neuroprotective effects in NDAs[24].

Autophagosome formation, cargo sequestration and delivery to lysosomes

Activation of Ulk1 triggers autophagosome nucleation through phosphorylation-activation of Beclin 1 within the autophagy-specific Vps 34 kinase complex[10] **(Figure 3).** LC3 and other family members like "GABARAP" covalently conjugate with phosphatidylethanolamine and assist in elongation of the isolation membrane and closure of autophagosomes[1,3,10,33]. They also serve as docking sites for autophagy receptors that selectively capture ALN substrates (Box 1)[3].

Compared to glia, the complex structure of neurons complicates ALN degradation of neurotoxic proteins[1,8,10,18]. Autophagosomes formed in synaptic terminals and neurites must be retrogradely transported with the aid of microtubules and dynein-dynactin motor complexes to the perikarya where lysosomal fusion occurs[10,16,34]. Indeed, many autophagosomes fuse with late endolysomal compartments containing membrane-localised Rab7 protein (a GTPase) and Lysosome-Associated Membrane Protein (LAMP)1 *before* reaching the perikaryon. This implies that the ALN process is partly intiatiated in advance of fusion with mature lysosomes and full luminal acidification, a process completed upon arrival in the perikaryon (Figure 2)[10,16,34,35].

Autolysosome formation is facilitated by the retromer complex, itself retrogradely transported to cell bodies[36,37]. "SNARE" proteins and the "Homotypic Fusion and Vacuole-Protein Sorting" complex bridge mature autophagosomes/amphisomes to lysosomes to initiate fusion[4,19]. Rab proteins and LAMP1/2 collectively aid in autophagosome maturation and lysosomal fusion, which is also dependent on membrane constituents like **Phospholipase D1**, phosphoinositols and other phospholipids like cholesterol[10,19,38].

Lysosomal digestion of cargo

Autophagosomes fuse with lysosomes that provide the hydrolases required for cargo degradation[3,4,9,39]. Hydrolases are dependent on a low pH, and lysosomal acidification is promoted by vacuolar-type H^+-ATPase complex (v-ATPase) which pumps protons into the lysosomal lumen. The electrogenic potential created by proton import is mediated by multiple ion channels that influence lysosomal pH[40]. Underpinning the importance of acidity, digestion can be halted by v-ATPase inhibitors like bafilomycin A[41] and lysosmotropic basic amphiphiles like chloroquine which alkalinize the lysosomal lumen[42]. Further, a deficiency of lysosomal cathepsins (B, L and D etc) prevents protein degradation and leads to accumulation of undigested cargo[16,17,39]. Lysosomal dysfunction blocks flux across the *entire* ALN, as evidenced by **lysosomal storage diseases** (**LSDs**) like **Niemann-Pick Type C** which manifest with neuropathological phenotypes (**Suppl Box 1)**[43].

In addition to ALN function, the importance of maintaining lysosomal activity reflects a broader role in, for example, regulation of cytosolic Ca^{2+} and energy homeostasis[44].

Chaperone-Mediated Autophagy

Like autophagy, CMA is important for amino acid recycling during periods of poor nutrient availability but, in contrast, it involves transfer of protein substrates for

degradation into the lysosomal lumen without enclosure by any membrane structure (Figure 2)[45-47]. With the help of heat shock protein90 (Hsp90) and other co-chaperones, heat shock cognate protein70 (Hsc70) recognises soluble, cytosolic proteins bearing a KFERQ or equivalent motif and guides them to the transmembrane LAMP2A receptor[1-5,10,47]. The substrate complex binds to the cytosolic tail of LAMP2A leading to LAMP2A stabilization and oligomerization: following unfolding of the protein cargo, it is then translocated into the lysosomal lumen. This process is aided by a specific, low pH-dependent lysosomal form of Hsc70 (Lysine-Hsc70), which promotes dissociation of the LAMP2A multimer so that the monomeric form is again available for substrate recognition and import. The level of LAMP2A determines the rate of CMA. In contrast to ALN, CMA is not devoted to the degradation of higher-order neurotoxic proteins and aggregates, but it is important for clearing oxidized proteins. Tau, α-synuclein and TDP-43 are substrates for CMA degradation, as well as APP but not Aβ42 itself[3,45-47,48]. Htt is not efficiently cleared by CMA, and the same appears to hold for its fragments, mutant and post-translationaly modified forms, although the precise role of CMA in Htt elimination remains to be more fully defined[2,45-47].

The Ubiquitin-Proteasomal System

The UPS mainly targets soluble and monomeric proteins rather than aggregates, using a process involving Hsp70 and the sequential actions of three classes of ubiquitin ligase (E1, E2, and E3). They effect the addition onto targeted proteins of ubiquitin residues, often as polyubiquitin chains, at single or multiple lysine sites (Figure 2)[2,3,8,48,49]. Ubiquitinated substrates are recognised by the 19S regulatory particle of the UPS complex. After binding to the Rpn subunits of the 19S ring, ubiquitin motifs are removed by three enzymes, Usp14, Uch37 and Rpn11. Rpn11 removes ubiquitination chains only *after* substrates are committed to destruction, whereas Ups14 and probably Uch37 act *before* commitment and hence can *rescue* substrates[49]. Following removal of ubiquitin moieties, proteins are unfolded by the Rpt1-6 subunits (ATPases) of the 19S component. The substrate then passes the α-subunit gate of the 20S core particle to enter its central β-subunit which possesses peptidase activity (trypsin, chymotrypsin and caspase-like) and effects proteolysis.

In addition to ubiquitinated substrates, the UPS can also handle oxidized proteins which may accumulated under conditions of cellular stress[8,50]. Further, as well as cytosolic proteins, the UPS degrades mitochondrial proteins that build up upon failure of mitochondrial import or

sorting[51]. It also operates in the nucleus. Interestingly, the UPS is important for elimination of tau and other neurotoxic proteins in post-synaptic dendritic compartments (a key site of spreading), where it plays a more general role favouring synaptic plasticity, dendritogenesis and memory formation[49,52]. **Susceptibility of neurotoxic proteins to ubiquitination is modified by phosphorylation and other post-translational modifications[3,8,49,51].**

Defective ALN, CMA and UPS mediated clearance of neurotoxic proteins

NDA-related impairments

Neurons adopt multiple strategies to deal with potentially-dangerous proteins. With the aid of chaperones like Hsp70, anomalously-configured proteins may be refolded or, if clumped in aggregates, disassociated[2,3,53]. Neurotoxic proteins may also be sequestered in insoluble tangles (tau) or in microtubule-associated aggresomes[2,4]. This intracellular lock-up may, at least initially, be neuroprotective, but continuing accumulation eventually poses a threat to cells underscoring the importance of elimination[2,4]. While clearance systems are, at least initially, recruited in NDAs, they eventually become unable to cope with the additional neurotoxic burden (**Table 1**)[1,5,9,18,54,55]. The partly common and partly disease-specific patterns of ALN, CMA and UPS disruption in NDAs are superimposed upon a generalized, age-related decline in clearance both in neurons and in other cell types like microglia[1,2,7,18,46,47,55,56]. Insufficient neuronal ALN flux is frequently manifested by lysosomal accumulation of **lipofuscin**[18].

For optimisation of therapy in NDAs, accurate interpretation of the causes of impaired elimination is paramount. This is challenging since it may be a repercussion of *upstream* anomalies like protein overproduction, misfolding or an excessive cytosolic **Unfolded Protein Response (UPR)** (**Suppl Box 2**)[57]. Further, it is difficult to identify the exact nature of UPS, CMA and **ALN dysfunction** (**Box 1**). While inadequate ALN flux is a common problem for all NDAs, under certain conditions ALN *overactivity* may contribute to pathology and even **autosis**[4] in ALS (**Suppl Box 3**).

The following paragraphs and **Table 1** summarize the complex patterns of defective neurotoxic protein clearance seen in specific classes of NDAs.

Alzheimer's disease

While induced in the early phase of AD[1,3,47,58], ALN, UPS and CMA-mediated clearance eventually becomes overwhelmed and impaired. *First*, autophagosomes and autophagic vacuoles indicative of failed maturation, transport and/or fusion with lysosomes are abundant, particularly in dystrophic neurites. Their accumulation may be linked to impaired lysosomal

elimination of cargo[18]. *Second,* while decreases in Beclin 1 levels in AD remain to be confirmed, Sirtuin-1 expression is diminished[24]. *Third,* **apolipoprotein E4** allele (ApoE4), a major risk allele for sporadic AD, is associated with increased generation and accumulation of Aβ42[59,60]. ApoE4 slows lysosomal Aβ42 clearance and, like Aβ42 itself, destabilizes lysosomal membranes. In addition to decreased degradation, one consequence is **leakage of asparaginyl endopeptidase into the cytosol where it generates toxic fragments of tau[61]**. Moreover, **ApoE4 impairs the elimination of Aβ42 and tau by astrocytes and microglia**, additionally compromised by decreased activity of Triggering Receptor Expressed on Myeloid cells (TREM)2[7,62]. *Fourth,* genetic mutations and anomalies of **presenilin-1,** a dominant-negative gene linked to AD, are associated with reduced lysosomal v-ATPase-mediated acidification[40,63], a compromised ALN and deficient mitophagy[64]. Presenilin-2, likewise an autosomal-dominant risk gene, is enriched in late endosomes/lysosomes where its **dysfunction provokes lysosomal accumulation of insoluble Aβ42 and possibly tau[65]**. *Fifth,* mutations in **Amyloid precursor protein (APP),** similarly disrupt endosomal and lysosomal function, in part due to accumulation of the β-secretase-generated, carboxyl-terminal and Aβ42–containing fragment of APP called C99[66]. *Sixth*, Aβ42 compromises the function of AMPK to impede initiation of the ALN[67]**. *Finally*, Aβ42 obstructs the UPS and CMA[47,68]. Both aggregates and mutant forms of tau likewise block the proteasome, and its efficacy for degrading hyperphosphorylated and oligomeric tau is reduced compared to the physiological form[3,55,68]. Finally, while physiological tau possesses KFERQ motifs and is degraded by CMA, aggregates, mutant forms and fragments interfere with CMA[45,47].**

Parkinson's disease

By analogy to AD, disrupted proteostasis is a major feature of PD, with the efficiency of ALN, CMA, UPS and other modes of clearance compromised by multiple cellular anomalies. *First,* autosomal-recessive forms of early-onset PD are associated with mutations in Phosphatase and Tensin Homolog-induced Putative Kinase (PINK1) and E3 ubiquitin ligase **Parkin:** these mutations lead to deficits in the mitophagic removal of damaged mitochondria (**Box 2**)[69,70]. *Second,* Leucine-Rich Repeat Kinase-2 GTPase is the most commonly "mutated" protein in late-onset, familial PD. Its role is complex, but mutations lead to an impairment of the ALN due to reduced activation of Beclin 1: another repercussion may be altered processing of APP, providing an unexpected link to AD[69,71-73]. *Third*, α-synuclein mutations, triplication or excess amplify the ALN burden, interfere with autophagosome formation and irreversibly disrupt the lysosomal membrane[1,3,44,56]. *Fourth*, homozygous mutations of lysosomal β-

glucocerebrosidase provoke the LSD, **Gaucher's Disease,** which is linked to decreased ALN flux, α-synuclein accumulation and a five-fold increase in risk for PD (**Suppl Box 1**)[43]. Decreased β-glucocerebrosidase activity also occurs in sporadic PD leading to the build-up of glucosides, lipid dyshomeostasis, poor clearance of α-synuclein and impaired lysosomal activity[43,74,75]. *Fifth*, defects in several genes disrupt lysosomal acidification[40]. For example, disruption of the ATPase, ATP13A2 (PARK9), which is also depleted in sporadic PD, leads to lysosomal alkalisation and digestive failure[76] together with accumulation of α-synuclein and other ubiquinated proteins[76-78]. *Sixth*, **aggregates and mutant forms of α-synuclein disrupt the proteasome in dopaminergic neurons. Further, mutations in Parkin and several other genes are linked to reduced UPS activity[2,56,69,79,80]. *Finally*, oligomeric and mutant forms of α-synuclein impair LAMP2A-mediated cargo transport for CMA, while levels of both LAMP2A and Hsc70 are reduced in PD brain[45,47,55,80]. In addition, CMA is disrupted by several genetic mutations occurring in PD, including Leucine-Rich Repeat Kinase-2[2,3,45-47,55,69,80]. CMA dysfunction in PD favours the accumulation of α-synuclein and leads to inactivation of the dopaminergic neuron survival factor, "MEF2D"[2,45,47,55].**

Frontotemporal dementia

As FTD was initially associated with tau mutations, it was originally considered a "tauopathy"[81,82]. However, in light of common risk genes like p62 (Sequestome1) and "C9orf72" (Chromosome 9 Open Reading Frame-72), FTD is increasingly linked to ALS[82,83]. Genetic anomalies in FTD are closely related to a deficient ALN, and, like ALS, the disease is characterised by aggregates containing tau, TDP43, Fused-in-Sarcoma and other ubiquitinated proteins insufficiently cleared by the ALN[82,84]. **Aggregates interfere with the UPS to create a vicious circle that further overloads the ALN[1,18,55,56,68,84]. Recently, it was found that poly-glycine/alanine tracts linked to mutant forms of the C9orf72 gene form twisted ribbon aggregates that sequester and stall the activity of proteasomes[85].** MAPT (tau) is a distinctive risk gene for FTD *vs* ALS, and dissociation of tau from microtubules disrupts retrograde transport of autophagosomes to the lysosome[81,82]. In addition, lysosomal dysfunction and loss of acidification is caused by tau fragments and a deficit of progranulin[40,82,83,86], while an interrelated deficiency of endosomal trafficking is linked to mutations in "CHMP 2B" (Charged Multivesicular Body Protein 2B) as well as C9orf72[82,83].

Amyotrophic lateral sclerosis

ALS shares many causal genes with FTD, including p62, CHMP2B, "TBK1" (Tank-Binding Kinase 1), optineurin and others associated with deficits in ALN and mitophagy. For example, mutations in optineurin and TBK1 interfere with cargo loading[82,84,87]. Mutations in C9orf72 (the most prevalent risk gene for familial ALS and FTD) are likewise linked to disruption of the ALN, including interference with dynactin-dynein coordinated transport of autophagosomes along axons of motor neurons to the perikarya[82,88]. They also lead to deregulation of Rab-GTPases and a failure of autophagosome elongation[89]. Paradoxically, however, certain anomalies of C9orf72 may *stimulate* the ALN and, under conditions of severe cellular stress, high ALN activity may be detrimental (**Suppl Box 3**)[48,88,90]. In any event, depending on their genetic profiles, ALS patients reveal aggregates of risk gene-encoded proteins like TDP-43, optineurin, Fused in Sarcoma and/or **superoxide dismutase (SOD1)**[48,82,84,87,89]. **Aggregated SOD1 and TDP-43 disrupt CMA and the UPS - with the latter also impaired by mutations in the C9orf72 gene[2,8,47,48,55,85,91].** Thus, mirroring other classes of NDA, a *failure* to clear neurotoxic proteins is characteristic of ALS[48,82,84].

Huntington's disease

In this autosomal-dominant, polyglutamine disorder, an increase in **CAG-expansion repeats** in the HTT gene encoding Htt protein magnifies its propensity to oligomerise[2,3,55,80]. Mutant Htt is cleared by autophagy but it compromises the ALN because of decreased poor cargo loading and impaired autophagosome formation and transport[55,56,68,92]. Further, ALN disruption in the striatum (a region strongly impacted in HD) involves altered activity of the striatal-specific Beclin 1 and Htt-interacting protein "Rhes"[93,94]. In addition, loss of physiological Htt and abnormal polyQ-Htt perturb neuronal cilia, important sites of cellular communication and signaling which reciprocally interact with autophagic mechanisms controlling their formation and growth[92]. **CMA only poorly handles mutant and post-translationally modified forms of Htt, which interfere with its activity[2,45,47,95]. While LAMP2A and Hsc70 are upregulated in early HD to compensate for decreased ALN clearance, CMA eventually fails in parallel with neuronal loss[47,96]. The status of the UPS in HD is currently unclear, but it only poorly cleaves mutant forms of Htt (and other polyglutamine tracts), while animal models suggest that impairment in HD, which would further lead to reduced clearance of Htt[97].**

Strategies for enhancing neurotoxic protein clearance by the ALN

Ultimately, any strategy that improves protein quality control and reduces excessive generation, aberrant processing and/or abnormal folding of neurotoxic

proteins should moderate the ALN burden and facilitate clearance. For example, agents that promote folding of nascent proteins, prevent misfolding, refold aberrantly-configured proteins, dissociate aggregates, counter ER stress and/or blunt an excessive UPR might *pre-empt* the build-up of neurotoxic proteins (Suppl Box 2)[1,2,54,56,57,84,98-100]. However, the present review focuses on strategies for *elimination* of neurotoxic proteins (**Table 2** and **Figure 4**). It should be noted that the precise mechanisms of drug action are not invariably well-defined[4] and that certain agents exert multiple beneficial (or deleterious) actions. For example, methylene blue counters tau oligomerization as well as promoting autophagy (**Suppl Table 1**)[101,102]. In addition, several drugs like resveratrol interact at *multiple* nodes of the ALN. Indeed, future drugs designed to act in a multi-modal manner may prove to be the most effective for enhancing clearance in NDAs.

The following paragraphs mainly evoke classical "small molecules": innovative treatment modes for reinforcing clearance are outlined in **Box 3**.

Modulators of sensing, initiation and regulation

Direct and indirect activators of AMPK-induced autophagy

Ligands inhibiting GPCRs coupled to the AC-cAMP-PKA axis are potential activators of AMPK[27,29]. Indeed, clonidine and rilmenidine, two Gi/o coupled α_2-adrenoceptor agonists, stimulate autophagy and clear Htt in cellular[103] and animal models of HD[104], although their precise mechanisms of action await further elucidation[21,103,104]. Calpains, Ca^{2+}-activated cysteine proteases, are elevated in ageing and proteolytically generate various neurotoxic peptides[54,81]. They stimulate the AC-cAMP-PKA axis to inhibit AMPK by activation of $G_S\alpha$[103]. Genetic knockdown of calpain or overexpression of its endogenous inhibitor, calpastatin, increased autophagy and cleared aggregates in SK-N-SH cells overexpressing a mutant form of Htt[103]. Efficacy was also seen in mutant *Drosophila* and mouse models of HD[54]. Calpeptin, a cell permeable calpain inhibitor, can also reduce Htt proteinopathy *via* induction of autophagy[103,105]. Calpain inhibition by calpastatin or pharmacological agents also confers neuroprotective effects in other NDAs models, including enhanced clearance of tau, α-synuclein and SOD1[54,106,107].

The aminoimidazole derivative, "AICAR," undergoes intracellular transformation to an AMP analog that triggers AMPK-mediated autophagy[21,108]**.** It conferred neuroprotection upon exposure of astrocytes to Aβ or oxidative stress[109] and countered α-synuclein toxicity in cultured rat neurons[110]. Another direct facilitator of AMPK, A769662, elicited autophagy and reduced the burden of Htt in a striatal cell line derived from knockin mice expressing a humanized form of mutant Htt (Exon 1 containing 7 polyglutamine repeats[111]). Selenium deficits have been linked to

AD, so it is interesting that selenomethionine boosted ALN flux from AMPK recruitment through autophagosome formation to lysosomal degradation in the 3xTgAD mouse model[112].

The "anti-ageing" drug, resveratrol, is thought to indirectly recruit AMPK *via* activation of Calmodulin-Kinase-Kinase-β which, acting in synergy with Ca^2, exerts its effects *via* Thr172 phosphorylation[113]. This action, amongst others (below), is involved in its reduction of Aβ levels in N2a cells and neurons[114] and the elimination of Aβ and Htt in animal models of AD and HD[114,115].

The anti-diabetic drug, metformin, a prototypical activator of AMPK, induced autophagy and increased longevity in mice[116]. Like AICAR, metformin abrogated α-synuclein toxicity in primary cultures of cortical neurons, though the precise contribution of autophagy requires clarification[110]. Moreover, reductions in levels of hyperphosphorylated tau and Aβ were seen in metformin-treated neurons[117,118], while it blunted neuronal loss in a neurochemical-lesion model of PD in mice[119].

The di-glucose derivative, trehalose, inhibits the "SLC2A" family of glucose transporters to promote AMPK-induced autophagy and reduce neurotoxic protein load, though it also exerts other actions downstream in the ALN[4,120]. Trehalose promoted autophagy and reduced disease progression in a SOD1 mouse model of ALS[120]. It also proved effective in cellular models of PD, HD and AD,[121,122] as well as in mouse models of HD, AD and tauopathies where it cleared aggregates, reduced neurodegeneration and ameliorated motor and cognitive performance[123-125].

Lithium ions inhibit inositol monophosphatase to deplete inositol phosphate-3. This mechanism may be involved in its promotion of autophagy and reduction in cellular levels of α-synuclein, SOD1, Htt and tau[126], amelioration of motor function in a P301L mouse model of tauopathy[127], and slowing of disease progression in SOD1 mice[128]. However, its precise mechanism of action awaits further elucidation[126].

Other drugs that act through AMPK activation include the anti-aggregant, methylene blue **(Suppl Box 1),** which elevated levels of Beclin 1, p62 and LC3, induced autophagy and suppressed tau in organotypic neuronal cultures and a mouse model of FTD[101,102]. In addition, calcitriol (the active metabolite of vitamin D3) elicited AMPK-dependent autophagy in a neurochemical lesion-induced model of PD[129].

Modulators of mTORC1 and its transcriptional control of the ALN

One major strategy for promoting autophagy is relief of repression by mTORC1. This kinase is classically inactivated by rapamycin that binds to the modulatory protein, "FKBP12"

(12-kDa FK506-binding protein). Enhancing autophagy with rapamycin reduced levels of α-syn, Fused-in-Sarcoma and Htt[130-132]. It also diminished polyglutamine aggregates and countered motor impairment in a *Drosophila* model of HD[133]. In addition, rapamycin abrogated pathology in murine models of AD and FTD, as well as countering neuronal loss in MPTP-treated mice[134-136]. Likewise, temsirolimus reduced the accumulation of phosphorylated tau in SH-SY5Y cells and P301S tauopathy mice[137]. It also removed cellular aggregates of mutant Htt and improved motor performance in a mouse model of HD, reduced α-synuclein aggregation and afforded neuroprotection in a lesion-based model of PD, and depleted mutant Ataxin-3 in a mouse model of supraspinal cerebellar ataxia-3[133,138,139]. Interestingly, several "small molecule enhancers of rapamycin" promoted autophagy and eliminated Htt in cellular and *Drosophila* models, but the precise role of mTORC1 in their actions remains to be clarified[140].

The naturally-occuring compound, curcumin, induced macroautophagy and neuroprotected rotenone-treated dopaminergic neurons[141] as well as accelerating elimination of mutant A53T-α-synuclein by repression of mTORC1 in a cellular model of early-onset PD, although it also exerts other actions such as modulation of protein acetylation and aggregation[142,143]. Pro-autophagic effects of curcumin are reflected in improved function, as well as reduced levels of α-synuclein aggregates[144] and Aβ/tau oligomers in cellular and animal models of PD and AD[145,146].

Inasmuch as phosphorylation by mTORC1 blocks translocation of TFEB from lysosomes to nuclei, mTORC1 inhibitors should promote the coordinated synthesis of proteins driving the ALN[20,22,147]. Indeed, TFEB over-expression reduced amyloid plaques in a APP/PS1 mouse model[148]. Moreover, the flavonol, fisetin, stimulated autophagic degradation of phosphorylated tau in cortical neurons *via* mTORC1-dependent activation of TFEB and the cytoprotective transcription factor, Nuclear factor Erythroid-2-Related factor 2 (Nrf2)[149]. Fisetin also reduced Aβ accumulation in an APP/PS1 mice model of AD[150]. Thus, mTORC1 - and, possibly, AMPK *via* poorly-characterised cascades[21] - offer channels into TFEB. It remains, nonetheless, a challenging target for induction[22,151].

"C-Abl" tyrosine kinase is a proto-oncogene that negatively regulates autophagy, partly acting upstream of the Akt-mTORC1 axis. It is over-activated in AD and tauopathies like FTD[152]. Inactivation of c-Abl with brain-penetrant nilotinib conferred neuroprotective autophagy in mouse models of PD[153]. It also reduced aggregates in cell and mouse models expressing TDP-43 protein[154]. Nilotinib recently underwent a Phase I safety study for treatment of PD[155].

<u>Modulators of Sirtuin-1 and inhibitors of acetyl transferases</u>

Activity of the deacetylase Sirtuin-1 declines with age, partially due to limited availability of its co-factor, nicotinamide[24,56,156]. Therefore, it is interesting that nicotinamide and its analogues promoted autophagic removal of damaged mitochondria in fibroblasts[157] and reduced Aβ toxicity in rat cortical neurons[158]. They also improved mitochondrial energy generation and, partly as a consequence, reduced plaques in Aβ-expressing neuronal cells and AD mice, while improving cognitive function[58]**.** Nicotinamide analogues similarly slowed cognitive decline and neuropathology in a 3xTgAD mouse model of AD[159].

Resveratrol can stimulate Sirtuin-1 *via* AMPK (see above), and it also possesses an AMPK-independent mode of Sirtuin-1 recruitment that participates in blunting of the neurotoxicity of Aβ25-35 fragments in PC12 cells[160]. This possibly involves a role for the DNA-repair protein, poly(ADP-ribose)polymerase-1 ("PARP**"). It's pharmacological inhibition elevates levels of the substrate, nicotinamide, with an enhancement of mitochondrial energy generation contributing to neuroprotective properties in an animal model of AD[160,161].**

Cilostazol (a phosphodiesterase-3 inhibitor) clears Aβ42 from neuronal cell lines by promoting autophagy, upregulating Beclin 1, Atg5 and LC3, down-regulating mTORC1, and inducing lysosomal cathepsin B: these actions of cilostazol involve activation of Sirtuin-1 as well as upstream Tyr-172 phosphorylation of AMPK[108,162,163]. Cilostazol improved cognition and reduced levels of A42 and hyperphosphorylated tau following intracerebroventricular injection of Aβ(25-35) into mice[162,163].

Protein deacetylation, as effected by inducers of Sirtuin-1, is of broader relevance to the ALN as reflected in activation of Atg gene transcription[20,24,164]. Further, acetyl transferases like p300 are druggable[20,165] and their inhibition (by garnicol) protected against autophagic deficits in a rodent model of PD[166]. Another p300 inhibitor, spermidine, has attracted attention by virtue of its autophagy-related increase in longevity[164,167]. Spermidine inhibited the acetylation of Atg proteins 7, 11 and 15 as well as that of histone 3, while inducing Beclin 1 *via* blockade of its cleavage through caspase-3[168]. Spermidine also decreased disease progression in a mouse model of FTD[169] and reduced α-synuclein toxicity in *C. elegans*[170]. Depletion of acetyl coenzyme-A would be worth exploring in models of NDAs[171]. Underpinning interest in inhibitors of acetyl transferase, p300 expression is increased in AD brain and involved in the aberrant acetylation of tau[165,167,172,173].

Inducers of autophagosome formation

As outlined in **Box 3**, the cell-permeable peptide, **Tat-Beclin 1**, acts at the Beclin 1/Vsp 34 complex to increase autophagy and promote the clearance of Htt aggregates in cell lines[174].

In addition, the plant-derived alkaloid, isorhynchophylline, upregulated Beclin 1 independently of mTORC1 and promoted autophagic clearance of α-synuclein, although its precise mechanism of action remains to be clarified[175]. Beclin 1 bears a "BH3" element on its N-terminus that is subject to inhibition by the anti-apoptotic protein, B-cell lymphoma (Bcl)-2[19,165,176]. Disruption of this Bcl2/Beclin 1 complex is an alternative approach for promoting autophagy, as achieved in mouse fibroblasts by the BH3 mimetic, ABT-737[177]. A knockin, gain-of-function Beclin 1 mutant with reduced repression by Bcl-2 also increased autophagy, promoted Aβ sequestration and improved cognition in a 5XFAD mouse model of AD: this pattern of effects was reproduced with ML246, a novel autophagy potentiator, with an uncertain mode of action[178]. Other potential approaches to Beclin 1 activation include inhibitors of (tau-phosphorylating) cyclin-dependent kinase-5[179].

The multi-modal agent, resveratrol, induced the expression of Atg4 and promoted autophagosome formation. This led to accelerated degradation of polyQ-Htt aggregates and protected SH-SY5Y cells from toxicity[180]. An unusual approach to augmenting autophagosome formation is represented by brain-penetrant "Autophagy Enhancer-99" (AUTEN-99) which blocks "Jumpy", a phosphatase that inhibits the phosphotidyl-inositol-3-kinase-mediated generation of the autophagosome membrane (**Figure 3**). Auten-99 augmented autophagic flux in isolated neurons, increased markers of autophagy in mouse brain and slowed neurodegeneration in *Drosophila* models of PD and HD[181].

Promoters of autophagosome transport and lysosomal fusion

Disruption of cytoskeletal networks and loss of microtubule function in NDAs compromises the transport of autophagosomes, late endosomes, amphisomes and retromers to perikaryal lysosomes, and hence impedes degradation of neurotoxic proteins[34-36]. Accumulation of autophagosomes and lysosomes in axonal swellings is associated with local APP processing into Aβ42, as well as plaque formation[16,34]. The microtubule stabilizers, paclitaxel and epothilone A, countered Aβ42-induced cytoskeletal disruption - and moderated excessive UPR - in neurons[182]. Further, epothilone D countered microtubule disruption and cognitive deficits in aged P301S/P19 AD mice[183]. However, it is unclear to what extent these agents promote ALN in the perikaryon, and a risk of cytoskeletal over-rigidity should not be neglected. Thus, mechanisms that promote microtubule/actin *dynamics* and cytoskeletal shuttling of autophagosomes/endosomes to lysosomes present alternative strategies for evaluation[184].

Several other, potentially-targettable mechanisms might also aid autophagosome delivery to (and fusion with) lysosomes[185]. These include Rab and Rab-effector proteins which facilitate the assembly of Synataxin17-SNARE complexes critical for fusion[186]. **Interestingly, genetic or pharmacological activation of Rab5 countered neurodegeneration in mouse C9orf72 models of ALS and FTD[187].** There is also growing interest in the stabilization of retromers for promoting fusion. This appears feasible based on modulation of their role in diverting APP out of endosomes and hence curtailing its cleavage into Aβ42[37,188]. Finally, inducers of histone deacetylase-6, broadly implicated in cytosolic transport and the fusion of autophagosomes, might be an option[3].

Facilitators of lysosomal digestion

Maintaining optimal intraluminal acidity is critical for activating lysosomal hydrolases and digesting cargo. There are several ways that a loss of lysosomal acidity in NDAs might be countered. *First*, lysosomal acidification could be favoured by stabilised cAMP analogues: in human fibroblasts bearing a Presenilin-1 mutation, cAMP acidified lysosomes and augmented the availability of cathepsins[189]. *Second*, the TFEB inducer, 2-hydroxypropyl-β-cyclodextrin promoted the acidity of lysosomes in neurons[190]. *Third*, acidic nanoparticles like polylactic acid and poly(lactide)co-glycolide increase acidification (**Box 3**). *Fourth*, activation of the lysosomal Ca^{2+} channel, "transient receptor potential mucolipin-1," with a synthetic agonist (ML-SA1) increased intralysosomal Ca^{2+} and lowered pH[191,192]. Other approaches include the enhancement of v-ATPase activity, and countering deficiencies in progranulin activity[40,63,86,193-195].

Dysfunction of PARK9 (ATP13a2) leads to an imbalance in the handling of zinc, a disruption of lysosomal activity and accumulation of α-synuclein[77]. Clioquinol, which acts as a metal-chelator, reverses these deficits and may reinforce lysosomal function (and acidification) in NDAs where the regulation of zinc and other metals is abnormal[77,196]. Indeed, clioquinol countered disruption of autophagy by chloroquine in retinal cells, reduced Aβ42 accumulation in CHO cells expressing APP and mutant Presenilin-1, and diminished amyloid-misfolding and aggregation in Tg2576 AD mice[196,197]. Cystatin B and C are endogenous antagonists of the cysteine-active site on lysosomal cathepsins and their genetic down-regulation ameliorated deficits in lysosomal proteolysis, synaptic plasticity and amyloid clearance in TgCNRD8 AD mice[198]. Pharmacological blockers of cystatins are currently being sought. In addition, upregulation of retromer complex might stimulate provision of hydrolases to the lysosome[37,188].

Lysosomal enzyme replacement is a staple treatment for primary LSDs: for example, β-glucocerebrosidase supplementation for Type I (non-neuropathic) Gaucher's Disease (Suppl

Box 1)[43]. Due to BBB impermeability, enzyme supplementation does not appear promising in PD. However, inhibition of substrate (glucosylceramide) synthesis by brain-penetrant GZ/667161 and GZ/SAR402671 reversed synucleinopathy in A53T-SNCA mice[199]. Another glycosphingolipid synthesis blocker, miglustat,[43] showed activity in cellular and *in vivo* models of PD[75], although its ability to downregulate target sphingolipids in the brain is limited.

One might also act upstream to promote lysosomal function by accelerating the import of functional enzymes. β-glucocerebrosidase again provides a good example. Ambroxol acts as a molecular chaperone to promote folding of β-glucocerebrosidase and aid its transit from the ER to lysosomes[43]. It increased expression of β-glucocerebrosidase, normalised autophagy and accelerated degradation of α-synuclein in a stem-cell model of dopaminergic neurons derived from PD patients bearing mutations for β-glucocerebrosidase[200]. Ambroxol, which also decreased ER stress in *Drosophila*[201], reduced α-synuclein levels in overexpressing, transgenic mice[202]. It is being evaluated for use in idiopathic PD (Suppl Table 1). A downside of ambroxol is that it occludes the catalytic site of β- glucocerebrosidase, but novel agents like NCGC607 avoid this untoward effect[203]. Intriguingly, while enhancement of β-glucocerebrosidase conferred therapeutic benefit in animal models of PD, its *inhibition* by conduritol-β-epoxide was beneficial in a mouse model ALS, underpinning the apparently distinctive nature of ALS as regards ALN function and energy balance (**Suppl Box 3**)[90].

Finally, a more global approach for harnessing lysosomal activity would be the induction of TFEB[20,22]. Harnessing TFEB by 2-hydroxypropyl-β-cyclodextrin promoted clearance of proteolipid aggregates and α-synuclein in a cellular model of PD[195,204]. It also augmented the elimination of Aβ in a Tg19959/CRND8 mouse model of AD[173]. The protein kinase C activator, "HEP14", stimulated nuclear translocation of TFEB to boost lysosomal gene transcription and reduced Aβ plaques in APP/PS1 AD mouse brains[151]. Modulation of DNA methylation and post-translational histone marking offer further opportunities for transcriptional control of lysosomal activity, while miRNAs could intervene at the level of translation **(Box 3)**[20,165].

Clinical studies of agents that modulate the ALN

Certain of the above-discussed agents have been clinically evaluated, alone or in association, in NDAs **(Suppl Table 1).** For example, metformin for cognitive function and energetic status in AD; resveratrol for functional decline and Aβ load in AD; rilmenidine for motor performance in HD; and ambroxol for β-glucocerebrosidase activity and motor function in PD. **To**

date, despite some positive observations, unequivocal proof for symptomatic improvement and/or course-altering effects has *not* been provided for any drug (Suppl Table 1). Nonetheless, long-term effects remain under study, no medication that *specifically* and exclusively induces the ALN has as yet been therapeutically characterized, and proof of target engagement in clinical trials remains challenging. Hence, it is premature to draw conclusions as regards therapeutic efficacy.

In fact, the anti-oxidant, edavarone, which *decreased* autophagy in ischaemic brain and macrophages[205], was recently authorized for use in a subset of ALS patients (**Suppl Box 3**)[206]. This appears paradoxical, but fits with the suggestion that *high* ALN flux is *detrimental* under conditions of severe cellular stress in ALS[90]. Whether decreased ALN flux is genuinely implicated in its clinical actions remains to be confirmed(Suppl Box 3)[3,206].

Caloric restriction and exercise mimetics for promoting ALN clearance

Anti-ageing and lifespan-extending benefits of "caloric restriction mimetics" expressed across a range of multicellular organisms are related, at least in part, to the induction of AMPK and Sirtuin-1 leading to promotion of autophagy[21,24,164,207]. These mimetics are generally safe yet encompass drugs that reduce ATP availability by interfering with cerebral/neuronal glucose uptake. This may pose problems since compromised neuronal energy is itself a risk factor for NDAs like AD and PD[25,164]. Nonetheless, efforts to find well-tolerated, autophagy-inducing mimetics are continuing[164] and clinical trials should prove instructive[25,164]. Further, there is increasing interest in pharmacological exercise mimics that exert putative neuroprotective properties *via* the modulation of AMPK, mTORC1, beclin 1 and other regulators of the ALN[21,207].

Strategies for enhancing neurotoxic protein clearance by the UPS and CMA

Opportunities for pharmacological manipulation of the UPS and CMA are less well-established than those for the ALN, but there are encouraging routes of progress[2,45-47,55,56,68]. Furthermore, the UPS inhibitor bortezmib is approved as a first-in-class treatment for multiple myeloma, indicating that clinical application of UPS modulators is possible[3].

Facilitation of chaperones acting on client proteins

One approach for reinforcing the UPS focuses on agents that target chaperones involved in the handling and recognition of neurotoxic proteins[2,68,208]. Of particular interest is Hsp70 which interacts with the E3 ubiquitin ligase "CHIP" to aid ubiquitination of proteins destined for proteasomal destruction[208]. Hsp70 binds to **heat shock factor** 1 (HSF1) and, under conditions of

neurotoxic protein stress, their dissociation leads to mutual activation, with HSF1 driving transcriptional generation of Hsp70 and other chaperones that facilitate proteostasis[208,209]. Hsp70 also exerts a more general role in the refolding and disassociation of aggregated proteins[2,3].

One promising agent is the hydroxylamine derivative, arimoclomol, which increases the activity of Hsp70 by augmenting transcriptional activity of HSF1[210]. Arimoclomol rescued cultured motoneurons from oxidative stress and from the pro-apoptotic actions of staurosporine[211]. It also mediated the removal of mutant SOD1 aggregates and improved motor function in a mouse model of ALS[212]. Supporting interest in arimocomol, it mimicked recombinant Hsp70 in reversing lysosomal pathology in fibroblasts from patients with LSDs **(Suppl Box 3).** In an alternative approach, the rhodocyanine derivative, YM-1, allosterically promoted the activity of Hsp70 to enhance degradation of polyglutamine (polyQ) proteins: these findings suggest potential utility in HD[213]. Further, Hsp70 has been co-administered with inhibitors (IU1 and its more potent derivative, IU1-47) of the deubiquitinating enzyme, USP14, to enhance proteasomal degradation of tau[214-216]. USP14 inhibitors act by preventing deubiquitination rescue of tau and other UPS substrates like TDP43 and Ataxin-3. They may also effect allosteric changes in proteasomal subunits[217]. Interestingly, USP14 inhibitors promote the ubiquitination activation of Beclin 1 to recruit the ALN[216]

Hsp90 counters the effects of Hsp70 by forming a complex with it to impede substrate ubiquitination: it likewise exerts a suppressive influence on HSF1[210,218]. Amongst compounds that inhibit Hsp90, geldanamycin promoted elimination of both hyperphosphorylated tau and oligomeric α-synuclein in cell lines[219,220]. Moreover, geldanamycin reduced Lewy-like bodies[221] and Htt aggregates in *Drosophila* neurites[222] and reduced tau in AD mice[219]. The less cytotoxic analogue of geldanamycin, 17-AAG, has improved brain penetrance. It decreased Aβ levels,[223] improved memory[224] and lowered tau in transgenic AD mice[224]. 17-AAG also reduced α-synuclein oligomers in H4 cells[220]. Another Hsp90 inhibitor, HSP990, has shown promise in lowering Htt aggregates and improving motor performance in two mouse models of HD[225]

Modulation of the phosphorylation status of the proteasome

Numerous classes of kinase phosphorylate the proteasome[68,226,227]. Phosphodiesterase inhibitors protect cAMP from degradation to recruit protein kinase A and boost UPS activity. Accordingly, rolipram protected rat cortical neurons from Aβ-induced synaptic disruption[228]. Further, in a transgenic tau mouse model of FTD where 26S proteasomal activity was impaired, rolipram attenuated markers of tauopathy, improved memory and protected synaptic integrity by

strengthening protein kinase A-mediated phosphorylation of the Rpn6 component of the 26S proteasomal subunit[229,230]. Rpn6 activation may also be involved in the anti-ageing effects of caloric restriction[56,164]. Interestingly, resveratrol inhibits phosphodiesterase-4, suggesting that proteasomal recruitment may be yet another component of its global impact on neurotoxic protein clearance[113]. One concern with phosphodiesterase inhibitors/protein kinase A inducers is their huge range of targets (including AMPK), but it may be possible to target proteasome-specific isoforms. Further, acting upstream of cAMP is an alternative strategy. Chronic administration of CGS21680, a selective agonist of AC-coupled adenosine-2A receptors, restored proteasomal activity in cellular and murine models for HD *via* **protein kinase A-mediated Ser-120 phosphorylation of the Rtp6 component of the 19S subunit[231].**

Another kinase that activates the proteasome (Rpt6 subunit) - and directs it to dendritic spines - is calmodulin-dependent kinase II[227]. It's recruitment may account for proteasomal activation by the $GABA_A$ receptor antagonist, bicuculline[52,232]. Protein kinase G similarly activates the proteasome, and inhibition of cGMP breakdown by sildenafil reduced neurotoxic protein aggregation in cardiomyocytes, encouraging studies in NDAs[68,226,227]. P38 mitogen-activated protein kinase *indirectly* influences the phosphorylation status of the proteasome, likely *via* cAMP signalling[3,68,226,227]. P38 depletion, or its blockade by PD169316, accelerated the degradation of ubiquinated proteins, promoted α-synuclein clearance and improved cell survival[233].

Phosphorylation is a dynamic process, and small molecule inhibitors of the nuclear proteasome phosphatase, "UBLCP1" suggest that calcineurin and other phosphatases represent hitherto-unexploited targets for enhancing UPS-driven clearance of neurotoxic proteins[227].

Selective elimination of specific classes of neurotoxic protein

An important question is whether the UPS can specifically clear neurotoxic proteins while safeguarding those that function normally. Several strategies are under exploration. *First*, cereblon is the substrate receptor for the E3 Ubiquitin ligase, Cullin Ring Ligase 4. It is specifically recognised by the immunomodulatory drug, pomalidomide, the binding of which changes ligase specificity to encourage degradation of discrete classes of protein[234,235]. *Second*, **PRO**teolysis **TA**rgeting **C**himera**S** ("PROTACS") and related multi-functional compounds simultaneously bind a E3 ubiquitin ligase and a defined neurotoxic protein like tau to enhance polyubiquitination and UPS-driven removal (**Box 3**)[234,236]. Certain agents amplify PROTAC-mediated breakdown of α-synuclein[233], while other classes of bifunctional ligand bind a target

protein plus Hsp70 to direct UPS degradation[235]. *Third*, target proteins can be bound by agents bearing bulky, hydrophobic adamantyl tags which provoke conformational instability and encourage proteasomal elimination[234]. *Fourth*, the cytosolic antibody receptor, "Tripartite Motif Protein 21" binds to protein-coupled antibodies, then recruits the UPS for substrate degradation. This has been demonstrated for tau and could be adapted for degradation of other classes of neurotoxic protein[237]. *Finally*, "cellular inhibitor of apoptosis protein" specifically binds mutant SOD1 and drives it to proteasomal degradation. This provides another potential path to discrete elimination of unwanted proteins in NDAs[238].

Control of transcription factors generating UPS components

The transcription factors, Nrf1 and Nrf2, are both substrates of proteasomal degradation, as well as inducers of proteasomal synthesis, and the latter has been specifically linked to NDAs[239,240]. Further, Nrf2 is a master regulator of the anti-oxidant response and drives synthesis of lysosomal and anti-inflammatory proteins in addition to 26S proteasome components[149]. Translocation of Nrf2 to the nucleus is promoted by triterpenoid derivatives that counter the ageing-related diminution of UPS activity[241]. In addition, sulforaphane elevates proteasome levels *in vivo* by inducing Nrf2, protects neurons against oxidative stress, and has been proposed for the treatment of HD[242]. Several other agents promote the proteolytic competence of proteasomes and facilitate clearance of Aβ and/or tau in cellular models, including betulinic acid. Enhanced transcription has been implicated in their actions, but this remains to be clarified[242]. **Finally, mirroring its inhibitory influence on the ALN, mTORC1 suppresses the UPS by impeding the formation and assembly of proteasomal subunits. Correspondingly, pharmacological blockade of mTOR may promote UPS degradation as well as ALN elimination of neurotoxic proteins[243].**

Enhancement of CMA-mediated clearance

Certain mechanisms outlined above for the UPS, like increasing chaperone-driven delivery of client proteins to degradative machinery, are also relevant to the CMA[47,48,95]. In fact, *specific* induction of CMA has received little attention, possibly since the rate-limiting element LAMP2A has, to date, proven intractable for small molecule chemistry. **Nonetheless, over-expression of LAMP2A accelerated CMA clearance of α-synuclein and afforded protection of dopaminergic neurons[45], and several routes to potential pharmacological exploitation may be mentioned. *First*, cathepsin A cleaves LAMP2A, resulting in its lysosomal degradation, so selective inhibitors of cathepsin A should reinforce CMA[39,47,48]. *Second*,**

LAMP2A is stored in cholesterol-rich membrane regions: hence, cholesterol depletion might enhance transfer to regions where it is functionally active[46]. *Third*, the dynamics of the LAMPA2A-client protein translocation complex are (oppositely) controlled by mTORC2 and the phosphatase "PHLPP1", offering potential targets for augmenting CMA[244]. *Fourth*, CMA is under the negative control of retinoic acid receptorα and their blockade by synthetic, all-trans retinoic acid derivatives resulted in upregulation of CMA, including the activity of LAMP2A[245]. Mouse fibroblasts treated with these agents showed improved resistance to combined over-expression of α-synuclein and oxidative stress[245].

Importance of early intervention

There are, then, emerging opportunities for intensifying the elimination of neurotoxic proteins by the UPS and CMA[47,68,227]. However, it is important that they are homeostatically regulated since - mirroring the ALN - *excess* activity is potentially dangerous[241]. As the UPS and CMA are disrupted by neurotoxic proteins like Aβ42 and tau, their early and preventative reinforcement may be critical. UPS potentiation might be particularly efficacious when enacted in dendritic sites of neurotoxic protein spreading to counteract NDA-related deficits in synaptic plasticity and learning[1,3,5,8,47,52,68,227].

Interplay between the ALN, CMA and the UPS: therapeutic relevance

As pointed out above, there is evidence of coordinated regulation of the ALN and UPS *via* mTORC1[1,3,5,243]. Furthermore, studies of a mutant tau allele that increases the risk for FTD and AD showed that upregulating the ALN compensated for the impairment of proteosomal activity[246]. This finding underscores the reciprocal interplay between these clearance systems[3]. Indeed, the ALN can "sense" UPS failure and compensates by upregulating its own activity. For example, proteasomal failure exacerbates ER stress and leads *via* the UPR to the expression of Sestrin-2 which recruits AMPK to down-regulate mTORC1 upstream of the ALN: Nrf2 is also upregulated[3]. Supporting the relevance of Sestrin-2, it protects dopaminergic neurons from the neurotoxin, rotenone, *via* AMPK-transduced autophagy[247]. Sestrin-2 overexpression also prompted mTORC1-dependent autophagy in cortical neurons in a presenilin-knockout model of AD[248]. Proteasomal degradation of Ulk1, LC3 and other ALN regulatory proteins may prevent ALN over-activity, an observation of particular relevance to ALS (**Suppl Box 3**)[3]. **By analogy, subunits of the catalytic core of the proteasome are regulated by CMA-mediated degradation[47,55].**

Extracellular elimination of neurotoxic proteins and its impairment in NDAs

Exosomal liberation of neurotoxic proteins from neurons

When intracellular pathways of protection prove insufficient, neurons may alleviate the burden of harmful proteins by discharging them into the extracellular space. This may be a self-preservation mechanism and an attempt to acquire glial support for elimination. However, the "release" of neurotoxic proteins contributes to trans-cerebral spread of pathology. That is, abnormal conformers of proteins originating in donor cells enter recipient cells to promote protein misfolding and disrupt clearance, diffusing in a domino, snowball-like fashion across the brain[81,249].

Exosomes are involved in the release of tau, APP/Aβ-42 and α-synuclein. Accordingly, they are linked to the progression of NDAs [55,77,81,250,251]. Intriguingly, when the ALN is overwhelmed and cargo accumulates, a process of "*autophagic*" exocytosis participates in the neuronal liberation of neurotoxic proteins. This discharge of neurotoxic proteins adds to the extracellular burden from dying cells, accelerates spreading, and underpins the imortance of clearance mechanisms *extrinsic* to neurons[250,252]. In this light, capture and digestion of extracellular proteins by glial cells is primordial[7,8]**.** However, there exist several other, therapeutically-pertinent mechanisms for ridding the brain of extracellular pools of neurotoxic proteins.

Clearance of neurotoxic proteins by proteases in the extracellular space

Neurons and glia contain diverse classes of protease, and they are localized in all those compartments where neurotoxic proteins accumulate - cytosol, mitochondria and even the nucleus[39,253-256]. However, certain intracellular proteases in the cytosol generate *toxic* fragments, notably of tau (calpains and caspases) and Htt (matrix metalloproteinases (MMPs)[39,257]. Accordingly, their *inhibition* rather than induction is of interest for the treatment of disorders like AD and Huntington's disease. Indeed, the inducible (extracellular) proteases most relevant to promoting neurotoxic protein clearance in NDAs are actively secreted by neurons and glia, located on exosomes and/or expressed on plasma membranes (**Figure 1**)[254]. They include several classes of MMP, neprilysin, insulin-degrading enzyme (IDE) and plasmin[253,256,258,259].

Aβ42 and amylin (a pancreas-derived, AD-associated protein found in brain) are substrates for degradation by IDE, which also irreversibly "traps" Aβ42 and α–synuclein, preventing their aggregation and promoting ALN and UPS elimination[259]. Cerebral levels of IDE are reduced in early AD and in mouse models of AD while, mirroring AD amyloidosis, Aβ42

accumulates in mice genetically depleted of IDE. In a vicious circle, Aβ42 itself decreases IDE expression, although it may prompt its release from glia[254,259]. IDE also degrades and prevents the formation of α-synuclein fibrils[259]. By analogy to IDE, neprilysin catabolizes Aβ42 and its loss in mouse models of AD and patients alike also contributes to levels Aβ42 accumulation[253,256,260].

Another Aβ42-degrading protease, plasmin, is derived from inactive plasminogen by the actions of tissue-type plasminogen activator (urokinase), which is used to treat stroke. It is secreted by neurons (and possibly glia) into the extracellular space. Like IDE and neprilysin, plasmin degrades Aβ42 and blocks Aβ42-induced toxicity, suggesting that the decrease in its levels in AD is involved in the evolution of AD[254,256,261]. Plasmin also degrades α-synuclein to retard intercellular spreading[262].

Interestingly certain isoforms of MMPs cleave *fibrillar* as well as monomeric Aβ42[254], while extracellular α-synuclein is also a substrate for MMP-3[256,258]. Another protease with pharmacotherapeutic potential is angiotensin-converting enzyme which contributes, albeit less prominently, to degradation of neurotoxic proteins in NDAs[263]. Finally, the extracellular and intracellular serine protease, neurosin (kallikrein 6), cleaves α-synuclein. Levels are reduced in Lewy body dementia and, based on lentivirus transduction studies, it is a potential treatment for clearing α-synuclein in PD[264].

Clearance of neurotoxic proteins by the blood-brain barrier and the glymphatic system

In AD, HD and other NDAs, disruption of the structure and function of the dynamically-regulated BBB is driven, at least in part, by detrimental actions of neurotoxic proteins like Aβ42. This permits the otherwise-restricted entry of immune cells and toxic substances *into* the brain. In addition, the active elimination of neurotoxic proteins like Aβ42 and tau (possibly encapsulated in exosomes) *from* the brain may be compromised (**Table 1** and **Figure 1**)[265-273].

Dysregulation of BBB integrity is serious since it normally transfers neurotoxic proteins to the circulation using both generalized and specialized receptors and transporters (**Figure 1**)[265-267,270-272]. In addition, proteins are degraded by vascular smooth muscle and endothelial cells of the BBB itself[265,271,272]. In ageing, AD and PD, a diminution of BBB-localized P-glycoprotein efflux transporters compromises elimination of neurotoxic proteins[267,273]. There are also decreases of low-density lipoprotein receptor-related protein1 (LRP1) transporters in AD, whereas receptor for advanced glycolation end-products (RAGE) receptors are induced. These changes would respectively contribute to retention in, and return of, Aβ42 to the brain[270-272]. An

ApoE4 genotype in AD exacerbates poor Aβ42 clearance by reducing its transport to the BBB and diminishing efflux[270-272].

Arterial pulsing aids CSF/ISF flow in flushing out interstitial extraneuronal proteins *via* the complementary glymphatic system (**Figure 1**)[265,269,274,275]. Its regulation is not well understood, but roles for aquaporin-4 water channels, other astrocytic mechanisms and noradrenaline have been documented[265,276,277]. Deletion of Aquaporin-4 in astrocytes markedly reduced glymphatic flow and aggravated Aβ42 accumulation in a genetic mouse model of AD[276,278] while aquaporin-4 expression is altered in the ageing, AD and PD brain[276,277]. Loss of sleep has been linked to an impairment of glymphatic clearance and Aβ42 accumulation[274]. This is significant since "rapid eye-movement sleep-behavior disorder" is the most robust predictor of PD, while insomnia and anomolous sleep patterns occur in other NDAs like early-onset AD, where disrupted sleep is correlated with alterations in Aβ levels[279].

Strategies for promoting extracellular clearance of neurotoxic proteins

Increasing protease-driven degradation

Overexpression of neprilysin or IDE reduces levels of Aβ42 and amyloid plaque burden in senescence-accelerated mice[256]. As regards pharmacological manipulation, substances like epigallocatechin and somatostatin promote the expression, secretion and - allosterically - catalytic activity of IDE and neprilysin in parallel with an increase in the degradation of Aβ peptides[259,280]. Further, expression of progranulin in the hippocampus of AD mice reduces the density of amyloid plaques by enhancing the activity of neprilysin[281]. Epigenetic regulation of neprilysin at the level of histones, as exemplified by valproate, offers another potential approach to proteolytic potentiation[253]. As regards other proteases, augmentation of plasmin clearance by blockade of the plasminogen inhibitor "PAI-1" (the expression of which increases with ageing and in murine models of AD) reduced Aβ levels and restored memory deficits in mouse models of AD[261,282].

These observations underscore the interest of proteases as targets for degradation of neurotoxic proteins[253]. Further, several drugs evoked above like resveratrol and curcumin induce IDE and/or neprilysin, suggesting a contribution to their actions[253]. Nonetheless, structure-activity relationships for small molecules that enhance the catalytic activity (or production) of proteases are not well-characterised[253,283]. Further, there are issues of substrate specificity. For example, IDE degrades insulin and glucagon as well as Aβ42 and interacts with many other proteins, including the proteasome[259]. Neprilysin targets a range of substrates like atrial natriuretic peptides and substance P, and *inhibitors* are employed in the therapy of heart failure,[253] while

MMP activators exert deleterious as well as beneficial effects reflecting their influence on microglia and the BBB[258,284]. Additional questions centre on whether any protease inducer alone could comprehensively and enduringly clear the burden of neurotoxic proteins in NDAs.

Thus, further work is needed to determine to what extent potentiation of extracellular, glial and endothelial/BBB-localized proteases is a viable strategy for safely enhancing neurotoxic protein clearance in NDAs[253,259].

Immunotherapies for neurotoxic protein sequestration

Immunotherapies for neurotoxic protein clearance in NDAs have been pursued for over a decade. As reviewed elsewhere[81,285], the most advanced approach is currently antibodies for sequestering extracellular pools of Aβ and tau (AD) or α–synuclein (PD)[7,286]. BBB antibody penetration is limited, but they may generate a "peripheral sink" in addition to exerting actions centrally. Although Aβ-immunotherapy has not yet yielded an approvable treatment (examples being AN1792-NCT00676143 and bapineuzumab-NCT00112073), more refined cohort selection, amyloid imaging for selection of early-disease patients, and the use of monoclonal antibodies from human patients such as aducanamab (NCT01397539/02782975/02434718 in MCI, and recruiting for Phase III-NCT02484547/0247780) offers hope for progress[287].

There are at least 5 antibodies under investigation for clearing tau, including a Phase II trial (NCT02880956) for C2N8E12 in AD[288]. Another trial (NCT02985879) is underway in post-cerebral palsy employing a single-chain antibody. This is the second tau-based Phase II trial after AADvac-1 (NCT02579252) to use an active immunotherapy approach[288]. Passive tau immunity approaches are also being tested using the PHF1 (Ser396/Thr404) epitope (ACI-35; ISRCTN13033912) and Ser409 epitope (RG1600; NCT03289143)[81,288]. Targeting extracellular tau to block intercellular spreading[249] should preclude the need for high antibody inclusion into cells. Antibodies like PRX002[289] have also shown promise for reducing extracellular α-synuclein and propagation of pathology, and Phase I testing has been completed (NCT02157714 and NCT02095171)[285].

Potential problems should not be ignored, including the deposition of immune-complexes in vascular tissue, inaccessibility of tau in exosomes, and antibody-driven *import* of Aβ into the brain. Nonetheless, employing more effective antibodies and appropriate biomarkers, there are still reasonable prospects for achieving course-alteration with immunotherapy.

Improving BBB-mediated and glymphatic transfer to the circulation

The BBB is equipped with potentially-targetable transporter proteins, channels and receptors (**Figure 1**)[265-267,270-273]. Inhibition of the α-secretase, "ADAM10" was found to drive LRP1-mediated extrusion of Aβ42 into the circulation[290]. In addition, LRP1 might be indirectly modulated by aquaporin-4 channels[276-278] and epigenetically *via* miRNAs[165]. Further, the hydroxymethylglutaryl-coenzyme-A inhibitor, fluvastatin, upregulated LRP1 in the BBB to provoke Aβ42 extrusion[291]. The antibiotic, rifampicin, likewise promoted Aβ42 clearance by inducing BBB-localised LRP1 and P-glycoproteins[273,292]. Whether LRP1-driven uptake of Aβ42 by microglia (and hepatocytes) is involved in the favourable effects of LRP1 up-regulation remains to be clarified[271]. Interestingly, both fuvastatin and rifampicin have additional actions - including a probable induction of the ALN - that contribute to beneficial actions in models of AD[291,293]. As for RAGE receptors, their blockade should temper re-entry of Aβ into the brain - and exert anti-inflammatory properties[294,295]. Phase III studies are underway with azeliragon (TTP488) in AD (NCT02080364; 02916056) following promising improvement in cognition in a Phase II trial[296]. Interestingly, resveratrol downregulated RAGE as well as MMP-9, actions related to decreased hippocampal load of Aβ42[297]. Finally, at least in murine models of AD, agonists of retinoid-X receptors induce the BBB-localized P-glycoprotein "ABCB1" transporter, and this may account for bexarotene-mediated Aβ clearance from the brains of AD mice[298]. Data with bexarotene remain controversial, but the principle of acting *via* BBB-localised transporters to encourage neurotoxic protein extrusion is clearly valid.

Focused ultrasound therapy has mainly been used to enhance the entry of proteins and vectors into the brain. For example, siRNA probes for knocking down Htt or, in principle, genes encoding clearance-promoting mechanisms[299,300]. However, it acts *bi-directionally*, so CNS-to-periphery transfer of neurotoxic proteins might likewise be accelerated. By targeting selective brain areas like the hippocampus/entorhinal cortex in AD, neurotoxic proteins could be driven into the periphery. Safety is obviously an issue, but it is reassuring that gap junctions close within 6 hours or less[301].

Activation of aquaporin-4 channels on perivascular astrocytes to aid the glymphatic elimination of cerebral Aβ and other toxic proteins is a potential strategy for stimulating clearance. Both antagonists as well as positive modulators have been identified, so this seems "chemically" feasible[269,272,275,278]. A contrasting approach is represented by dobutamine which stimulates arterial pulsation and the perivascular/glymphatic CSF flushing of neurotoxic proteins from the ISF *via* lymphatic conduits into the blood[269,275]. Deposition of Aβ42 in cerebral vessels impairs vascular function-flexibility and is accompanied by an upregulation of

phosphodiesterase-3 in smooth muscle cells[302]. Cilostazol, a phosphodiesterase-3 inhibitor clinically approved for peripheral vascular disease (and an UPS activator), restored vascular reactivity, increased perivascular drainage of Aβ and promoted cognitive performance in a mouse model of cerebral β-amyloidogenesis[302]. Intriguingly, a retrospective clinical analysis suggested that cilostazol (added onto dozenapil) abrogates cognitive decline in patients with modest dementia[303]. Adrenergic mechanisms influence ISF volume and hence neurotoxic protein clearance[274], and additional pharmacological opportunities for promoting glymphatic efflux will likely emerge from an improved understanding of its regulation by astrocytic, neurotransmitter and other mechanisms[269,272,274].

Disruption of sleep impedes glymphatic clearance of neurotoxic proteins, so encouraging sleep hygiene should promote CSF/ISF transfer to the periphery[274,275]. The atypical antidepressant and sleep-promoting agent, trazodone, is of interest since it normalized an over-protracted UPR and accordingly reversed pathology in animal models of tauopathies (**Suppl Box 2**)[99]. Other therapies that favour sleep in NDAs may improve glymphatic clearance of proteotoxic substrates and hence abate disease progression[265,269,279]. **Interestingly, alcohol displays a J-shaped curve, with low/high consumption respectively enhancing/reducing glymphatic function - and moderating/aggravating the risk of dementia[304].**

Finally, in a recent study in human subjects, peritoneal dialysis cleared peripheral Aβ from the circulation, while parallel experiments in APP/PS1 mice showed that peritoneal dialysis reduced ISF and brain Aβ load and ameliorated behavioural deficits[305].

Therapeutic perspectives and open questions

Accumulation of neurotoxic proteins unquestionably contributes to the onset and progression of NDAs. Accordingly, agents that promote their elimination are attractive as potential therapeutic agents. Nonetheless, several issues remain to be resolved prior to successful and safe clinical exploitation.

First, improved knowledge of the causes, characteristics and chronology of poor clearance in NDAs, and of similarities and differences amongst them, would be important for clarifying which therapeutic strategy is best adapted to the treatment of specific classes of NDA and subsets of patients. This would also help determine the optimal mode, timing, pattern and dosage of treatment[4].

Second, it is important to better understand the interplay between neurotoxic protein clearance and other pathophysiological processes, such as neuroinflammation. Moreover, hub proteins like AMPK, mTORC1 and Sirtuin-1 impact both the ALN and manifold other processes

implicated in NDAs, such as epigenetic regulation and energy homeostasis[21,24,25,306,307]. Hence, drugs that modulate their activity may have beneficial and/or deleterious actions *beyond* their influence on clearance. Indeed, potential side-effects should not be ignored. This is exemplified by mTORC1 antagonists like rapamycin which possesses immune-suppressive actions and affect memory formation, although studies in oncology and neurodevelopmental disorders are reassuring[5,307].

Third, numerous mechanisms remain to be pharmacologically harnessed. These include receptor tyrosine kinases for the ALN and "upstream" GPCRs *potentially* for all modes of elimination[26,27,29]. For the ALN, additional targets include the Vps34 complex, histone deacetylase-6[3], Rab proteins implicated in autophagosome-lysosome fusion[186] and v-ATPase, crucial for lysosomal acidification[40]. There has been much recent progress towards manipulation of the UPS, whereas exploitation of the CMA remains a major challenge[2,3,45-47,68,80]. For certain targets, non-small molecule strategies like PROTACS, aptamers and RNA probes, as well as nanoparticles and nucleic acid-based therapeutics, may prove useful (**Box 3**). Novel technologies will also be of importance for achieving the specific clearance of neurotoxic *vs* "normal" proteins, and for directing actions to discrete cells and brain regions, like dopaminergic pathways in PD[8,45]. Further research is needed to confirm, clarify and potentially exploit the role of glymphatic clearance in the elimination of neurotoxic proteins in NDAs[308]. Another line of research could focus on the blood-*CSF*-barrier which bears parallels and differences to the BBB, is impacted in ageing, and also represents a potential site for acceleration of neurotoxic protein elimination: its contribution to clearance of Aβ42 is diminished in AD[269,272,309,310]. [309,310] [272].

Fourth, to improve the preclinical characterization of candidate medicine, we need more refined cellular and animal models, including induced pluripotent stem cells from patients **(Box 1)**[1,3,4,10,23]. This will help to determine precisely which components of the ALN, CMA and UPS are impacted by specific classes of medication, and to quantify their influence on overall ALN flux. improved models should also help determine the influence of therapeutic agents on clearance in discrete classes of neuron in comparison to astrocytes and microglia, which may well require contrasting modes of manipulation. **Improved models and measures should also facilitate the development of translational readouts for clinical trials. Studies of the multi-functional ALN promoter and aggregation inhibitor, methylene blue, exemplify challenges faced in patient selection, trial design, dose-response relationships, readouts of efficacy and optimal time of intervention (Suppl Table 1).**

Fifth, improved clearance may well have a broad therapeutic time-window, yet *early* treatment would be advantageous, especially as regards reinforcement of the UPS and CMA

before aggregation predominates. Hence, reliable biomarkers of clearance will be important for detecting pre-symptomatic subjects for early intervention[81,311]. Biomarkers are likewise crucial for demonstration of target engagement and as surrogate signals of disease-slowing and long-term efficacy. While we cannot directly monitor ALN, CMA or UPS in human brain, quantification of CSF and plasma levels of neurotoxic proteins like Aβ42 and tau is instructive. Further, imaging of neurotoxic protein load is helping enrollment of subjects into clinical trials[311]. In addition, retinal imaging offers a window on cerebral clearance of tau[312] while biomarkers of neurovascular flow from the brain to the circulation are under development[265,275].

Sixth, the therapeutic strategies evoked herein are pertinent to other classes of NDA. For example, Machado-Joseph disease (spinocerebellar ataxia type-3) is an autosomal-dominant, polyglutamine disease provoked by over-repetition of a CAG sequence in the ataxin3 gene. The mutant protein destabilizes beclin 1[94]. Accordingly, studies in transgenic mice and fibroblasts from patients suggest that reinforcing beclin 1-dependent ALN flux would be beneficial[313,314]. Blockade of mTOR1 to induce autophagy (and the UPS) may likewise be useful.

Finally, reinforcing clearance might best be undertaken in association with other strategies like suppression of protein misfolding, amelioration of cerebral energetics, or moderation of neuroinflammation[2,3,7,25,164,181,]. Drug associations or multi-target agents possessing complementary mechanisms of action are both viable options. In addition, medication for promoting neurotoxic protein clearance will likely prove most effective when used in conjunction with lifestyle changes like improved sleep hygiene, exercise and a healthy diet.

Concluding comments

An excessive neurotoxic protein load is a core pathophysiological feature underlying and driving NDAs. Amongst several potential strategies for alleviating this burden, an enhancement of clearance is particularly attractive in view of the range of options available, and because insufficent elimination is itself implicated in the pathogenesis of NDAs. While challenges remain, ALN, CMA, UPS, proteolytic, neurovascular and lymphatic mechanisms of clearance offer potentially important strategies for preventing the onset and progression of diverse classes of NDA. Intensive work in this field will hopefully soon be translated into clinical benefits for patients.

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Glossary

Neurodegenerative disorders of ageing (NDA): A suite of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and frontotemporal dementia that typically are diagnosed in the elderly. Most cases are sporadic, but rare forms are associated with mutations (**Table 1**). Huntington's disease is an exception in being purely genetic and having a somewhat earlier onset at 30-50 years of age.

Proteinopathy: General term for disorders characterised by the buildup of excess, anomalously-marked, misfolded and/or aggregated neurotoxic proteins like Aβ, tau or α-synuclein.

Amyloidβ: The major neurotoxic product of APP processing, including amyloidβ42, that deposits into extracellular plaques in Alzheimer's disease. It is toxic as a soluble monomer or low-order oligomers by, for example, disrupting synaptic transmission, damaging mitochondria and impeding proteosomal clearance.

Tau: A protein that stabilizes axonal microtubules. It is prone to cleavage, hyperphosphorylation and other modifications that trigger and/or follow microtubule dissociation. This leads to misfolding, oligomerisation, synaptic mislocalization and inter-neuronal spreading. Aggregates, fibrils and initracellular neurofibrillary tangles are also formed.

α-Synuclein: A phospholipid-binding protein abundant in pre-synaptic terminals and involved in the release and regulation of synaptic vesicles. α-synuclein is a major component of Lewy bodies (protein and lipid aggregates) in PD. Its spread and accumulation in dopaminergic cell bodies and other cell types is a typical feature of the disease.

TAR DNA Protein-43: A normally nuclear protein that is associated with FTD and ALS. In these diseases, it is found in the cytoplasm where it aggregates.

Glymphatic System: CSF-driven mechanism for flushing extracellular pools of neurotoxic protein into the circulation: it involves perivascular drainage, astrocytes and the lymph system.

Blood-brain barrier: Physical and functional barrier that isolates the brain from the rest of the body. Certain nutrients, lipid vesicles and small molecules enter, yet it excludes toxic elements

that may damage the brain. It also ejects neurotoxic proteins and other unwanted material. Active transfer of neurotoxic proteins from the brain to the periphery involves specific classes of receptor and transporter.

Aggresomes: Microtubule-associated inclusions located in the perinuclear region that contain mainly oligomeric, aggregated and ubiquitinated neurotoxic proteins together with p62 and chaperones that aid in their formation. Often generated when UPS activity is insufficient. Protective when short-lived, yet may be harmful in the long-term and can morph into Lewy bodies in PD. Cleared by the ALN.

Stress granules: Non-membrane enclosed, cytoplasmic agglomerates of ribonucleoproteins that store and protect mRNA during short-term cellular stress. Chaperones like Hsp70 are involved in assembly and unfolding. In NDAs, neurotoxic proteins prolong the presence of stress granules and decrease their solubility, leading to aggregation or transformation into aggresomes.

Peroxisomes: Small (100nm-1μM) organelles which oxidize long-chain fatty acids and aid in detoxification. They can be generated by budding-off the endoplasmic reticulum and replicate *via* fission. Pexophagy refers to the autophagy of peroxisomes.

Lysosomes: An acidic compartment for the degradation of proteins and other cellular constituents. Their breakdown yields products like amino acids, sugards and lipids which are recycled. Christian de Duve received the Nobel Prize in Physiology or Medicine for their discovery in 1974.

Autophagy-related genes: Genes and the molecular machinary for autophagy were characterised in yeast by Y. Ohsumi (Nobel prize in Physiology or Medicine, 2016) and others. The associated genes, identified using mutants, were originally termed Apg1-15, yet Atg is now used. In view of conservation across species, this terminology is used for genes/proteins that regulate autophagy in humans as well.

AMP-kinase: 5'-adenosine monophosphate-activated protein kinase, an enzyme involved in energy and nutrient sensing. When activated, AMPK triggers glucose uptake, lipogenesis and triglyceride synthesis. It is a major protein for sensing ATP deficits and initiating the autophagic-lysosomal network.

Mammalian target of rapamycin: Multi-tasking serine/threonine protein kinase that inhibits autophagy, mitophagy and proteosomal degradation. It also has other roles in, for example, controlling mRNA translation and protein synthesis. Comprises part of a complex (mTORC1) together with several other regulatory and effector proteins.

Nicotinamide adenine dinucleotide: Dinucleotide co-enzyme necessary for energy generation in all types of cell. It is a co-factor for activation of Sirtuin-1, and is required for operation of the ALN. The oxidised and active form is NAD^+.

Acetyl coenzyme A: Cofactor involved in protein, carbohydrate and lipid metabolism. it is formed during glycolysis. It provides the acetyl used by acetyl transferases like p300 to acetylate Agt proteins, histones and other substrates like tau.

Rab proteins: Members of the Ras superfamily of monomeric G-proteins that participate in vesicular trafficking, vesicle formation, vesicle movement (actin/tubulin-mediated) and vesicular fusion, as in autophagosomal fusion with lysosomes.

SNARE: SNARE (Soluble N-ethylmaleamide-sensitive factor Attachment protein REceptor) refers to a complex of proteins including Synaptobrevin, Syntaxin, “SNAP-25” and Synaptogamin. SNARE contributes to vesicle fusion by “zippering” a donor vesicle (like an autophagosome) onto the recipient compartment (like the lysosome).

Phospholipase D: Enzyme involved in the transformation of various lipids: it participates in the fusion of autophagosomes with lysosomes.

Lysosomal storage disorders: Diseases resulting from genetic mutations that lead to failure of lysosomal digestion and consequent accumulation of lipids, proteins and other non-digested material. Pathology not restricted to the brain. Age of onset much earlier than for sporadic, age-related neurodegenerative disorders.

Niemann-Pick Type C disease: Lysosomal storage disorder triggered by a defect in the NPC1 gene responsible for cholesterol transport. Patients often display $A\beta 42$ and tau pathology, underpinning parallels to AD in which cholesterol transport is likewise disrupted.

Hsc70: Hsc70 (Heat shock cognate 70kDa protein) is a constitutively-expressed chaperone also known as Heat Shock Protein Family A member 8 which effects ATP-dependent nascent/unfolded protein folding. It specifically recognizes proteins with an exposed KFERQ-like sequence and delivers them to LAMP2A on lysosomes where, aided by other proteins, substrates are translocated to the lumen for degradation by CMA.

KFERQ: The KFERQ motif on a protein is the principal criterion for capture followed by CMA. Q refers to glutamine - although this sometimes may be an asparagine (N). The other residues are acidic (D), basic (K, R) or basic/hydrophobic (F). There are, however, variations and post-translational modification can modify susceptibility of proteins bearing a KFERQ signal for CMA.

Lipofuscin: Pigmented cellular inclusion composed of undigested lysosomal contents, including oxidised and cross-linked proteins. This electron-dense, autofluorescent material is characteristic of ageing and NDAs, and can be seen in all types of cerebral cell.

Unfolded protein response (UPR): Protective response to help cells recover from cellular and ER stress. Acts *via* three key effector proteins to modify gene transcription/mRNA translation. The UPR interrupts bulk protein synthesis, promotes the generation of chaperones for protein folding, and increases degradation of misfolded proteins. Over-activation and protracted engagement of the UPR is harmful for neurons and implicated in NDAs.

ALN dysfunction: *Underactive autophagy* - term used when rates of autophagosome formation and cargo sequestration decrease below basal levels, or fail to upregulate sufficiently under stress. *Impaired autophagy* - lysosomal delivery, fusion or digestion of autophagosomes is compromised. *Overactive autophagy* - over-production of autophagosomes and excess ALN activity: can lead to autosis.

Autosis: Autophagy-mediated cell death mediated principally by the Na^+/K^+-ATPase pump. Can occur with prolonged and excessive autophagy. Triggered by hypoxia-ischemia (as in stroke or traumatic brain injury) but occurrence in NDAs is uncertain.

Apolipoprotein Epsilon 4 (ApoE4): Robust genetic risk factor for AD *vs* the more common ApoE2 and E3 alleles. ApoE is secreted by astrocytes and binds lipids like cholesterol which are carried to neurons. Also involved in transport of cholesterol-bound Aβ to the blood-brain barrier (ApoE4 *less* efficient than 2/3), and in driving synthesis of Aβ42 (ApoE4 *more* potent than 2/3).

Presenilin-1: Catalytic unit of the γ-secretase complex that processes APP into β-amyloid. Mutations are associated with familial AD, and in part reflect altered APP processing. In addition, reduced lysosomal acidification and ALN function may be involved due to mutant Presenilin-1 driven deficits in maturation and translocation of vATPase subunits to the lysosome.

Amyloid precursor protein: Transmembrane protein highly expressed in neurons and involved in maintaining cell-cell contact. Successive cleavage by β- and γ-secretases results in the formation of APP terminal fragments like C99, as well as Aβ42 and related species of neurotoxic peptide.

Parkin: Component of the E3 ubiquitin ligase complex that binds to its partner PINK1 to facilitate the autophagic removal of dysfunctional mitochondria that have lost their membrane potential.

Gaucher's disease: Primary, autosomal-recessive lysosomal storage disease caused by mutations in the GBA1 gene which encodes β-glucocerebrosidase: 5-fold higher risk for PD in affected carriers. The activity of β-glucocerebrosidase is impaired in a sub-population of non-familial PD patients, many of whom show genetic mutations related to lysosomal disruption.

Superoxide dismutase (SOD1): Mitochondrial enzyme dedicated to the reduction of free radicals (reactive oxygen species). SOD1 mutations and dysfunction are seen in a subset of patients with amyotrophic lateral sclerosis.

CAG-expansion repeats: Proteins containing multiple CAG repeats - CAG encoding glutamine (symbol "Q"). When the number of CAG repeats is supra-normal (for example, >35 for Htt protein), proteins aggregate, provoke cellular damage and trigger inherited, polyglutamine (polyQ) diseases like Huntington's disease, spinocerebellar ataxia 3/Joseph-Machado disease (ataxin-3 protein), and spinal and bulbar muscular atrophy (androgen receptor protein).

TAT-beclin 1: A synthetic peptide comprising 11 amino acids of the Human Immunodeficiency Virus Tat protein transduction domain, a diglycine linker and a (commonly 11-mer) sequence derived from amino acids 267–284 of beclin 1. Cell-penetrant and triggers ALN-mediated neurotoxic protein clearance without causing cytotoxicity, although higher concentrations may carry the risk of autosis.

Heat Shock Factor 1 (HSF1): Protein that occurs as a monomer in the nucleus and cytoplasm, being repressed by heat shock proteins like Hsp70. Following disruption of proteostasis, heat shock proteins dissociate from HSF1 in order to aid protein-folding. HSF1 then trimerizes and acts as a transcription factor to increase production of Hsp70 and other neuroprotective proteins.

Exosome: Small (30-150nm), ceramide-rich vesicles formed from cytosolic endosomes, multivesicular bodies and lysosomes. Released with contents (proteins, lipids, sugars and nucleic acids) into extracellular space upon fusion with plasma membrane. Contribute to spread of neurotoxic proteins. Exosomes in CSF, blood and urine are stable and useful as biomarkers.

Immunotherapy: A “biological” therapy that passively or actively boosts the body's natural defenses. Specific classes of antibody aim to neutralise neurotoxic proteins like Aβ42 or tau. Entrance to the brain is limited, but they may also act as a peripheral sink for neurotoxic proteins in the circulation. In the brain, antibodies probably act for the most part extrinsically to neurons.

Box 1: Autophagic-lysosomal flux and its measurement: cellular and animal models

Characterisation of the ALN and its therapeutic restitution in NDAs necessitates accurate interpretation of autophagic states both *in vitro* and *in vivo*[10,23]. While electron microscopy has traditionally been used to observe key features of autophagosomes, recently-introduced approaches allow for more refined analysis of the ALN: for example, whether increases in autophagosome number (the most common measure undertaken) reflect an increase in their synthesis or, rather, decreased ALN flux[23].

Since membrane-bound LC3-II (called Atg8 in zebrafish) is covalently conjugated to phosphotidylethanolamine on the outer and inner autophagosomal membranes (**Figure 3**), its expression and localisation is widely used to track autophagic kinetics. Calculating the ratio of LC3-II to tubulin is a popular method for measuring cellular autophagosome levels by immunoblot[17]. Green fluorescent protein (GFP)-tagged LC3 has proven especially useful for quantifying autophagosomes**,** but self-aggregation of cytosolic GFP-LC3 and the quenching of GFP fluorescence in acidic lysosomes complicates interpretation in cytological assays[23]. To overcome GFP quenching, tandem constructs containing GFP and an acid-resistant red fluorescent protein (DsRed or mCherry) can be used to discriminate autophagosomes (and amphisomes) from autolysosomes (**Figure 3**). To show that increased levels of LC3-II genuinely represent accelerated ALN flux, it is useful to use compounds like bafilomycin A or chloroquine which neutralise lysosomal pH and produce an additive elevation in LC3-II levels under conditions where ALN flux is indeed high. Levels of p62 or other cargo acceptors are also useful readouts: a decrease in p62 often accompanies accelerated autophagic flux, while its accumulation may indicate a decrease. Potential variables that complicate this measure include proteasomal degradation of p62, alterations in transcription and reduced protein synthesis in degenerating cells[315]. Therefore, parallel monitoring of p62 mRNA levels and UPS status is recommended[229]. Phospho-specific antibodies that detect activation states of key autophagy-regulatory kinases like AMPK, mTORC1 and Ulk1 are also instructive indicators of ALN status.

As regards *in vivo* models, Zebrafish (*Dano rio*) larvae are transparent and permit visualization of ALN reporters like GFP-LC3-II constructs and neurotoxic proteins[316]. Further, targeted gene transduction, deletion or editing can easily be performed by morpholinos and the "CRISPR/Cas" system. Comparatively high-throughput screening can also be undertaken with compounds added to water that are absorbed transdermally[103]. For example, stimulating autophagy and TFEB nuclear translocation by trifluoperazine prevented neuronal loss in PINK1-deficient zebrafish[317]. Fruitflies (*Drosophila melanogaster*) are also useful. They can be rendered autophagy-deficient, resulting in spontaneous neurodegeneration, while restoration of autophagy

is neuroprotective in PINK1 mutants[318]. In addition, genetic tools are available for manipulating each step of ALN disruption, while somatic, mutant clones in subsets of *specific* neurons permit evaluation of ALN status in impacted cells surrounded by wild-type tissue[319]. *Drosophila* have also been used to validate the effects of drugs regulating the ALN: for example, rapamycin had beneficial effects in a polyglutamine model of HD[133]. Nonetheless, mice remain the most common, *in vivo,* pre-clinical model for modulation of the ALN in NDAs[23] and a broad range of pharmacological agents has been studied, as summarized in **Table 2**. Apart from the brain, retinal tissue has also proven instructive: for example, in evaluating axonal transport of acidic vesicles to lysosomes[312,320].

Finally, for *in vitro* and *in vivo* studies of the ALN, overexpression of mutant proteins associated with NDAs is often used as a model of proteinopathy burden. However, this may not faithfully recapitulate sporadic forms of disease and the importance of other factors influencing the ALN, like ER stress, the cytosolic and mitophagic UPR (Suppl Box 3) and diminished energy supply, should be borne in mind[25,57,58,99,321].

Box 2. Defective mitophagy and its restoration for treatment of NDAs

Mitochondria support the high energetic costs of a complex and dynamic neuronal architecture, synaptic transmission and, last but not least, operation of the ALN. Indeed mitochondrial function and the ALN are reciprocally interlinked. For example, generation of radical oxygen species and ATP depletion induce the ALN *via* AMPK which will, in turn, eliminate damaged mitochondria[21,322]. In fact, there are several quality control mechanisms that preserve healthy mitochondrial populations: fusion and fission cycles to redistribute mitochondrial content and isolate damaged mitochondria; chaperones for ensuring maturation and folding of mitochondrial proteins; proteases for degrading misfolded mitochondrial constituents; lysosome-dependent pathways for destruction of damaged mitochondria; and a specific mode of UPR that preserves mitochondrial proteostasis[57,255,323].

Mitophagy refers to a type of macroautophagy that leads to degradation of mitochondria (**Figure 2**)[9,70,323]. While crucial for many developmental programmes, mitophagy has a more generalized, protective role in preventing the accumulation of reactive oxygen species and the release of pro-apoptotic factors. Of particular significance to NDAs is a stress-responsive, mitochondrial degradation cascade co-regulated by two genes known to be mutated in familial PD: the mitochondrial kinase, PINK1 and the E3 ubiquitin ligase, Parkin[69,70]. This cascade, driven by PINK1-dependent activation of Parkin and ubiquitylation of proteins in dysfunctional mitochondria, is a well-characterised pathway of mitochondrial clearance, and studies using fluorescent reporter systems to track mitochondria in autophagosomes and lysosomes have highlighted its important role in neurons[324]. PINK1 may also clear damaged mitochondria independently of Parkin by recruiting autophagy receptors like optineurin: for example, in AD where PINK1 appears to be deficient[325].

Whether driven by the PINK1/Parkin system or other ubiquitin-dependent or independent mechanisms, mitophagy decreases with age. Further, while mitophagy may be compensatorily augmented at the onset of NDAs, in later phases it is generally disrupted[9,75,323]. There is a complex interplay between protein aggregation, mitochondrial dysfunction and mitophagy. Aggregation-prone proteins, such as Aβ, SOD-1 variants and α-synuclein are imported into mitochondria[326]. This may reflect an adaptive mechanism, using mitochondria to clear aggregates[255]. However, in the long run, aggregation-prone proteins like α-synuclein provoke mitochondrial dysfunction and block mitochondrial protein import. Stimulating mitophagy may, thus, improve both mitochondrial function and cytosolic proteostasis[58,255,326].

As for pharmacological approaches for promoting mitophagy in NDAs[327], certain are common to those inducing cytosolic autophagy. More specifically, several strategies aim to activate

PINK1/Parkin-driven mitophagy, for example, by the neo-substrate, kinetin triphosphate, which enhances PINK1 kinase activity[328]. Small-molecule transcriptional activators of Parkin have also been proposed[329]. Other approaches use iron chelators to induce PINK1/Parkin-independent mitophagy. The ubiquitin-specific deubiquitinase, USP30, negatively regulates the initiation of Parkin-mediated removal of damaged mitochondria: its structurally-distinct features compared with other deubiquitinases are encouraging interest as a Parkin-related drug target[227,330]**. Two other deubiquitinases, USP8 (delays Parkin binding to damaged mitochondria) and USP15 (suppresses Parkin-driven mitophagy) are also under scrunity as targets for promoting mitophagy in NDAs[217].**

The inner mitochondrial membrane protein, prohibitin-2, directly binds LC3-II to target ruptured mitochondria for degradation and is depleted in human PD brain[11]. Since Prohibitin-2 overexpression is protective in cellular models of PD, it is an interesting target for potential therapy[331]. Compounds that stabilise Nrf2 are also of interest, since Nrf2 triggers Parkin-independent mitophagy by a mechanism involving activation of p62[332]. Replenishment of nicotinamide, which declines with age[56], may promote mitochondrial clearance by activating Sirtuin-1 driven mitophagy[333]. Further, in promoting mitochondrial proteostasis, nicotinamide derivatives opposed the deposition of Aβ in cellular and mouse models of AD[58]. The plant flavanol, kaempferol, induces autophagy and exerts protective effects on mitochondria, for example against toxins triggering PD-like dysfunction. Its actions involve induction of Akt upstream of mTORC1[334]. Other natural compounds, such as urolithin A, promote mitophagy by mechanisms that remain to be determined[335]. Finally, lifestyle factors, like exercise and intermittent fasting, favour mitochondrial and neuronal health by a combination of mechanisms that include the stimulation of mitophagy[9,25,164,207].

Box 3: Novel, non-small molecule strategies for enhancing neurotoxic protein clearance

Classical ”small molecules” cannot explore all potentially-available chemical space and may not be suitable for some targets like protein-protein interfaces and lipids. They are also not ideal for discrete delivery to specific brain regions. Thus, it is important to outline a suite of novel, non-small molecule approaches for eliminating neurotoxic proteins in NDAs.

Protein-protein interactions like Beclin-Bcl2 can be disrupted by a “Tat” strategy that homes in on a unique peptide sequence in one protein partner, and incorporates the addition of a short, basic, arginine-rich sequence to improve cell penetrance. A Tat-Beclin 1 construct triggered autophagy and cleared polyglutamine expansion protein aggregates *in vitro*[174], **while also promoting long-term memory in rats**[336]**.**

Aptamers are small oligonucleotides that recognise specific proteins. They offer another chemically-distinctive strategy for modulating clearance. Using this technology, the de-ubiquitinase, USP14[49,217] could be inhibited to facilitate tau clearance[214]. Inhibiting ubiquitin carboxyl-terminal hydrolase37, another proteosome-linked de-ubiquitinase, may also facilitate proteasomal clearance of neurotoxic proteins[337]. Similarly, aptamers moderated the ALN burden by blocking the misfolding and oligomerisation of tau[338] and α-synuclein[339].

Numerous classes of miRNA are deregulated in NDAs[165], including an increase of miR-34a in AD which neutralizes mRNAs encoding Sirtuin-1 and TREM2[165]. Conversely, miR-132, which likewise interacts with Sirtuin-1, is down-regulated in AD[165]. Another example is the loss of miR-124 in a lesion model of PD[340]. Selective targeting of miRNAs in NDAs is becoming possible using modified oligonucleotides like antagomiRs, locked nucleic acids and miRNA sponges[165]. In addition, stabilized antisense oligonucleotides are showing promise not only for silencing miRNAs like miR-34, but also for knocking out or altering the aberrant splicing of specific neurotoxic/aggregating protein like tau, mutant Htt, CRorf72 and SOD1[341].

PROTACs permit *selective* proteosomal elimination of unwanted proteins. They are composed of two motifs joined by a linker: one recognises a specific protein like tau[236], whereas the other encodes an E3-ligase binding site[234]. This allows the target protein to be poly-ubiquitinated, captured and degraded by proteasomes (and the ALN): addition of TAT-like motifs can increase efficacy[234]. In the 3XTgAD mouse model, PROTACs moderated levels of tau in the cortex and hippocampus suggesting target engagement in key pathological regions[234]. Interestingly, PROTACs may also be useful for orienting proteins towards CMA since the E3-ligase binding site can be substituted by a “KFERQ” CMA-recognition motif. This approach was used to clear α-synuclein *in vitro*[233]. Smaller PROTAC variants offer improved stability, higher potency and better structure-activity relationships[342].

Restoring lysosomal acidification using poly(DL-lactide-co-glycolide) acidic nanoparticles proved neuroprotective in preclinical models of PD[343] Though they are poorly brain-penetrant, nanoparticles with improved pharmacokinetic profiles are being developed. Encouragingly, intranasal delivery reduced 6-hydroxydopamine-induced neurotoxicity in rats[344]. Another dimension of nanotechnology is represented by engineered nanorods which, when internalized by Hela cells, accelerated the ALN and cleared Htt aggregates in synergy with trehalose *via* a mTORC1/ERK-signalling pathway: *in vivo* actions and safety remain to be established[345].

One strategy for *locally* enhancing intracellular clearance is virally-produced gene delivery to the pathological site, avoiding autophagic induction in "healthy" areas[346]. A target protein might be expressed in restricted areas using neuronal-type-specific promoters, like the dopamine transporter in dopaminergic neurons[347]. Invasiveness of delivery is a drawback, but peripheral administration employing exosomes together with the use of focused ultrasound to favour local BBB passage may offer a solution[348]. The latter approach enhanced access of siRNA to the striatum for knocking down mutant Htt[300]. Further, localised clearance was achieved with striatal lentivirus transfer of the proteasome activator, "PA28γ", that binds the 20S subunit to form an immunoproteasome. It enhanced clearance and improved motor performance in an Htt mouse model[349]. Another example is provided by intranigral gene delivery of Beclin 1 or TFEB that stimulated the ALN and alleviated pathology in α-synuclein overexpressing mice[350].

Finally, recurrent exposure of mice to a non-invasive, 40Hz flicker regime that entrained GABA interneuron-driven oscillations in visual cortex reduced Aβ40/42 load: this resulted from a suppression of amyloidogenesis and a shift in microglial activation status leading to enhanced uptake and clearance[351].

Figure 1: Overview of intra and extracellular mechanisms for the clearance of neurotoxic proteins from the brain.

Neurotoxic proteins (NTPs) are eliminated by a broad suite of specific and non-specific mechanisms expressed in neurons, glial cells and endothelial/vascular smooth muscle cells of vessels. The three major modes of intracellular clearance are shown for neurons, but they are also active in other cells like microglia ("clearance"). Under conditions of inflammation, proteasomal β-subunits in glia are switched and substrate specificity changes: the precise role of these "immunoproteasomes" - specialized in peptide production for antigen presentation - for neurotoxic protein elimination in NDAs is debated[8]. Clearance also occurs in the extracellular space, the interstitial fluid (ISF) of the brain parenchyma that surrounds neurons, and the CSF with which the ISF exchanges. **Intraneuronal mechanisms of clearance are illustrated for NTPs in general, but only Aβ42 is shown for extracellular clearance, since it has generated the vast majority of currently available data. Extracellular pools of NTPs are derived from passive diffusion, active release from terminals, extrusion by exocytosis, and dispersion upon cell death.** NTPs disrupt neuronal and synaptic function and are taken up by other neurons and glial cells ("spreading"). Therapeutically-relevant proteases degrading NTPs include endothelin-converting enzyme and insulin degrading enzyme (IDE) (mainly cytosolic), neprilysin and matrix metalloproteinases (MMP) (intracellular and extracellular), and plasmin (mainly extracellular). NTPs that escape glial capture and proteases are driven into the circulation. *First*, blood-brain barrier (BBB) localised receptors and transporters actively eject them into the blood, including P-glycoproteins like "ABCB1" transporters and low-density lipoprotein receptor related protein 1 (LRP1). Conversely, the Receptor for Advanced Glycation End-product (RAGE) receptor returns Aβ into the CNS. Similar mechanisms operate at the blood-CSF-barrier in the choroid plexus; for example, LRP2 transfer of transthyretin-bound Aβ from CSF into blood. *Second*, transfer of NTPs to the periphery is mediated through the glymphatic system. CSF runs along the peri-arterial space, transverses Aquaporin 4 receptor-bearing circumvascular astrocytes to enter the ISF. Convective flow driven by arterial pulsing flushes NTPs *via* glial cells and the peri-venous space back into the CSF. Glymphatic-cleared, CSF-derived NTPs mainly reach the circulation mainly *via* the cervical lymph nodes, but also *via* the dural venous sinus. Within the blood, specific proteins sequester Aβ, such as the soluble fragment of LRP1 and immunoglobulins (IgG). NTPs are ultimately eliminated in the kidneys and liver. Abbreviation not in main text or above: s, soluble.

Figure 2: Overview of intracellular mechanisms for the elimination of neurotoxic proteins from neurons and other classes of cell in the brain.

Within neurons and other classes of cell, the UPS and CMA clear non-aggregated forms of neurotoxic protein, and the UPS also deals with substrates of Endoplasmic Reticulum Associated Degradation of incorrectly-folded proteins ("ERAD"). Proteins destined for the proteasome are poly-ubiquinated and guided to the proteasome by chaperones. They are deubiquinated by Rpn11 once committed to entering the proteosome pore: other deubiquitinases like USP14 may rescue them before entry[49]. Unfolding is followed by degradation. The CMA operates on proteins bearing a KFERQ-like motif. This sequence is found in, for example, tau but not Aβ. Hsc70 recognises the KFERQ sequence and, together with co-chaperones, transports the protein to the LAMP2A receptor on lysosomes: LAMP2A then coordinates protein translocation into the lumen. The ALN is the major system for removing misfolded, higher-order, aggregated proteins as well as damaged organelles. Autophagosomes bearing cargo fuse with acidic lysosomes leading to degradation of contents. In addition, some autophagosomes fuse with late endosome. The resultant amphisomes then likewise fuse with lysosomes. See also **Figure 3**. Abbreviation not in main text: Co-chap, co-chaperone; Lys, lysine and Ub, ubiquitin,

Figure 3 Organization, operation and regulation of the autophagic-lysosomal network

The top part of the schema illustrates the sequence of steps associated with operation of the ALN, while the bottom part shows the main regulatory proteins involved, focusing on potential targets for pharmacotherapy. "Sensing", both extrinsic (*e.g.* glucose levels) and intrinsic (*e.g*. ATP/AMP levels), can determine whether or not autophagy is initiated by activation of AMPK and/or inhibition of mTORC1 - which leads to TFEB-driven transcription of ALN-requisite proteins. The pre-autophagosome (phagophore) structure first emerges from diverse membrane sources, and its formation is promoted by Atg9 (not shown). Nucleation is accomplished with the help of a complex cluster of proteins. Phosphatidylinositol-3-phosphate (PtdIns3P) is recognised by WIPI (WD-repeat-protein-interacting-with-phosphoInositides) proteins that help induce autophagosome elongation in association with several classes of Atg protein and small GTPases like Rab5. With the aid of LC3 and cargo acceptors, autophagosomes take up cytoplasmic material like aggregated proteins and dysfunctional mitochondria (**Box 2**). Autophagosomes and other autophagic vesicles are transported with the help of dynactin and dynein along microtubules towards acidic lysosomes. Autophagosomes fuse with lysosomes containing resident hydrolases that degrade their contents into amino acids, sugars and lipids etc for

recycling. Exosomal release/secretion of neurotoxic proteins ("exocytosis)") may occur upon reduced ALN flux and accumulation of autophagosomes. For details, see main text. Abbreviations not in main text or Glossary: FIP, family interacting protein; HOPS; Homotypic fusion and protein sorting complex; NAD^+, nicotinamide adenine dinucleotide; PE, phosphoethanolamine; PI3K/Akt: phosphoinositol-3-kinase/atypical kinase and PLD, phospholipase D.

Figure 4: Major molecular sites of action of agents that enhance neurotoxic protein clearance in NDAs

Representative agents are shown for diverse modes of intracellular (ALN and UPS), extracellular (immunotherapy and protease-driven) and vascular (BBB extrusion and glymphatic) clearance. The principal loci of drug actions are depicted, yet precise mechanisms of action remain to be more fully deciphered for many drugs while several agents like resveratrol act at *multiple* sites (main text). As illustrated, a broad range of drugs exert their actions *via* AMPK, mTORC1 or sirtuin-1 (which also influences downstream events like autophagosome formation). Certain agents exert their effects *via* other components of the ALN, up to and including lysosomal catabolism. In addition, ambroxol acts as a chaperone to help transport β–glucocerebrosidase to lysosomes. Diverse class of agent likewise promote UPS activity, including chaperones that assist in protein refolding and triage, modulators of proteasomal phosphorylation, and agents acting *via* the transcription factor, Nrf2, to induce coordinated synthesis of proteasomal subunits. Extraneuronal clearance of full-length, truncated, post-translationally-modified, monomeric and/or higher-order neurotoxic proteins can be promoted by: stimulating proteases like neprilysin; immunotherapies targeting specific neurotoxic proteins; and increasing BBB-mediated and glymphatic extrusion into the circulation. For details, see main text. Abbreviations not in main text or **Figure 3**: AT, acetyl transferase; DUB, deubiquitinase; GBA; β–glucocerebrosidase; G-synthase, glucoceramide synthase; PDE, phosphodiesterase; PKA/G, protein kinases A/G and RAR, retinoid acid receptor.

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Table 1: Neurodegenerative disorders of ageing: major clinical and pathophysiological features, disruption of proteostasis, and impairment of neurotoxic protein clearance.

Clearance mechanisms are recruited early in disease, yet eventually become dysfunctional and/or inadequate to cope with neurotoxic burden. Not all changes can be shown, and NDAs are associated with neuroinflammation/immune deregulation, glial anomalies, disruption of cerebral bioenergetics, mitochondrial dysfunction and ER/oxidative stress. Several variants of frontotemporal dementia (FTD) include behavioural, progressive non-fluent aphasia and semantic forms. ALS shares common pathological hallmarks and risk genes with FTD like C9orf72 (Chromosome 9 Open Reading Frame 72). This and other NDA-associated risk genes linked to impaired clearance, are indicated in column one. Examples of genes/proteins incriminated in pathological processes are given in columns 3-6. APOE4 (apolipoprotein E4); PARK9 (ATPase13A2); CHMP2B (chromatin-modifying protein 2B); DCTN1 (dynactin); FUS (Fused in sarcoma); GBA1 (β-glucocerebrosidase); GRN (progranulin); HTT (huntingtin); LRRK2 (leucine-rich repeat kinase 2); MAPT (microtubule association protein, tau) ; OPTN (optineurin); PARK2 (parkin); PICALM (phosphatidylinositol binding clathrin assembly protein); PINK1 (PTEN-induced putative kinase 1); PS (presenilin); SNCA (α–synuclein); SOD1 (superoxide dismutase 1); SQSTM1 (sequestome 1, p62); TBK1 (TANK-binding kinase 1); TARDBP (TAR DNA binding Protein 43); TMEM106, transmembrane Protein 106B; TREM2 (triggering receptor expressed on myeloid cells 2); UBQLN2 (ubiquilin 2); UCH-L1, Ubiquitin carboxy-terminal hydrolase L1 (a deubiquitinase) and VCP (valosin-containing protein). Aβ refers to Aβ42 and related neurotoxic fragments of APP. See text for further information and citations. Abbreviations not above or in text: FKBP, FK-binding protein; SNPC, substantia nigra, pars compacta and RBD, rapid eye movement sleep behavioural disorder.

Disease (age of onset) ***% Familial*** ***Main risk genes related to poor clearance***	**Clinical and pathophysiological phenotype**	**Disruption of proteostasis**	**Autophagic-lysosomal network impairment**	**Impairment of CMA and of the UPS**	**Impairment in other modes of neurotoxic protein clearance**
Alzheimer's (usually over 70) ca. 5% *APOE4, APP, PS1, PICALM, TREM2*	Cognitive deficits; psychiatric symptoms; disorganized language; disrupted sleep/circadian rhythms. Neurodegener-ation (entorhinal cortex, medial temporal lobe, hippocampus etc); ↓axonal transport; axonal and synaptic degeneration; altered microglial phenotype.	Aβ oligomers disrupt neurones, synapses, aggravate tau toxicity; Aβ aggregates in extra-cellular plaques/vessels; aberrant tau cleavage, post-translational marking, folding and oligomerisation; ↑tau release and spreading; intra-cellular tau tangles (with p62 and other Ub-proteins). α-syn neuropathology in subpopulation.	↓Sirtuin-1; ↓Neuronal ALN flux; ↓Autophagosome maturation, transport (MAPT) and fusion with lysosomes; ↓APP loading (PICALM); APP and C-terminal fragment accumulation in endo-lysosomes; ↓Lysosomal acidity and digestion (PS-1/2, APP ApoE4); ↓Glial ALN (TREM2, ApoE4). ↓Mitophagy (PS1).	**↓ CMA (disrupted by Aβ/tau aggregates); Anomalous mutant tau at LAMP2A impedes CMA; ↓ UPS clearance (perturbed by Aβ and tau oligomers); FKBP51 binds Hsp90 to interfere with UPS substrate loading.**	↓Proteolytic Aβ clearance (↓IDE, Neprilysin, Plasmin); ↓BBB clearance of Aβ and, probably, tau (↓LRP1; ↓P-glycoprotein; ↑RAGE); ↓Aβ provision to BBB (ApoE4); ↓glymphatic clearance of Aβ and, probably, tau.

Parkinson's (usually over 60) ca. 5-15% *SNCA, PINK1, GBA, PARK2, LRRK2, PARK9, UCH-L1*	Motor impairment (poor gait, rigidity, bradykinesia, tremor); ↓olfaction; gastrointestinal problems; cognitive deficits; pain; depression; prodromal RBD. Neuronal loss (Dopaminergic cells in SNPC etc).	α–Syn inclusions and Lewy Bodies (contain lipids, α–syn, Tau, other neurotoxic proteins, ubiquitin); ↑α-syn release and spreading in brain - possibly earlier, in gut. Tau neuropathology in subpopulation.	Many α–syn related anomalies of ALN: ATG9 mislocalisation; ↓Formation, maturation, axonal transport and lysosomal fusion of autophagosomes;↓Lysosomal function (LRRK2, PARK9, GBA); ↓beclin 1 (LRRK2); ↓Mitophagy (PINK1, PARK2).	**↓LAMP2A/Hsc70 levels; ↓ CMA activity (aggregated α-syn and mutant forms of α-syn and LRRK2 block);** Slow α-syn dissociation from LAMP2A.**↓UPS clearance (aggregates and mutant forms of α-syn block);** Impaired α-syn traffic to UPS (UCH-L1).	↓BBB α-syn clearance; likely ↓α-syn elimination by glymphatic system.
Frontotemporal dementia (~40-60) ca 10-15% *MAPT, C9ORF72, GRN, VCP, FUS, TARDBP, TREM2, CHMP2B, TMEM106, UBQLN2*	Cognitive impairment; altered personality; mood and language deficits; cell loss prominently in inferior frontal and anterior temporal cortices, asymmetrically or bilaterally.	Misfolded and aggregated forms of tau, TDP-43 and/or (more rarely) FUS; Often found with p62 and ubiquitin in inclusions.	Autophagosome accumulation; ↓Cargo loading into autophagosomes by p62; ↓Axonal autophagosome transport (MAPT); ↓Endosomal trafficking (CHMP2B); Lysosomal dysfunction (GRN, TMEM106); ↓Glial ALN flux (TREM2).	↓CMA and UPS clearance (impeded by aggregates of tau, TDP-43 and FUS); **poly-GA aggregates (caused by C9orf72 mutations) sequester and stall proteasomes**; p62 dysfunction.	Not well defined, but likely similarities to AD as regards altered BBB permeability and ↓ glymphatic flow.
Amyotrophic lateral sclerosis (~50-60) *ca 10%* *SOD1, TARDBP, FUS, C9ORF72, VCP, SQSTM1, UBQLN2, OPTN, TBK1, DCTN, GRN, TREM2*	Motor impairment (cramps, muscle weakness, spasticity); cognitive impairment; mood disturbances (especially late-phase); ventral horn motoneuron loss; brainstem and cortical neuron degeneration.	Misfolded and aggregated TDP-43 and (more rarely) SOD1 and FUS inclusions in brain, spinal cord and motoneurons; inclusions may contain ubiquitin and ubiquitin-ligases.	Mainly ↓ALN, but if cellular stress severe, high ALN may actually be detrimental; ↓Autophagosome maturation (C9ORF72); ↓Cargo loading (SQSTM1, UBQLN2, OPTN, TBK1); ↓Autophagosome retrograde transport (DCTN, C9ORF72); ↓Lysosomal function (CHMP2B/GRN); ↓Glial ALN flux (TREM2).	**Aggregated proteins block proteasome;** ↓Hsp70 and Hsp40; ↓ Provision of SOD1 and other proteins for UPS degradation (VCP); **↓ CMA clearance of TDP-43.**	BBB disruption; ↓glymphatic flow may impede efflux of neurotoxic proteins.

Huntington (~30-50) Inherited (ca. 8-10% = de novo mutations) *HTT*	Motor dysfunction (chorea, dystonia, slurred speech); cognitive impairment; sleep disturbances; basal ganglia neuron loss, especially striatal medium spinal neurons; disruption of corticostriatal pathway; failure of axonal transport.	Aggregates of mutant (excess CAG repeat number) Htt; mutant Htt inclusions with ubiquitin, beclin 1, mTOR1, p62 and other cargo-loading proteins; Mutant Htt and fragments of Htt are cytotoxic.	Mutant Htt poor substrate of and disrupts ALN - and mitophagy; interference with beclin-1; ↓Autophagosome formation and cargo recognition/loading; ↓Axonal transport of autophagosomes.	**Mutant Htt poor substrate of CMA and UPS; LAMP2A and Hsc70 initially upregulated, but CMA less efficient in late stages;** **Possible ↓ UPS (blocked by mutant forms of Htt?);** **↓Hsp70.**	BBB disruption due to accumulation of Htt, but role in Htt clearance uncertain; potential ↓glymphatic clearance to establish.

Table 2: Pharmacotherapeutic strategies for promoting intracellular clearance: actions in cellular and animal models of neurodegenerative disorders of aging.

↓Indicates reduced, and ↑ increased levels. Cell line/species is followed by drug action in procedure/model. SK-N-SH, its sub-line SH-SY5Y and M17 are human neuroblastoma cell lines, H4 is a human neuroglioma cell line, and RPE denotes human retinal pigmented cells. Pheochromocytoma-12 (PC12) and neuro 2a (N2a) are mouse neuroblastoma cell lines, while HT-22 is a mouse hippocampal cell line. Cells were transfected with mutant protein, treated with Aβ peptides, or exposed to cytotoxic stressors like serum deprivation, okadaic acid (phosphatase inhibitor), rotenone (mitochondrial complex I inhibitor), staurosporine (protein kinase A/C inhibitor), hydrogen peroxide (H_2O_2), lipopolysaccharide (pro-inflammatory) or prostaglandin J2 (neurotoxic). Mutant protein variants in superscripts: e.g., Syn^{A53T}. YFP signifies yellow-fluorescent protein tagged (fluoresce when oligomerised). For *in vivo* models, Table shows overexpression of mutant neurotoxic proteins, in some cases tagged with Green Fluorescent Protein (GFP) for visualization. Models employing transgenes and/or mutations (^{superscript}) listed as, e.g., R6/2-Htt^{150}. Transgenic models for polyglutamine disorders express pro-aggregant proteins bearing multiple CAG repeats. For example, the R6/2 HD mouse expresses exon 1 of the human HTT gene containing 144-150 CAG repeats. In a model of Joseph-Machado disease, mice overexpressed Ataxin 3(Q^{70})) with 70 CAG repeats. TDP43 and FUS (Fused in Sarcoma) refer to mice overexpressing these proteins as models for FTD and/or ALS. FLTD-U mice show Ubiquitin-inclusions upon TDP43 overexpression. The SOD1 mutant mouse, G93A, is a model of ALS. Tau (MAP gene)-based models related to FTD (and AD) include mice with P301L (JNPL3 line) or P301S (PS19 line) mutations. RTg4510 mice have regulatable tau (P301L) expression. HTau signifies overexpression of human, wild-type tau. Mouse models for AD are based on overexpression of Tau and/or APP (Swedish and Swedish/Indiana) mutations: Tg2576 mice overexpress mutant APP (isoform 695) with the Swedish mutation (KM670/671NL); J20, TgCRND8 and Tg19959 mice overexpress mutant APP with Swedish plus Indiana (V717F) mutations; APP/PS1 mice bear APP-Swedish plus PS1-L166P mutations; 3XTgAD mice contain 3 mutations (APP-Swedish, PS1-M146L and tau-P301L) and 5XFAD mice encode 3 APP mutations (Swedish, Florida and London) plus 2 PS1 mutations (M146L and L286V). Models for PD are overexpression of wild-type or mutant (A53T, A30P) human α–synuclein, in one case on a α-syn knockout background (*$SNCAKO^{tm1Nbm}$*). R275W is a mitophagy-linked Parkin (PARK2) mutant mouse. GBA (β–glucocerebrosidase) mice embrace lines with natural (N370S and L444P) and induced mutations (D409V). Lesion-based models of PD employed the dopaminergic neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), okadaic acid or H_2O_2. Abbreviations not above or in text: CaMKK2, Calmodulin Kinase Kinase 2; DA, dopaminergic; HAT, Histone acetyl transferase; MAP Kinase, Mitogen Activated Protein Kinase; MCI, Mild cognitive Impairment; PE, phosphotidylethanolamine; PrP, prion protein; PS, presenilin; and PtdIns, phosphatidyl-inositol-3-kinase.

Agent	Clinical indication (or other use), and mechanistic influence on clearance mechanisms		Influence on neurotoxic proteins: *In vitro* procedures	Influence on neurotoxic proteins: *In vivo models*
Autophagy activators: modulation of sensing, initiation and regulation				
AMPK facilitation **Clonidine, Rilmenidine**	Antihypertensives	α_2-adrenergic agonists/AC inhibition, ↓AC-AMP/↑AMPK	PC12: ↓α-syn (Syn^{A53T}) / ↓Htt (Htt^{Q74})[103]	Mice: ↓Htt, ↑motor function (Htt^{82Q})[104]

Calpastatin, Calpeptin	Investigational compounds (endogenous peptides)	Calpain inhibitors: ↑AMP/AMPK induction, ↓ cleavage Atg proteins	SK-N-SH: ↓Htt (Htt^{Q74})[103]	*Drosophila*: ↓Htt, ↓neurodegeneration (Htt^{Q46})[54] Mice: ↓Htt aggregates, ↑motor function ($Htt^{171\text{-}82Q}$)[54]; ↓motoneuron loss ($SOD1^{G93A}$)[107], ↓tauopathy (JNPL3-$MAPT^{P301L}$)[106]
AICAR	Experimental agent. Potential treatment for myocardial ischaemia	AMP analogue - allosteric inducer of AMPK	N2a: ↑AMPK[108]; Glia: ↓toxicity(Aβ/LPS)[109]; SH-SY5Y: ↓α-syn (wild-type protein)[110]	-
A-769662	Experimental agent	Allosteric AMPK inducer	Striatal neurones/mouse fibroblasts: ↑LC3 and p62, ↓mHtt and ↑cell viability[111]	-
Resveratrol	Polyphenol found in grapes etc (dietary supplement). Clinical evaluation in AD, MCI	CaMKK2 potentiator, upstream of AMPK; Upstream inducer of Sirtuin-1	N2a: ↑AMPK[108]**;** ↓Aβ (APP695)[114]; Cortical neurones: ↓Aβ (J20)[114]	*C. elegans*: ↓polyglutamine (Htt^{Q128})[115]; Mice: ↓Aβ (APP/PS1)[114]
Metformin	Antidiabetic. Clinical evaluation for MCI	AMPK activator	SH-SY5Y: ↓α–syn[110]; ↓tau phosphorylation[117], ↓Aβ toxicity[118]	Mice: ↓TH neuronal loss, ↑motor function (MPTP)[119]
Trehalose	Disaccharide. Abiotic stress protectant. Food-additive	Glucose transporter inhibitor, ↑AMP/AMPK activator	PC12 : ↓α–syn (A30P/A53T) / ↓Htt (Q74)[121]; Cortical neurones: ↓tau (Tau_{RD}ΔK280)[122]	Mice: SOD1 ($SOD1^{G93A}$)[120] ; ↓Htt (R6/2-Htt^{150Q})[124], ↓tauopathy (PS19-$MAPT^{P301S}$)[125], ↓Aβ (APP/PS1)[123]
Lithium	Mood stabiliser, anti-epileptic Evaluated in FTD and ALS	↓Inositol monophosphate, AMPK activator?	SK-N-SH: ↓Htt (Htt^{Q74})[126]	Mice: ↑survival ($SOD1^{G93A}$)[128]; ↓tau/filaments, ↑motor function, ↑autophagy (JNPL3)[127]
Methylene blue	Dye. Treatment of methemoglobinemia. Development for AD/FTD (various formulations)	AMPK activator, ↑beclin 1 (also inhibitor of tau aggregation)	HT-22: ↑AMPK, ↓cell death (serum deprivation)[102]; Organoypic Hippocampal Slice/Neurones: ↓tau (JNPL3, $MAPT^{P301L}$)[101]	Mice: ↓tau (JNPL3)[101]
Calcitriol (Vitamin D metabolite)	Treatment of Ca^{2++} deficiency.	CaMKK2 potentiator upstream of AMPK	–	Mice: ↓neurodegeneration (C57BL/6/MPTP)[129]
mTOR1 Inhibition **Rapamycin**	Macrolide. Immunosuppressant (organ transplants). Potential chemotherapy	mTOR1 inhibitor	PC12: ↓α–syn (MPTP)[130], ↓Htt (Htt^{Q74})[131] Cortical neurones: ↓FUS, ↓stress granule (FUS^{R521C})[132]	Drosophila: ↓Htt,↓neurodegeneration (Htt^{Q74})[133]; Mice: ↓Aβ/tau (3XTgAD)[136], ↓TDP43/p62 (FTLD-U/TDP43)[134] and ↓neuronal loss (MPTP)[135]

Temsirolimus	Renal cell carcinoma	mTOR1/2 inhibitor	SH-SY5Y: ↓hyperphosphorylated tau (okadaic acid)[137]	Mice : ↓tau (MAPTP301S)[137], ↓α-syn/ neuroprotection(MPTP)[138], ↓Ataxin3 (Ataxin3Q70)[139];↓Htt/ ↑motor skills (R6/2)[133]
Curcumin	Tumeric extract. Food colour. Dietary supplement. Clinically evaluated in MCI	Indirect mTOR1 repressor, p300 HAT inhibition causing Atg deactylation	SH-SY5Y: ↓α-syn aggregation (Syn^{A53T})[142,143]; DA neurones: ↑neuroprotection (rotenone)[141]	Mice: ↓Aβ aggregation (Tg2576)[146], ↓tau dimers (hTau)[145], ↓α-syn (GFP-Syn)[144]
Fisetin	Plant polyphenol. Anti-oxidant	mTOR1-dependent activator of TFEB	Cortical Neurones: ↓phospho-tau[149]	Mice: ↓Aβ (APP/PS1)[150]
Nilotinib	Resistant chronic myelogenous leukemia. Clinically evaluated in PD	C-Abl kinase inhibitor, upstream recruitment of mTOR1	M17: ↓TDP43 (GFP-TDP43)[154]	Mice: ↓α–syn, ↑motor function (Syn^{A53T})[153], ↓TDP43 (TDP43)[154]
Sirtuin1 facilitation **Nicotinamide**	Vitaminin in food. Treatment of niacin deficiency. Clinically evaluated in AD	Nicotinamide adenine dinucleotide precursor/sirtuin1 promoter, Atg deacetylation	Cortical Neurones: ↓Aβ toxicity (Aβ25-35/1-42)[158]	Mice: ↓Aβ and tau (3XTgAD)[159]
Cilostazol	Treatment of intermittent claudication. Platelet aggregation inhibitor.	Phosphodiesterase 3 inhibitor, Upstream recruiter of Sirtuin-1	N2a: ↓Aβ (APP_{SWE}); ↑AMPK, ↓mTOR1, ↑autophagosomes, ↑cathepsin B[108]	Mice: ↓Aβ, ↓phospho and acetylated-tau; ↑ cognition (icv Aβ25-35)[162,163]
Spermidine	Natural polyamine. Potential promoter of longevity	p300 HAT Inhibitor, Atg and Histone H3 deacetylator, ↑Beclin 1	Cortical Neurones/PC12: ↑survival, ↓toxicity (staurosporine)[168]	*Drosophila:* ↑motor function (α-syn) [170]; *C. elegans*: ↓α–syn toxicity (UAS-GAL4-α-syn)[170]; ↓TDP-43 (FTLD-U)[169]
Autophagy activators: enhanced autophagosome formation				
Isorhynchophylline	Plant alkaloid. Investigational compound	↑Beclin 1	DA Neurones/N2a: ↓α–syn (Syn^{WT}, Syn^{A53T}, Syn^{A30P})[175]	-
Auten-99	Investigational compound	↑ PtdnIns3P activity (*via* Jumpy phosphatase inhibition)	SH-SY5Y: ↑survival (H_2O_2)[181]	*Drosophila:* ↓neurodegeneration, ↓p62 ($Parkin^{R275W}$)[181]

Enhancers of autophagosome fusion/transport				
Paclitaxel, Epothilone D	Chemotherapy of several cancers (Paclitaxel). Potential treatment for cancer (Epothilone)	↑Cytoskeletal/microtubule transport of autophagosomes	SH-SY5Y: ↓Aβ-mediated cytoskeletal destabilization and ER stress (Aβ25-35)[182]	Mice: ↓tau (PS19, Tau^{P301S})[183]
Enhancers of lysosomal digestion				
2-Hydroxypropyl-β-cyclodextrin	Investigational compound. (binds cholesterol)	TFEB inducer; ↓endolysosomal cholesterol; ↓lysosomal pH; ↑ABCB1 transporters (astrocytes)	H4: ↓α-syn aggregates(α-syn-GFP)[195]; N2a: ↓Aβ (APP_{SWE})[173]	Mice: ↓tau, ↓Aβ plaques, ↑memory (Tg19959/CRND8)[173]
Clioquinol	Anti-fungal, anti-protozoal drug	Zinc (and iron) chelator; Increased lysosomal acidification.	Fibroblasts: ↓α–syn(ATP13a2/PARK9 knockdown)[78]	Mice: ↓Aβ(Tg2576)[197]
GZ/667161, GZ/SAR402671	Investigational compounds, Clinically evaluated in PD	Inhibitors of glucosylceramide synthesis, substrate reducers	-	Mice: ↓α-syn/ubiquitin/tau, ↑memory(GBA^{D409V})[199]
Miglustat	Gaucher's disease, Niemann-Pick Type C1 disease	Inhibitor of glucosylceramide synthesis, substrate reducer	Mesencephalic Neurones: ↓lipid accumulation in lysosomes (MPTP+ conditural-β–epoxide)[75]	Mice: ↓substrate storage, ↑longevity (MPTP)[75]
Ambroxol	Secretolytic for respiratory diseases. Clinically evaluated in PD and Gaucher's disease	Chaperone: aids β–glucocerebrosidase transport to lysosome	Dopaminergic Neurons: ↓α-syn (GBA^{N370S})[200]	*Drosophila*: ↓ER stress ($GBA^{N370S,L444P}$)[201]; Mice:↓α-syn (*$SNCAXSNCAKO^{tm1Nbm}$*)[202]
NCGC607	Salicyclic acid derivative. Investigational compound	Chaperone: aids transport of β–glucocerebrosidase to lysosome - no catalytic inhibition	Dopaminergic neurons from Gaucher's patients: ↓glycolipids, ↓α-syn ($GBA^{N370S+/+}$, $GBA^{N370S/c.84dupG}$)[203]	-
HEP14	Investigational compound	Protein Kinase C-mediated TFEB activation and possibly ZKSCAN3 inhibition	-	Mice: ↓Aβ(APP/PS1)[151]
Facilitators of proteosomal (UPS-mediated) degradation				
Arimoclomol	Niemann-Pick Type C1 disease. Clinical evaluation for ALS	Heat Shock Factor 1 stabilizer, ↑Hsp70 chaperone production	Motoneurones: ↑survival (staurosporine, H_2O_2)[211]	Mice: ↓SOD1, ↓motor loss, ↑longevity ($SOD1^{G93A}$)[212]

IU1/IU1-47	Investigational compounds	USP14 (deubiquitinase) inhibitors	Cortical Neurones: ↓tau, Ub-proteins (Prostaglandin J2)[215]; ↑tau degradation and ↑ALN flux[216]	-
Geldanamycin	Antibiotic. Potential anti-tumorigenic	Hsp90 inhibitor ↑Hsp70 chaperone activity	M17: ↓tau (tau transfected)[219]; H4: ↓α-syn (α-syn-YFP complementation)[220]	Drosophila: ↓ α-syn (α-synA306/504) [202] Drosophila: ↓insoluble (Htt^{Q93})[222]; Mice: ↓tau (JNPL3)[219]
17-AAG	Investigational compound. Potential anti-tumorigenic	Hsp90 inhibitor (improved brain entry), ↑Hsp70 chaperone activity	H4: ↓α-syn oligomers (α-syn-YFP complementation)[220]	Mice: ↓ Aβ and ↓synaptic toxicity/memory impairment (Tg2576)[223,224], ↓tau (JNP3L)[224]
HSP990	Investigational compound	Hsp90 inhibitor, HSF1 promoter, ↑Hsp70 chaperone activity	-	Mice: ↓Htt aggregates, ↑motor performance (R6/2)[225]
Rolipram	Investigational compound. Potential use in auto-immune disorders	Phosphodiesterase inhibitor, ↑Protein Kinase A-mediated proteasome phosphorylation	Cortical Neurones: ↓Aβ/α-syn synaptic damage (human brain extract)[228]	Mice: ↓tau, ↓ubiquitin, ↑improved cognition (rTg4510, JNPL3)[229]
PD169316	Investigational compound	p38 MAPK inhibitor, ↓p38 MAPK proteasome phosphorylation	↓α-syn (wild-type protein)[233]	-

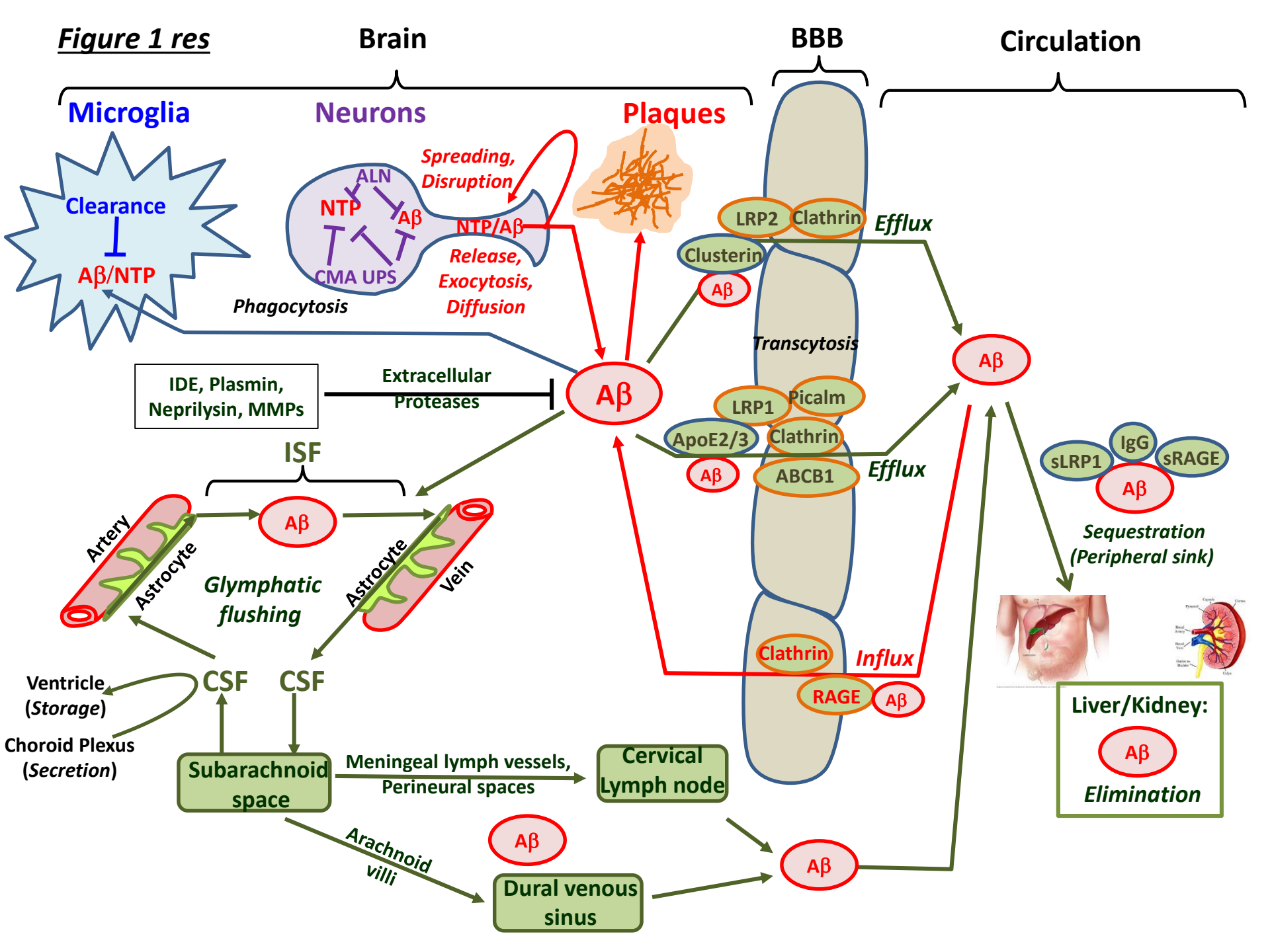


Figure 1 res
Brain
BBB
Circulation
Microglia
Neurons
Plaques
Clearance
Aβ/NTP
ALN
NTP
Aβ
CMA UPS
NTP/Aβ
Spreading, Disruption
Release, Exocytosis, Diffusion
Phagocytosis
LRP2
Clathrin
Efflux
Clusterin
Aβ
Transcytosis
IDE, Plasmin, Neprilysin, MMPs
Extracellular Proteases
Aβ
LRP1
Picalm
ApoE2/3
Clathrin
ABCB1
Efflux
ISF
Artery
Astrocyte
Aβ
Glymphatic flushing
Astrocyte
Vein
sLRP1
IgG
sRAGE
Aβ
Sequestration (Peripheral sink)
Clathrin
Influx
RAGE
Aβ
Liver/Kidney:
Aβ
Elimination
Ventricle (Storage)
Choroid Plexus (Secretion)
CSF
CSF
Subarachnoid space
Meningeal lymph vessels, Perineural spaces
Cervical Lymph node
Arachnoid villi
Aβ
Dural venous sinus
Aβ

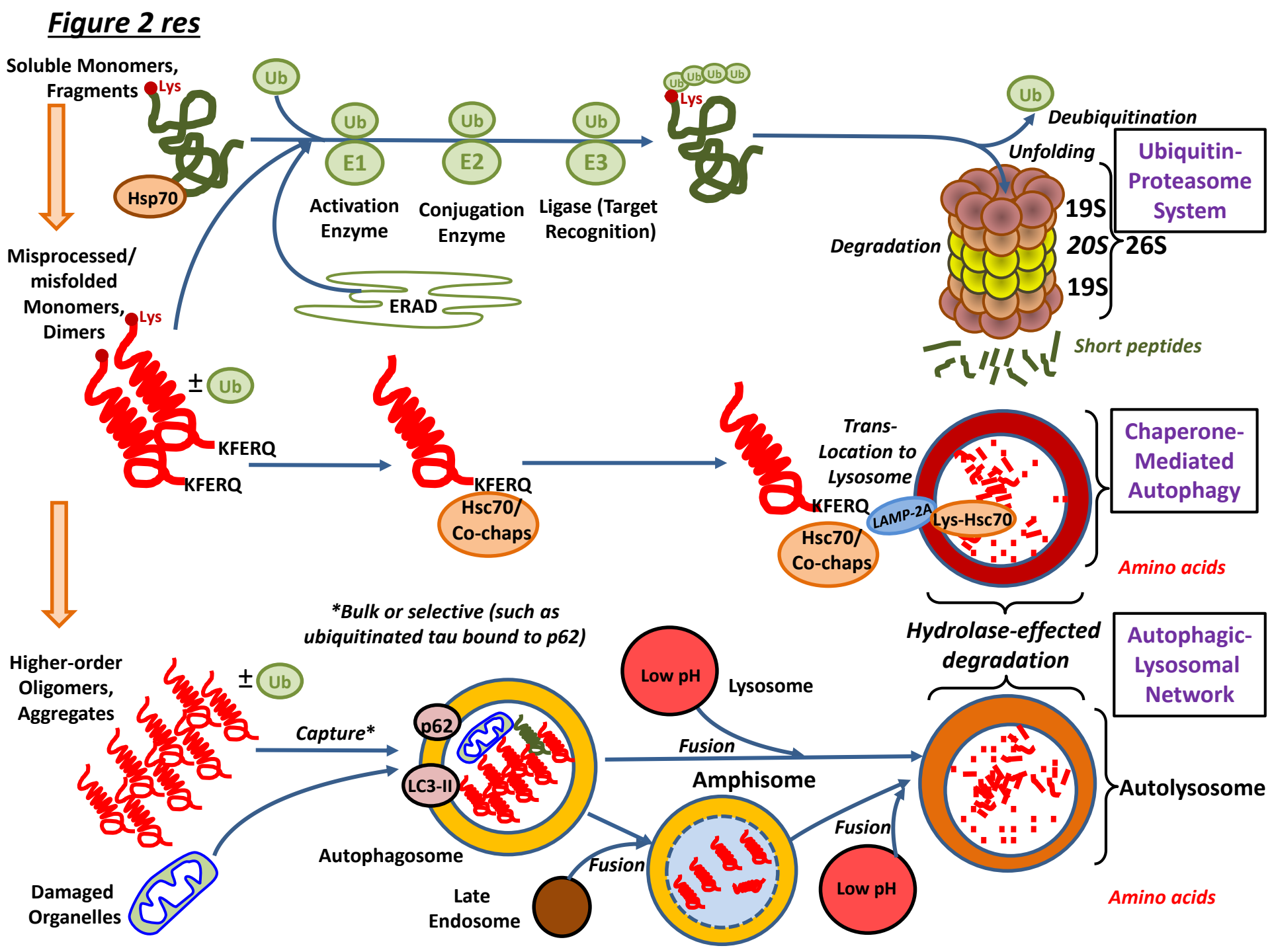
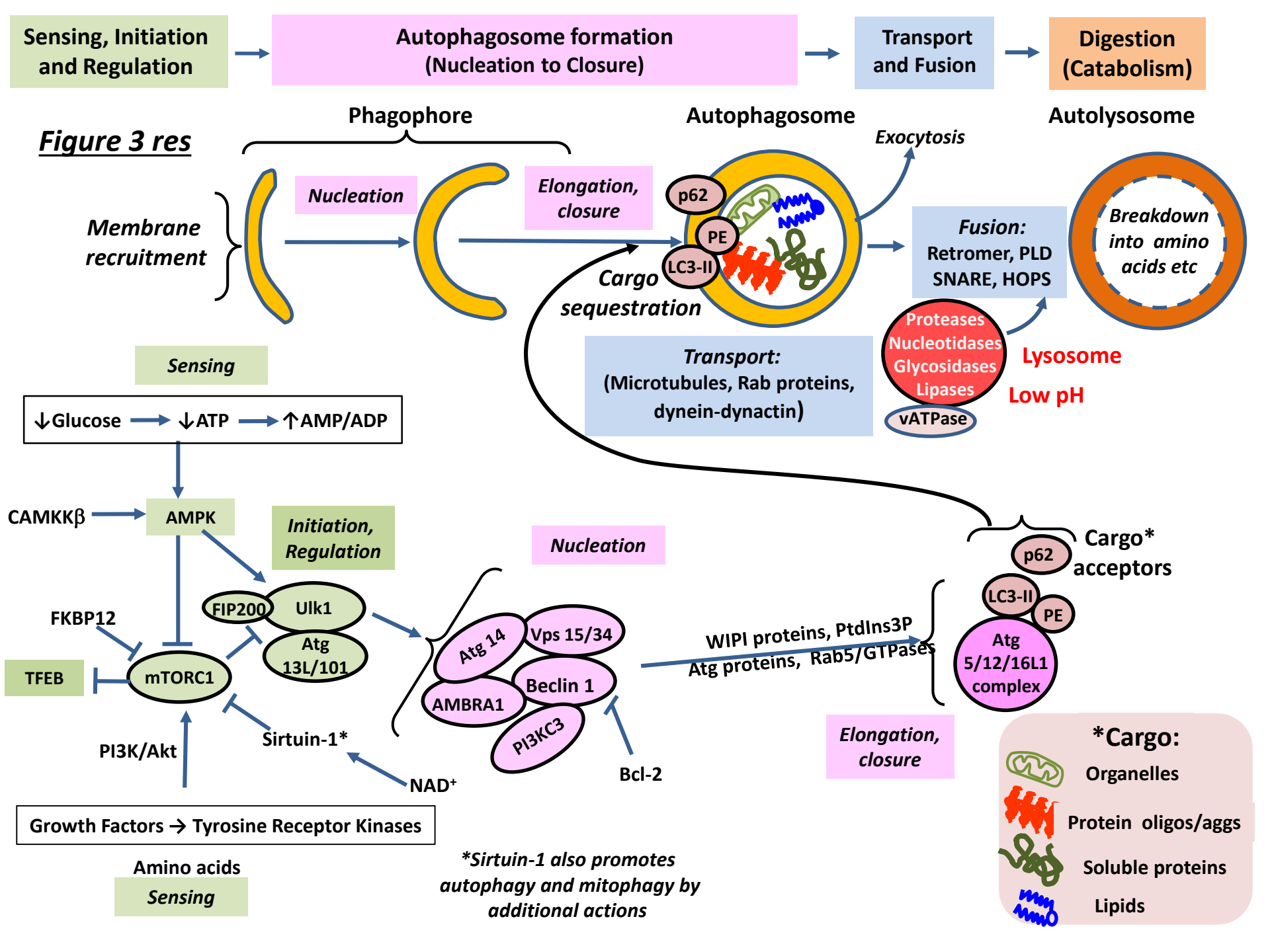
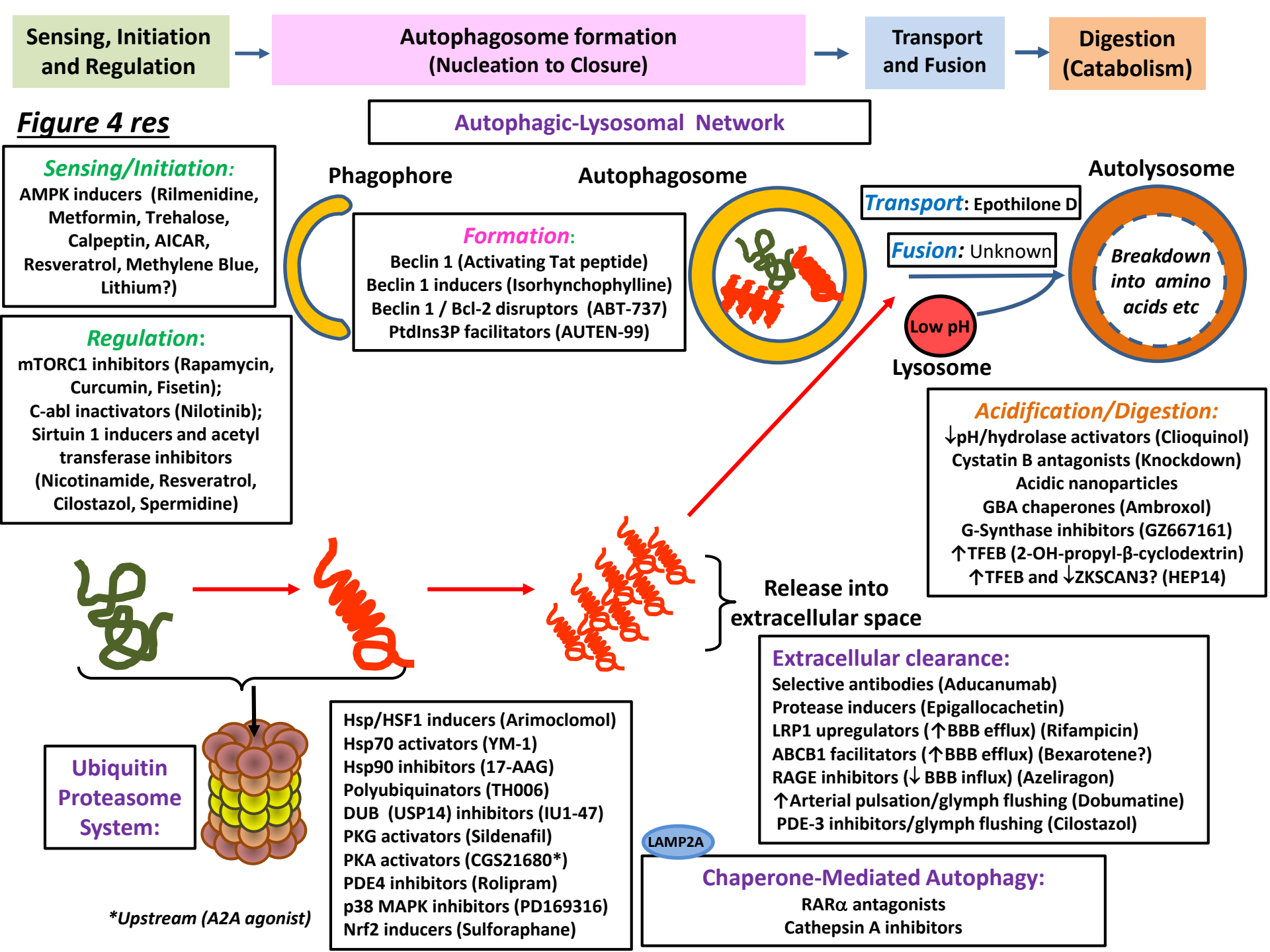


Figure 2 res
Soluble Monomers, Fragments
Lys
Hsp70
Ub
E1
E2
E3
Activation Enzyme
Conjugation Enzyme
Ligase (Target Recognition)
Deubiquitination
Unfolding
Ubiquitin-Proteasome System
19S
20S
26S
19S
Degradation
Short peptides
ERAD
Misprocessed/ misfolded Monomers, Dimers
± Ub
KFERQ
Hsc70/ Co-chaps
Trans-Location to Lysosome
LAMP-2A
Lys-Hsc70
Chaperone-Mediated Autophagy
Amino acids
*Bulk or selective (such as ubiquitinated tau bound to p62)
Higher-order Oligomers, Aggregates
Capture*
p62
LC3-II
Autophagosome
Low pH
Lysosome
Fusion
Amphisome
Late Endosome
Damaged Organelles
Hydrolase-effected degradation
Autophagic-Lysosomal Network
Autolysosome
Amino acids


Sensing, Initiation and Regulation
Autophagosome formation (Nucleation to Closure)
Transport and Fusion
Digestion (Catabolism)
Figure 3 res
Phagophore
Autophagosome
Autolysosome
Membrane recruitment
Nucleation
Elongation, closure
p62
PE
LC3-II
Exocytosis
Fusion: Retromer, PLD SNARE, HOPS
Breakdown into amino acids etc
Cargo sequestration
Proteases
Nucleotidases
Glycosidases
Lipases
Lysosome
Low pH
vATPase
Transport: (Microtubules, Rab proteins, dynein-dynactin)
Sensing
↓Glucose → ↓ATP → ↑AMP/ADP
CAMKKβ
AMPK
Initiation, Regulation
Nucleation
FKBP12
FIP200
Ulk1
Atg 13L/101
TFEB
mTORC1
Atg 14
Vps 15/34
Beclin 1
AMBRA1
PI3KC3
Bcl-2
WIPI proteins, PtdIns3P
Atg proteins, Rab5/GTPases
Cargo* acceptors
p62
LC3-II
PE
Atg 5/12/16L1 complex
Elongation, closure
PI3K/Akt
Sirtuin-1*
NAD+
Growth Factors → Tyrosine Receptor Kinases
Amino acids
Sensing
*Sirtuin-1 also promotes autophagy and mitophagy by additional actions
*Cargo:
Organelles
Protein oligos/aggs
Soluble proteins
Lipids


Sensing, Initiation and Regulation
Autophagosome formation (Nucleation to Closure)
Transport and Fusion
Digestion (Catabolism)
Figure 4 res
Autophagic-Lysosomal Network
Sensing/Initiation:
AMPK inducers (Rilmenidine, Metformin, Trehalose, Calpeptin, AICAR, Resveratrol, Methylene Blue, Lithium?)
Phagophore
Autophagosome
Autolysosome
Transport: Epothilone D
Fusion: Unknown
Breakdown into amino acids etc
Low pH
Lysosome
Formation:
Beclin 1 (Activating Tat peptide)
Beclin 1 inducers (Isorhynchophylline)
Beclin 1 / Bcl-2 disruptors (ABT-737)
PtdIns3P facilitators (AUTEN-99)
Regulation:
mTORC1 inhibitors (Rapamycin, Curcumin, Fisetin);
C-abl inactivators (Nilotinib);
Sirtuin 1 inducers and acetyl transferase inhibitors (Nicotinamide, Resveratrol, Cilostazol, Spermidine)
Acidification/Digestion:
↓pH/hydrolase activators (Clioquinol)
Cystatin B antagonists (Knockdown)
Acidic nanoparticles
GBA chaperones (Ambroxol)
G-Synthase inhibitors (GZ667161)
↑TFEB (2-OH-propyl-β-cyclodextrin)
↑TFEB and ↓ZKSCAN3? (HEP14)
Release into extracellular space
Extracellular clearance:
Selective antibodies (Aducanumab)
Protease inducers (Epigallocachetin)
LRP1 upregulators (↑BBB efflux) (Rifampicin)
ABCB1 facilitators (↑BBB efflux) (Bexarotene?)
RAGE inhibitors (↓ BBB influx) (Azeliragon)
↑Arterial pulsation/glymph flushing (Dobumatine)
PDE-3 inhibitors/glymph flushing (Cilostazol)
Ubiquitin Proteasome System:
Hsp/HSF1 inducers (Arimoclomol)
Hsp70 activators (YM-1)
Hsp90 inhibitors (17-AAG)
Polyubiquinators (TH006)
DUB (USP14) inhibitors (IU1-47)
PKG activators (Sildenafil)
PKA activators (CGS21680*)
PDE4 inhibitors (Rolipram)
p38 MAPK inhibitors (PD169316)
Nrf2 inducers (Sulforaphane)
*Upstream (A2A agonist)
LAMP2A
Chaperone-Mediated Autophagy:
RARα antagonists
Cathepsin A inhibitors

Suppl Table 1: Clinical trials undertaken in Neurodegenerative Disorders of Aging with drugs that experimentally modify the clearance of neurotoxic proteins.

The Table depicts those drugs affecting autophagic-lysosomal or ubiquitin-proteasomal clearance that have been, or are being, clinically evaluated for the treatment of neurodegenerative disorders of aging. The clinical trial identifier (with hyperlink to https://clinicaltrials.gov/ information) is shown, together with the phase of testing, doses under study (oral) and primary measures/readouts used. The drugs shown were not necessarily developed as modulators of neurotoxic protein elimination *per se* - for example, TRx0237[1]. However, based on experimental data, they are known to modulate clearance. In addition to the drugs and studies indicated in the Table, an open label investigation with rilmenidine was recently undertaken with a view of evaluating its efficacy in the treatment of Huntington's disease[2]. For mechanisms of drug action - which in several cases, like Lithium, are not entirely clear[3] - see main text and Table 2. While resveratrol did not reduce brain volume loss in the overall trial in AD and MCI[4,5] analysis of a small patient subset with CSF levels of Aβ1-42 of less than 600 ng/ml, provided evidence for a favourable influence on the blood-brain barrier (blocked leakage due to decreased levels of Matrix Metalloprotease 9), a reduction in immune-inflammatory markers, and a less marked decline in cognition and functional performance[5]. TRx0237 (LMTX or LMTM) is a new formulation of methylene blue (methylthioninium chloride) and a successor of Trx014 (Rember^{TM}). Further analysis of the AD trial suggested that it may have beneficial effects, notably on brain atrophy[1,6] but this has been disputed and another randomized trial would be needed to verify this post-hoc interpretation. Further, the focus is now largely on the anti-aggregation properties of Trx0237, so it is unclear to what extent induction of autophagy is involved in its clinical actions. Ironically, the only drug to have received FDA authorization is edaravone yet, as discussed in Suppl Box 3, edaravone may *reduce* ALN activity. However, this remains controversial and edaravone has other, therapeutically-useful actions like anti-oxidant and anti-aggregant properties. Abbreviations not in main text: ADAS-Cog, Alzheimer Disease Assessment Scale; ALSDRS-R, ALS Functional Rating Scale-Revised; CGIC, Clinician's Global Impression of Change; FDDNP-PET 2-(1-(6-[(2-[fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl)-ethylidene)malononitrile - Positron Emission Tomography; GBA, β-Glucocerebrosidase; MCI, Mild cognitive impairment; MOCS, Montreal Cognitive Score; MRI, Magnetic Resonance Imaging; NPI, Neuropsychiatric inventory; TBD,To be determined and UPDRS, Unified Parkinson's Disease Rating Scale.

Drug	Disorder	Clinical Trial	Phase	Dose	Primary Outcome Measures	Status
Lithium	FTD	NCT02862210	II	150-600 mg/d	Neuropsychiatric Inventory Scale; BDNF serum levels and changes in NPI score	Recruiting, negative in ALS[3]
Metformin	Ageing	NCT02432287	IV	1700 mg/d	Gene expression, insulin sensitivity	Ongoing[4]
Metformin	MCI	NCT00620191	II	1000 mg/2x/d	Memory recall, ADAS-cog, 2-deoxy-2-fluoro-D-glucose positron emission tomography	Completed, minor cognitive benefit; other markers negative[5]
Resveratrol	AD, MCI	NCT00678431	II	Grape juice	ADAS-cog, CGIC	Completed, unsuccessful
Resveratrol	AD	NCT01504854	II	500-1000 mg/2x/d	Aβ–amyloid 1-42 levels, brain MRI; Innate immune/inflammatory biomarkers; Cognitive and functional decline	Completed, no change in brain volume; positive signals in patient subset (see legend)[4,5]
Resveratrol	HD	NCT02336633	III	40 mg/2x/d	Caudate atrophy; Unified Huntington Disease Rating Scale; Total Functional Capacity; inorganic phosphate/phosphocreatine levels	Recruiting
Nicotinamide	AD	NCT00580931	I	1500 mg/2x/d	ADAS-cog	Completed, no report
TRx0237 (LMTX/M)	AD	NCT0162639	II	100 mg/2x/d	Safety and tolerability with Acetylcholinesterase Inhibitor or Memantine co-administration	Terminated[1,6]; Post-hoc analysis "positive" (see legend)
TRx0237 (LMTX/M)	FTD	NCT01626378	III	100 mg/2x/d	Whole brain volume (MRI); Addenbrooke's Cognitive Exam; Functional Activities questionnaire; Frontotemporal Dementia Rating Scale; Modified CGIC	Completed, unsuccessful[7]
Curcumin	MCI	NCT01383161	II	465 mg/6x/d	Cognitive testing, inflammation markers; Aβ–amyloid 1-42 levels; FDDNP-PET	Ongoing
Ambroxol	PD	NCT02941822	II	Escalating doses 60-420 mg/d	Glucosylceramide and ambroxol levels in CSF; GCase activity; Montreal Cognitive Assessment; UPDRS	Ongoing
Ambroxol	PD	NCT02914366	III	525,1050 mg/d	ADAS-cog; CGIC; MOCS; CSF (α-syn; tau; Aβ); MRI (atrophy)	Recruiting
Arimoclomol	ALS	NCT00706147	II/III	200 mg/3x/d	Rate of decline on ALSFRS-R, safety and tolerability	Well-tolerated; low adverse effects; possible increased survival; slower ALSFRS-R decline[8]
Arimoclomol	ALS	NCT00244244	II	75-300mg/3x/d	Safety, tolerability, pharmacokinetics; rate of decline on ALSFRS-R	Well-tolerated, low adverse effects; slower ALSFRS-R decline with Arimoclomol[9]
GZ/SAR402671	PD	NCT02906020	II	Escalating doses TBD	UPDRS, Parkinson's Disease Cognitive Rating Scale; Hoehn and Yahr score	Recruiting
Nilotinib	PD	NCT02281474	I	150, 300 mg/d	Safety, tolerability, pharmacokinetics and biomarkers (homovanillic acid in CSF)	Completed, *potential* benefits to confirm[10]
Nilotinib	PD	NCT02954978	II	150, 300 mg/d	Safety, tolerability, pharmacokinetics and biomarkers (homovanillic acid in CSF)	Recruiting
Nilotinib	AD	NCT02947893	II	150, 300 mg/d	Safety, Biomarkers and Clinical Outcomes	Recruiting
Edaravone	ALS	NCT01492686	III	60 mg/d	ALSFRS-R; time of death; health changes over time	Successful (ALSFRS-R)[11]; FDA approved

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Supplementary Boxes

Box S1: Lysosomal storage disorders: insights into the pathogenesis and treatment of neurodegenerative disorders of ageing (NDAs)

The more than 50 recognised classes of lysosomal storage disease (LSDs)[1], like Niemann-Pick disease type C, are genetically inherited disorders that arise from: 1), defects in the activity of lysosomal hydrolases that catabolise proteins and other autophagic cargo (primary storage disorders); 2), dysfunction of proteins involved in autophagic cargo sequestration and lysosomal delivery (secondary storage disorders)[2] or 3) aberrant forms of proteins involved in cargo handling and exocytosis (tertiary storage disorders)[3]. Deficiencies in lysosomal hydrolases disrupt the sequential degradation of cellular substrates like proteins, sphingolipids and glycosaminoglycans[2,4]. Several LSDs are associated with neurodegeneration that commences in early childhood: for example, Tay-Sachs disease where β-hexosaminidase deficiency causes G_{M2}-ganglioside accumulation. Further, since mutations are expressed body-wide, LSDs present with systemic complications like splenomegaly and cardiomyopathy[1].

Conceptually, NDAs might be considered, at least in part, as 'storage' disorders associated with the accumulation of incompletely-degraded proteins, such as α-synuclein in Parkinson disease (PD) and Aβ and tau protein in Alzheimer's disease (AD)[5]. In NDAs, a reduction in lysosomal clearance of aggregated proteins and dysfunctional organelles is, then, associated with characteristic pathophysiological features (main text, **Table 1**).

Although the lysosome is the final site of substrate degradation, and 'classical' LSDs are conventionally viewed as compartmentalized inborn errors of metabolism, the lysosome is part of a dynamic system integrating other cellular processes like endocytosis and autophagy (main text)[1,6,7]. Moreover, LSDs partly result from the sequestering of proteins and other substrates that would otherwise be recycled to serve as building blocks for meeting on-going cellular demands. A diversity of pathophysiologic events including decreased lysosomal acidity, neuroinflammation and dysregulation of calcium homeostasis are associated with LSDs and provide a mechanistic link to NDAs where similar pathological changes are seen[1,8]. As for the earlier age of symptom onset in LSDs *vs* NDAs, this is likely explained by the more radical impact of single gene defects that trigger from birth ON A loss of lysosomal (degradative) enzyme activity and rapid accumulation of substrates[1,9]. Conversely, in NDAs, the primary defect(s) are mainly extrinsic to the lysosomal compartment, the evolution of disease is slower and the rate of 'substrate' accumulation more protracted. Eventually, with the progressive accumulation of aggregated proteins, cellular storage surpasses a threshold ("inflection point") that initiates a cascade of damaging events.

Parallels between LSD and NDAs are exemplified by Gaucher's disease (GD) and PD. In GD, heterozygous mutations in GBA1, which encodes lysosomal β-glucocerebrosidase, markedly increase the risk of PD. Conversely, a subset of sporadic PD patients present with disrupted function of β-glucocerebrosidase and lysosomal accumulation of its substrate, glucosylceramide[10]. Moreover, as recently reported[11], around half of PD patients have at least one putatively deleterious mutation in genes associated with LSDs *other* than GBA1[1,12]. Lysosomal dysfunction is also of more general relevance to the pathogenesis of familial and idiopathic AD which, amongst other processes, can be linked to disruption of Presenilin-1, a protein driving lysosomal acidification[10,11]. Further comparisons of LSDs and NDAs should yield additional insights into their underlying and common causes, and also into potential therapies for restoring lysosomal function[1].

Approved disease-modifying treatments for LSDs include enzyme replacement therapies which deliver recombinant enzymes, such as β-glucocerebrosidase for GD disease, although this enzyme poorly enters the brain[1,12]. Substrate reduction therapy involves the inhibition of enzymes that normally synthesize the excess substrate: *e.g*., miglustat and eliglustat inhibit glucosylceramide synthase but, unfortunately, have limited access to the brain[9]. This type of therapy with better ability to penetrate the BBB (*e.g.,* GZ/SAR402671), together with the use of chaperones to promote β-glucocerebrosidase transfer to lysosomes, is potentially useful for PD - see main text[1]. Another drug undergoing clinical testing is arimoclomol which is being evaluated in Niemann-Pick disease type C. It mimicked the ability of recombinant Heat Shock Factor 70 (a protein chaperone that promotes the binding of sphingolipid-degrading enzymes to their co-factors) to counter lysosomal defects in primary fibroblasts from this and other classes of LSD[13].

Important differences between NDAs and LSDs should not be neglected: not least, a dysfunction of clearance mechanisms *other* than the ALN is a core facet of the former diseases. Nonetheless, Research and Development programmes devoted to LSDs are proving instructive in the elucidation of pathological mechanisms underlying NDAs, and in the elaboration of novel strategies for their treatment[1,14,15]. The first trial assessing the clinical benefit of GZ/SAR402671 has recently been launched in patients with PD bearing a GBA1 mutation (NCT02906020) (Suppl Table 1).

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Box S2: The Unfolded Protein Response: role in pathophysiology of neurodegenerative disorders of aging (NDA) and potential therapeutic targeting

In addition to clearance mechanisms discussed in the main text, maintenance of proteostasis requires effective folding and assembly of newly-synthesized proteins before they exit the endoplasmic reticulum (ER)[1]. When this process is insufficient and cells begin to accumulate misfolded and harmful proteins, the Unfolded Protein Response (UPR, see Glossary) is transiently triggered to let the cell recover. The UPR is integral to maintaining proteostasis and its modulation can affect autophagic processes[1,2].

Transient UPR activation results in increased chaperone expression for protein re-folding, and, *via* the kinase Protein Kinase RNA-like Endoplasmic Reticulum Kinase (PERK), a reduction in protein synthesis rates to reduce the ER burden. However, chronic over-activation of PERK signalling occurs in certain NDAs and has been implicated in neurotoxicity: hence, PERK down-regulation is a promising target for NDA treatment which can be considered as complimentary to the augmentation of clearance[3]. In mouse models, deregulated and excess PERK signalling causes a sustained and marked reduction in cerebral protein synthesis leading to synaptic dysfunction and neuronal death: this is reversible by genetic and pharmacological inhibition[4,5]. Similar beneficial effects were observed using trazodone, an antidepressant that inhibits PERK signalling and is being repurposed for clinical trials in human NDAs[3]. Genetic or pharmacological modulation of the UPR also results in the activation of autophagy and has shown promising results in models of Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis[6].

As mentioned above and discussed in the main text, disruption of proteostasis (and accumulation of neurotoxic proteins) is toxic, so cells initiate a series of adaptive responses. These include induction of clearance by the autophagic-lysosomal network (ALN) and the ubiquitin proteasome system (UPS), and reduced generation of proteins *via* activation of the UPR[2]. These adaptive responses (sometimes referred to as hormesis) can also be induced by a *sub-toxic*, low intensity stressor ("preconditioning") which results in cellular protection against further toxic injury[7,8]. Hormesis can be induced by many preconditioning stimuli like oxidative stress, ER stress and inflammatory stimuli[8-11]. It has been proposed that preconditioning of the UPR *via mild* ER stress may be neuroprotective. For example, in *Drosophila* models of apoptosis-induced retinal degeneration and human α–synuclein overexpression, ER-preconditioning elicited either genetically or with tunicamycin promoted neuroprotection by harnessing the ALN[12]. Similarly, in a 6-hydroxydopamine mouse model of Parkinson's disease, preventive injection of tunicamycin reduced both dopaminergic neuron loss and locomotor dysfunction[12]. The interconnection between the UPR and clearance is also critical for neuroprotection in models of ischemia reperfusion[2]. Finally, modest activation

of X-box binding protein 1, a major transcription factor triggered by the UPR, leads to autophagic activation and neuroprotection in mouse models of ALS and HD[13].

Collectively, the above comments indicate that two UPR-articulated strategies may provide neuroprotection in NDAs: *first*, abrogation of an over-persistent and extreme UPR and, *second*, a mild and transient recruitment of the UPR as a means to engage ALN-driven clearance of neurotoxic proteins

In addition to the cytosolic UPR discussed above, there is also a mitochondrial UPR which is activated to protect mitochondria from stressors like accumulation of unfolded proteins in their matrix[14]. These harmful, intra-mitochondrial proteins are digested by proteases and the resultant peptides exported to the cytosol: there, they trigger a cascade that restores proteostasis by inducing genes encoding, for example, mitochondrial chaperones. In addition, due to a decrease in the activity of mTOR, protein translation is reduced. Boosting the mitochondrial UPR and mitophagy with nicotinamide had beneficial effects on mitochondrial function in human cells expressing Aβ, as well as in mice models of AD where it helped to clear intracellular deposits of Aβ[14,15]. Finally, mitochondrial stress due to failure of protein import or sorting leads to the accumulation of proteins in the cytosol and to an overburdening of the UPS. Accordingly, agents that promote UPS activity may also be of therapeutic utility in NDAs associated with mitochondrial dysfunction[16].

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Box S3: Potentially detrimental effects of (excess) autophagic-lysosomal network (ALN) activity: relevance to amyotrophic lateral sclerosis (ALS)

Excess activity of the ALN under specific conditions and its consequences

While modest and time-locked ALN activation is protective, over-activation is potentially deleterious. Indeed, sustained and massive ALN activity in stroke or traumatic brain injury leads to tissue damage and even cell death ("autosis") (Glossary) due to excessive catabolism and cellular stress[1,2]. While there is little or no evidence for excess ALN in most classes of NDA, this may potentially occur in ALS although, as outlined below, not all observations support this - still controversial - possibility.

Evidence for a *reduction* in the activity of the ALN in ALS

In post-mortem tissues of ALS patients, protein inclusions are seen in upper and lower motoneurons, as well as in neuronal and glial cells in the midbrain, prefrontal neocortex and striatum[3-5]. This argues for defective clearance of neurotoxic proteins, and one possible cause of compromised ALN activity is release by reactive astrocytes of Transforming Growth Factor β which interferes with initiation of autophagy by stimulating mTOR[6]. Further, a low handling capacity of the ALN - as reflected in the scarcity of beclin 1 in the ventral horn of patients with sporadic or familial ALS - may be related to development of the disorder[7]. Indeed, by analogy to frontotemporal dementia[8] and other classes of NDA (main text), defective autophagy has been proposed as a point of convergence point of harmful events in ALS, such as endoplasmic reticulum stress, mitochondrial dysfunction, anomalous processing of mRNA/miRNAs and oxidative stress[9-11].

Mutated Fused in Sarcoma (FUS), which accounts for about 5% of familial forms of ALS, inhibits the formation of autophagosome in neuronal cells as visualized in the accumulation of Light Chain-3 (LC-3) positive vesicles[12]. Negative regulation of autophagy has also been reported in cultured neurons transfected with mutated TAR DNA-binding protein-43 (TDP-43), while superoxide dismutase (SOD) 1 overexpression impairs fusion of autophagosome and lysosomes[13]. These results support ALN impairment, although conflicting with another paper which reported an *upregulation* of macroautophagy in cells transfected with TDP-43 or SOD1[14]. Underpinning the pertinence of specific cell types. In symptomatic $SOD1^{G93A}$ (specific mutation) mice, expression of pro-autophagic markers is highly upregulated in muscle but not, or more moderately, in spinal cord[15]. These observations accord with the idea that muscle cells are more effective in clearing SOD1 by upregulating the ALN[16]. Nonetheless, muscles of $SOD1^{G93A}$ mice accumulate autophagosomes when compared to wild-type mice[17], presumably due to insufficient ALN flux.

Evidence for an *increase* in the activity of the ALN in ALS

Despite this evidence for *reduced* autophagic flux in ALS patients, this may not be true in all disease stages, for all ALS patients or, as intimated above, for all cell types. Moreover, ALN status may also depend on the precise genotype as highlighted by C9orf72. A hexanucleotide repeat expansion in the C9orf72 gene is a major cause of familial ALS. Although it is not clear whether C9orf72 mutations increase the propensity for ALS due to a loss and/or gain of function, carriers of expansion repeats present with reduced expression of C9orf72[18]. Their impact may, at least partially, be explained by a deregulation of autophagy[19]. Indeed, C9orf72 has been proposed as a negative regulator of mTOR/macroautophagy[20] and its silencing enhanced autophagy flux in mice[21]. Thus, lower expression of C9orf72, or loss of function, could translate into *higher* autophagy flux for ALS patients. Indeed, there is clinical evidence for an up-regulation of the ALN in ALS. Expression and activity of lysosomal enzymes were higher in the spinal cord of ALS patients and animal models[22,23]. Underlining complexity, genetic removal of Atg7 in motoneurons inhibited macroautophagy in SOD1^{G93A} mice and resulted in *earlier* disease onset yet *longer* survival[24]. The loss of macroautophagy in motoneurons was also associated with reduced glial cell reactivity. Finally, SOD1 mutations caused *overactivation* of autophagy and cellular degeneration in SOD1 mutant mice[25]. This appeared to involve AMPK stimulation of Ulk1, and a reduction of AMPK activity conferred some modest neuroprotection[26]. Accordingly, treatment of cultured motoneurons from SOD1 mice with a AMPK stimulator exacerbated cytotoxicity suggesting that autophagic activity was already elevated (and deleterious) and that further stimulation might induce autosis[27].

Therapeutic control of excess ALN activity

Collectively, the above observations are far from simple to reconcile (!), yet suggest a time, cause and cell-type dependent changes in specific components of the ALN. Mobilisation may well be beneficial for neuromuscular and neuronal integrity at disease *onset*, but "excess" autophagy in motoneurons may be toxic in late stages of the disease. As the main text focusses on efforts to promote ALN activity, the following comments evoke possibilities for tempering a putative hyperactivity under specific conditions in ALS.

As mentioned above, agents that dampen an overactive ALN are mainly conceived for therapeutic use in stroke, cerebral ischemia and traumatic brain injury[28,29] but data are emerging for ALS. Administration of n-butylidenephthalide, a natural compound which negatively regulates pro-autophagy proteins, extended survival of SOD1^{G93A} mice[30]. Moreover, inhibition of β-glucocerebrosidase, a lysosomal enzyme participating in the degradation of glycosphingolipids, preserved neuromuscular junction and delayed

neurodegeneration in SOD1^{G86R} mice[31]. Edaravone, a reactive oxygen species scavenger[32], reduced cerebral autophagy *in vivo* after ischemia[33] and also in macrophages[34]. This action is potentially linked to its utility for treating ALS[35] and edaravone was approved for use in a *subset* of ALS patients[36]. It would be interesting to know their ALN status compared to other subjects who were not helped by treatment. Underscoring interest in edaravone, it improved cognition and diminished Aβ levels in a rat model of Alzheimer's disease, and also displayed neuroprotective properties in a rotenone-induced model of PD[37,38]. However, its influence on Aβ appears to at least partly involve anti-aggregant properties, and it remains to be proven that ALN suppression is genuinely involved in the actions of edaravone[38]. Furthermore, not all data support the idea of promoting ALN activity in ALS. In this light, it is interesting to consider rilmenidine, a positive modulator of mTOR-independent macroautophagy (main text). In SOD1^{G93A} mice, rilmenidine increased ALN flux in the spinal cord but ultimately *exacerbated* motoneuron degeneration and the accumulation of insoluble, misfolded SOD1 protein[14].

Concluding comments

In conclusion, ALS exemplifies the complexity of ALN deregulation in NDAs. Baseline flux may be inherently *low* at the onset of disease, related to old age and deficient actions of proteins encoded by genes like dynactin and progranulin (**Table 1**). Hence, the cell attempts to promote the ALN and this may initially be protective. However, later in the disease, under conditions of chronic cellular stress, energy imbalance and inflammation, "excess" ALN activity may become deleterious. Accordingly, there may be a phase-dependent need to therapeutically enhance *or* moderate ALN activity, potentially explaining contradictory findings in the literature. This remark also suggests that the duration and pattern as well as the time of drug administration would be critical. Finally, accentuating complexity, while this seems to hold for motoneurons and neuromuscular junctions, it remains to be more fully established how events unfold in spinal and cerebral neurons impacted in ALN. Much further research will be needed to clarify how to optimally harness the ALN for clearing neurotoxic proteins in ALS.

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