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 which may 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 appear likewise to be 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 focusses on emerging mechanisms for enhancing neurotoxic protein clearance, a strategy that may curtail the onset and slow the progression of ageing-related neurodegenerative disorders.

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, **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]. Numerous therapeutic strategies for countering the generation, mis-processing, oligomerisation and accumulation of neurotoxic proteins are currently being explored. Amongst these, approaches for accelerating elimination are of particular interest since impaired clearance is a major factor in their buildup, aggregation and spread[1-5].

As summarized in **Figure 1**, several endogenous mechanisms are dedicated to 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 phagocytised by microglia and astrocytes. Within glial cells and neurones, 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 *alone* 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 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, large, double-membrane-bound autophagosomes that deliver their contents to **lysosomes** for digestion. Autophagic flux (**Box 1**) describes the process spanning formation of

the autophagosome isolation membrane through to cargo digestion in the lysosome (**Figures 2 and 3**). Autophagy is a fundamental feature of neurones, oligodendrocytes and endothelial cells. Further, astrocytes and several subtypes of microglia (some specific to NDAs) fulfil important functions in the phagocytosis **then ALN-driven elimination of of extracellular pools of neurotoxic protein aggregates** - although other beneficial and *deleterious* roles of microglia should be borne in mind[6,7]. **(Jansen et al, 2014**) In addition to bulk clearance of cytoplasmic contents, dedicated autophagy receptors promote sequestration of specific misfolded and/or aggregate-prone proteins, damaged organelles, **aggresomes, stress granules, peroxisomes,** endoplasmic reticulum (ER)/Golgi components, lipids, ribosomes, polysaccharides and nucleic acids[4,8]. LC3-II and adaptor/scaffold receptor proteins like optineurin and p62 recruit discrete classes of potentially neurotoxic protein like tau to autophagosomes[9]. Other scaffolds include "Nix", "BNIP1" and Prohibitin-2 for dysfunctional mitochondria (**Box 2**)[4,8-10]. **Ubiquitin and non-ubiquitin dependent autophagy occurs but, in general, ubiquitination of tau and other neurotoxic proteins enhances capture by autophagic receptors like p62: other post-translational modifications like acetylation (e.g., of htt) may also favour ALN degradation but await further evaluation (Khaminets et al, 2016).**

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 largely constant supply of **recycled amino acids, sugars, lipids and other products of ALN-mediated catabolism[3,11]**. That autophagy serves an essential role is demonstrated by genetic knockdown of **autophagy-regulating genes** (Atg). For example, mice with e-specific Atg 7 deletions develop early post-natal neurodegeneration[12], while knockdown of Beclin 1 (Atg6) exacerbates hippocampal neurone vulnerability to energy deprivation[13]. These findings highlight the crucial housekeeping role of autophagy in the maintenance of neuronal health. Moreover, since post-mitotic neurones *cannot* dilute harmful proteins *via* mitosis, they are uniquely vulnerable to its impairment[1,3,5,14-16].

Maintaining efficient ALN flux requires coordination of the actions of a suite of modulatory (and targetable) proteins - **and phospholipids** (**Figure 3**)[3,9] Changes in their amount, stoichiometry and function are charactertistic of **NDAs[1-3,5,16-18].**

Operation and regulation of the ALN

Sensing, initiation and regulation

Initiation of the autophagic cascade depends on the actions of sensors and regulators (**Figure 3**). The heterotrimeric serine/threonine kinase, **AMP-regulated Kinase** (AMPK) and **mammalian target of rapamycin complex (mTORC1)** have dual roles and they respectively trigger and repress autophagy, as well as mitophagy (**Figure 3, Box 2**)[3,9,18-21]. Unc-51-like kinase (Ulk1) is primarily an autophagy-initiating protein[3,9,17], and the same holds for mTORC1-suppressed Transcription Factor EB (TFEB) which orchestrates the synthesis of lysosomal and other proteins critical for maintaining ALN flux[18-21]. Since the Class III deactylase, Sirtuin-1 requires **nicotinamide adenine dinucleotide** to sustain its activity, this positive regulator of autophagy may also be considered a sensor[22].

Intrinsic sensing refers to detection of localised changes in intracellular levels of glucose, amino acids, fatty acids, AMP, inositol triphosphate (IP_3), cytosolic Ca^{2+}, reactive oxygen species and metabolic intermediates like **acetyl coenzyme A (Box 2)**[5,11,17,19,21,23]. 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[19]. Extrinsic sensing occurs ***via* inherently drug-targetable mechanisms situated** at the plasma membrane. *First*, receptor tyrosine kinases converge onto mTOR1, AMPK or the Beclin 1-Vps34 complex (Figure 3) to modulate autophagy following stimulation by growth factors[9]. (**Fraser et al, 2017**) *Second*, G-protein coupled receptors (GPCR) as well as ion-channel coupled receptors control autophagy *via* signalling pathways that likewise modulate AMPK and mTORC1[24-26]. GPCR-mediated generation of cAMP can negatively regulate autophagy *via*, for example, protein kinase A (PKA)-mediated phosphorylation of Atg proteins[24,26,27]. *Third*, specific classes of cytokine and cytokine receptor also modulate autophagy, although events in the brain remain poorly defined[21].

AMPK exerts a dual mechanism for triggering autophagy: phosphorylation-activation of of Ulk1/2 (Ser317 and Ser777) and phosphorylation-inhibition of mTORC1[19,28]. Conversely, mTORC1 inhibits Ulk1/2 by Ser757 phosphorylation[28]. MTORC1 also restrains autophagy by preventing nuclear translocation of TFEB[18]. Other transcription factors that positively regulate autophagy include Forkhead-Box O1 and O3[20]**.** Conversely, repression is effected by STAT3 (Signal Transducer and Activator of Transcription 3) and, possibly, "ZKSCAN3" although its role has been disputed[20,29]**.** Sirtuin 1 is recruited by AMPK-mediated increases in nicotinamide: it fulfils a pivotal role in driving the ALN in view of its 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 Sirtuin1 mediated neuroprotective effects in NDAs[22].

Autophagosome formation, cargo sequestration and delivery to lysosomes

Activation of Ulk1 triggers autophagosome nucleation through the phosphorylation-activation of Beclin 1 within the autophagy-specific Vps34 kinase complex[9] **(Figure 3).** LC3 and other family members like "GABARAP" covalently conjugate with phosphatidylethanolamine and, together with several other factors (Figure 3), assist in the elongation of the isolation membrane and the closure of autophagosomes[1,3,9]. They also serve as docking sites for autophagy receptors that selectively capture ALN substrates (Box 1)[3] Although autophagosomes form in the absence of LC3, efficiency is reduced[30]. **Compared to glia, neurons are very complicated cells for orchestrating ALN degradation of neurotoxic proteins 1, 16 (Jansen et al, 2014).** 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 most lysosomal fusion occurs[9,14]. **164 Some fuse with endolysosomal compartments containing membrane-localised Rab7 protein (a GTPase) and Lysosome-Associated Membrane Protein (LAMP)1 *before* reaching the perikaryon: this implies that a proportion of ALN degradation occurs *before* reaching the soma (Fig 2)[9,14] (Maday et al, 2016)**

Autolysosome formation is facilitated by the retromer complex, itself retrogradely transported to cell bodies (Tammineni et al, 2017)[17,31]. "**SNARE**" proteins and the "Homotypic Fusion and Vacuole-Protein Sorting" complex bridge mature autophagosomes/amphisomes to lysosomes to initiate fusion[4,17]. 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[9,17,32]. **Martens et al, 2016 to replace Dall'armi REF 32).**

Lysosomal digestion of cargo

Autophagosomes fuse with lysosomes that provide the hydrolases required for cargo degradation and nutrient recycling (amino acids, lipids and sugars) (**Kaminskyy and Zhivotovsky, 2012)**. 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[33]. Underpinning the importance of acidity, digestion can be halted by v-ATPase inhibitors like bafilomycin[34] and lysosmotropic basic amphiphiles like chloroquine which alkalinize luminal contents[35]. Further, a deficiency of lysosomal cathepsins (B, L and D etc) prevents protein degradation and leads to accumulation of undigested cargo[14,15]

Kaminskyy and Zhivotovsky, 2012). Lysosomal dysfunction **blocks flux across** the *entire* ALN, as evidenced by **lysosomal storage diseases** (**LSDs**) like **Niemann-Pick Type C** which are associated with neuropathological phenotypes (**Suppl Box 1)**[36].

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[37].

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 substrates for degradation into the lumen without enclosure by any membrane structure (Figure 2) (39, Xilouri and Stenfanis, 2015; Catarino et al, 2017). The protein chaperone, "Hsc70", recognises soluble, cytosolic proteins bearing a "KFERQ" or equivalent motive and, with the help of co-chaperones like Hsp90, guides them to transmembrane LAMP2A receptor[1-4,9],[3,38,39] CHANGE ORDER REFS The substrate complex binds to the cytosolic tail of LAMP2A leading to its stabilisation and oligomerisation and allowing - following protein unfolding - translocation into the lumen. This process is aided by a specific, low pH-dependent lysosomal form of Hsc70 which then promotes dissociation of the LAMP2A multimer such that the monomeric form is again available for substrate recognition. The level of LAMPA2A and translocation determines the rate of CMA. In contrast to the ALN, the 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 as well as APP are substrates for CMA degradation, but not Aβ42 itself[3,38,39].. Htt is not efficiently cleared by CMA, and the same holds for fragments, mutant and post-translationaly modified forms but the precise role of CMA in this respects is still debated (2, 39 Xilouri and Stefanis, 2015).

The Ubiquitin-Proteasomal System

The UPS mainly targets soluble and monomeric proteins using a process involving Hsp70 and the sequential actions of three classes of ubiquitin ligases (E1, E2, and E3). They effect the addition onto targeted proteins of Ubiquitin residues at single or multiple Lysine sites, often as polyubiquitin chains (Figure 2)[3,38,39] Ubiquitinated substrates are recognised by the 19S regulatory particle of the UPS complex. After binding to the 19S ring (Rpn subunits), ubiquitin motives are removed by three enzymes, Usp14, Uch37 and Rpn1. Rpn11 removes ubiquitination chains only after substrates are committed to destruction, whereas Ups14 and probably Uch37 act *before* commitment and hence can *rescue*

substrates[40]. Following removal of Ubiquitin moieties, proteins are unfolded by the Rpt 1-6 subunits (ATPases) of the 19S component. The substrate then passes the α-subunit gate of the 20S particle to enter its contral β-subunit core which contains peptidase (trypsin, chymotrypsin and caspase-like) sites and effects proteolysis.

In addition to ubiquitinated substrates the UPS can also handle oxidized proteins under conditions of cellular stress - **which itself damages the 19S subunit (Bonet-Costa et al, 2016).** The UPS degrades not only cytosolic proteins, but also mitochondrial proteins that accumulate owing to a dysfunction of mitochondrial import or sorting pathways, The UPS acts, then, in parallel to the ALN which clears damaged mitochondria themselves (Box 2)[41]. Further, 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[40,42]. **Susceptibility of neurotoxic proteins to ubiquitination is modified by phosphorylation and other post-translational modifications. However, i**n contrast to the ALN and mirroring the CMA, the UPS does *not* degrade oligomers and aggregates.

Defective ALN, CMA and UPS mediated clearance of neurotoxic proteins

NDA-related impairments

Neurones adopt several 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[2,3,43]. 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**)[1,5,16,44]. The partly common and partly disease-specific patterns of ALN, CMA and UPS dysfunction are superimposed upon a generalized age-related decline in all modes of clearance efficiency both for neurones and for other cell types like scavenging microglia[1,7] **2, 39, 67 (Catarino et al, 2017). Jansen et al, 2014)** Insufficient neuronal ALN flux is frequently manifested by lysosomal accumulation of **lipofuscin**[16].

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 and misfolding or an excessive cytosolic **Unfolded Protein Response** (**Suppl Box 2**)[45]. 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 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 likely induced in early phases of the disorders[1,3,46], several lines of evidence suggest that ALN, UPS and probably CMA-mediated clearance eventually becomes overwhelmed and impaired in AD. *First*, autophagosomes and autophagic vacuoles indicative of failed maturation, transport and/or fusion with lysosomes are abundant, particularly in dystrophic neurites, and similar profiles are seen in mouse models of AD. Their accumulation may be linked to impaired lysosomal elimination of cargo, and rescuing lysosomal function improves deficits[16]. **Maday, 2016.** *Second,* while putative decreases in Beclin 1 levels in AD remain to be confirmed, Sirtuin-1 expression is diminished[22]. *Third,* **Apolipoprotein E4** allele (ApoE4), a major risk allele for sporadic AD, is associated with increased generation and accumulation of Aβ42[47,48]. 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[49]. Moreover, ApoE4 impairs the elimination of neurotoxic proteins by astrocytes and microglia, additionally compromised by decreased activity of Triggering Receptor Expressed on Myeloid cells (TREM)2[7,50]. *Fourth,* genetic mutations and anomalies of **Presenilin-1,** a dominant-negative gene linked to AD, are associated with reduced lysosomal v-ATPase-mediated acidification[33,51] and deficient mitophagy[52]. Presenilin-2, likewise an autosomal-dominant risk gene, is enriched in late endosomes/lysosomes where its dysfunction provokes lysosomal accumulation of insoluble Aβ42[53]. *Fifth,* mutations in **Aβ42 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 ("C99 or CTFβ")[54]. *Sixth*, Aβ42 compromises the function of AMPK and obstructs the UPS and CMA[55,56]. **The efficacy of the UPS for degrading hyperphosphorylated and oligomeric forms of tau is reduced compared to normal tau, and both aggregates of tau and mutant forms block the proteosome 56, 67. Bonet-Costa et al, 2016 Jansen et al, 2014. Tau possesses KFERQ motives and is degraded by the CMA, but aggregates, mutant forms and fragments interfere with its activity (39, Xilouri and Stefanis, 2015).**

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, including reduced functionality of mitochondria (**Box 2**). *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,** both important for mitophagic removal of damaged mitochondria (**Box 2**)[57,58]. *Second,* Leucine-Rich Repeat Kinase-2 GTPase is the most commonly "mutated" protein in late-onset, familial PD. Its role is complex but, in addition to impairment of the ALN, in part due to reduced activation of Beclin 1, another repercussion may be altered processing of APP providing an unexpected link to AD[57,59-61]. *Third*, α-synuclein mutations, triplication or excess amplify the ALN burden, interfere with autophagosome formation and irreversibly disrupt the lysosomal membrane[1,62]**.** *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**)[36]. Decreased β-glucocerebrosidase activity also occurs in sporadic PD with the build-up of glucosides, lipid dyshomeostasis, poor clearance of α-synuclein and impaired lysosomal activity[36,63,64]. *Fifth*, defects in several genes disrupt lysosomal acidification[33]. For example, disruption of ATPase, ATP13A2 (PARK9), depleted in sporadic PD, leads to lysosomal digestive failure[65] together with accumulation and release of α-synuclein and other ubiquinated proteins[65,66]. *Sixth*, **aggregates and mutant forms of α-synuclein disrupt the proteasome in dopaminergic neurons. Further, loss of Parkin activity may also compromise the UPS and numerous mutations are also linked to reduced UPS activity (Table 1) 2, 68 Zondler et al, 2017. Oligomeric and mutant forms of α-synuclein also impair LAMP2A-mediated cargo transport for CMA: moreover, LAMPA2A and Hsc70 are reduced in PD brain (67, Xilouri and Stefanis, 2015). CMA dysfunction is particularly awkward in view of its importance for eliminating α-synuclein and clearing pathologically-oxidated proteins. Further, compromised CMA may lead to inactivation of the dopaminergic neuron survival factor, "MEF2D" 2, 39, 67 (Xilouri and Stefanis, 2015). Finally, CMA is disrupted by several mutations that occur in PD, including LRKK2[2,3,39,67,68] (Catarino et al, 2017; Xilouri and Stefanis, 2015).**

Frontotemporal dementia

As FTD was initially associated with tau mutations, it is classed with "tauopathies" like progressive supranuclear palsy[69,70]. However, classification is complex and, due to common risk

genes like p62 (Sequestome1) and "C9orf72" (Chromosome 9 Open Reading Frame-72), it is increasingly linked to ALS[70,71]. Genetic anomalies in FTD are closely related to a deficient ALN, and, like ALS, the disease is also characterised by aggregates containing tau, TDP43, Fused in Sarcoma and other ubiquitinated proteins which are insufficiently cleared by the ALN[70,72]. Aggregates interfere with the UPS, creating a vicious circle that further overloads the ALN[56,62,67,72]. **Recently, it was found that poly-glycine/alanine tracts linked to mutant forms of the C9orf72 gene (seen both in FTD and ALS) form twisted ribbon aggregates that sequester and stall the activity of proteasomes (Guo et al, 2018).** In addition, 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[69,70]. Lysosomal dysfunction and loss of acidification is caused by tau fragments and a deficit of progranulin[70,71,73], while an interrelated deficiency of endosomal trafficking is linked to mutations in "CHMP 2B" (Charged Multivesicular Body Protein 2B) as well as C9orf72 (see further below)[70,71].

Amyotrophic lateral sclerosis

ALS share many causal genes with FTD, including p62, CHMP2B, "TBK1" (Tank-Binding Kinase 1), optineurin and others associated with deficits in the ALN and in mitophagy. For example, mutations in optineurin and TBK1 interfere with cargo loading[70,72,74]. 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 neurones to the perikarya[70,75]. They may also lead to, for example, deregulation of Rab-GTPases and a failure of autophagosome elongation[76]. Paradoxically, however, certain anomalies of C9orf72 may *stimulate* the ALN while, under conditions of severe cellular stress, high ALN activity may potentially be detrimental (**Suppl Box 3**)[38,75,77]. In any event, depending on their genetic profiles, ALS patients reveal aggregates of risk gene-encoded proteins like TDP-43, Optineurin, Fused in Sarcoma (FUS) and **Superoxide Dismutase (SOD1)**[70,72,74] which disrupt operation of the UPS and CMA[39,78]. **In addition to the anomalous function of the ALN in ALS, aggregated SOD1 and TDP-43 cannot be cleared by, and may disrupt, both CMA and the UPS – with the latter also disrupted by mutations in the C9orf72 gene 2, 67 Guo et al, 2018 Jansen et al, 2014.** Thus, mirroring other classes of NDA, a *failure* to clear neurotoxic proteins is characteristic of ALS[38].

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[1], with chaperone-containing aggregates impairing the UPS[2,3,67,68]. Mutant Htt is autophagically cleared but it compromises the ALN with decreased poor cargo loading and impaired autophagosome formation and transport[56,62,67,79]. Further, ALN disruption in the striatum (strongly impacted in HD) involves altered activity of the striatal-specific Beclin 1 and Htt-interacting protein "Rhes"[80,81]. 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[79]. **CMA only poorly handles mutant and post-translationally modified forms of Htt, which may interfere with its activity (2, Xilouri and Stefanis, 2015; Bauer et al, 2010). While LAMP2A and are Hsc70 are upregulated in early HD to compensate for decreased ALN clearance, CMA eventually fails in parallel with neuronal loss (39, Xilouri and Stefanis, 2015). The status of the UPS in HD is currently unclear, but animal models suggest that it may be impaired which would reduce clearance of mutant Htt (Her et al, 2015).**

Strategies for enhancing neurotoxic protein clearance by the ALN

Ultimately, any strategy that improves Protein Quality Control and reduces excess 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 Unfolded Protein Response might *pre-empt* the build-up of neurotoxic proteins (**Suppl Box 2**)[44,72,82,83] **2 (Mogk et al, 2018).** However, the present review focuses on strategies for *elimination* of neurotoxic proteins once accumulated. As outlined in **Table 2** and depicted in **Figure 4**, there is a plethora of potential therapeutic targets. However, the precise mechanisms of drug actions are not invariably well-defined[4]. Further, many agents exert multiple beneficial (or deleterious) actions: for example, methylene blue counters tau oligomerization as well as promoting autophagy (**Suppl Table 1**)[84,85]. In addition, certain 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.

While the following comments 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 acting at GPCRs coupled to the AC-cAMP-PKA axis are likely activators of AMPK[24,26]. Indeed, clonidine and rilmenidine, Gi/o coupled α_2-adrenoceptor agonists, stimulate autophagy and clear Htt in cellular[86] and animal models of HD[87], although their precise mechanisms of action await further elucidation[19,86,87].

Calpains, Ca^{2+}-activated cysteine proteases, are elevated in ageing and proteolytically generate various neurotoxic peptides[44,69]. They also stimulate the AC-cAMP-PKA axis to inhibit AMPK by activating $G_S\alpha$[86]. 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[86]: efficacy was also seen in mutant *Drosophila* and mouse models of HD[44]. Calpeptin, a cell permeable calpain inhibitor, can also reduce Htt proteinopathy *via* induction of autophagy[86,88]. Calpain inhibition by calpastatin or pharmacological agents also comferred neuroprotective effects in other NDAs models, including enhanced clearance of toxic forms of tau, α-synuclein and SOD1[44,89,90].

The aminoimidazole derivative, "AICAR," undergoes intracellular transformation to an AMP analog that triggers AMPK-mediated autophagy[19,91]**.** It conferred neuroprotection upon exposure of astrocytes to Aβ or oxidative stress[92] and countered α-synuclein toxicity in cultured rat neurones[93]. 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[94]**.** Selenium deficits have been linked to AD, so it is interesting that its complementation with selenomethionine boosted ALN flux from AMPK recruitment through autophagosome formation to lysosomal degradation in the 3xTgAD mouse model[95].

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[96]. This action, amongst others (below), is involved in its reduction of Aβ levels in N2a cells and neurones[97] and the elimination of Aβ and Htt in animal models of AD and HD[97,98].

The anti-diabetic drug, metformin, a prototypical activator of AMPK, induced autophagy and increased longevity in mice[99]. Like AICAR, metformin abrogated α-synuclein toxicity in primary cultures of cortical neurones, though the precise contribution of autophagy requires clarification[93]. Moreover, reductions in levels of hyperphosphorylated tau and Aβ were seen in metformin-treated neurones,[100,101] while it blunted neuronal loss in a neurochemical-lesion model of PD in mice[102].

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[4,103]. Trehalose promoted autophagy and reduced disease progression in a SOD1 mouse model of ALS[103]. It also proved effective in cellular models of PD, HD and AD,[104,105] as well as in mouse models of HD, AD and tauopathies where it cleared aggregates, reduced neurodegeneration and ameliorated motor and cognitive performance[106-108].

Lithium inhibits 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[109], amelioration of motor function in a P301L mouse model of of tauopathy[110], and slowing of disease progression in SOD1 mice[111]. However, its precise mechanisms of action in modulating the ALN await further elucidation[109].

Other drugs that mediate their effects at least partly 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[84,85]. In addition, calcitriol (the active metabolite of vitamin D3) elicited AMPK-dependent autophagy in a neurochemical lesion-induced model of PD[112].

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[113-115]. It also diminished polyglutamine aggregates and countered motor impairment in a *Drosophila* model of HD[116]. In addition, rapamycin abrogated pathology in murine models of AD and FTD, as well as countering neuronal loss in MPTP-treated mice[117-119]. Likewise, temsirolimus reduced the accumulation of phosphorylated tau in SH-SY5Y cells and P301S tauopathy mice[120]. 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[116,121,122]. 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[123].

The natural compound, curcumin, induced macroautophagy and neuroprotected rotenone-treated dopaminergic neurones[124] as well as accelerating elimination of mutant A53T-α-synuclein by repression of mTORC1 in a cellular model of early-onset PD, although it may also exert other actions like modulation of protein acetylation and aggregation[125,126]. Pro-autophagic effects of

curcumin are reflected in improved function, as well as reduced levels of α-synuclein aggregates[127] and Aβ/tau oligomers in cellular and animal models of PD and AD[128,129].

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[18,20,130]. Indeed, TFEB over-expression reduced amyloid plaques in a APP/PS1 mouse model[131]. Moreover, the flavonol, fisetin, stimulated autophagic degradation of phosphorylated tau in cortical neurones *via* mTORC1-dependent activation of TFEB and the cytoprotective transcription factor, Nuclear factor Erythroid-2-Related factor 2 (Nrf2)[132]. Fisetin also reduced Aβ accumulation in an APP/PS1 mice model of AD[133]. Thus, mTORC1- and, possibly AMPK *via* poorly-characterised cascades[19] - offer channels into TFEB. It remains, nonetheless, a challenging target for (direct) induction[20,134].

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[135]. Inactivation of c-Abl with the brain-penetrant nilotinib conferred neuroprotective autophagy in lesion and α-synuclein-provoked mouse models of PD[136]. It also reduced aggregates in cell and mouse models expressing TDP-43 protein[137]. Nilotinib recently underwent a Phase I safety study for treatment of PD[138].

Modulators of Sirtuin-1 and inhibitors of acetyl transferases

Activity of the deacetylase Sirtuin-1 declines with age, partially due to limited availability of its co-factor, nicotinamide[22,62,139]. Therefore, it is interesting that nicotinamide and its analogues promoted autophagic removal of damaged mitochondria **in** fibroblasts[140] and reduced Aβ toxicity in rat cortical neurones[141]. 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[46]**.** Nicotinamide analogues similarly slowed cognitive decline and neuropathology in a 3xTgAD mouse model of AD[142].

Resveratrol can stimulate Sirtuin-1 *via* AMPK (see above), and it also possesses an AMPK-independent mode of Sirtuin-1 recruitment accounting for its ability to blunt the neurotoxicity of Aβ25-35 fragments in PC12 cells[143]. This involved a role for the DNA-repair protein, poly(ADP-ribose)polymerase-1 ("PARP"), of which the direct inhibition boosted nicotinamide levels to favours autophagy and mitophagy[144]**.**

Cilostazol (a phosphodiesterase-3 inhibitor) mimicked resveratrol in clearing Aβ42 from neuronal cell lines by promotion of autophagy, and it upregulated Beclin 1, Atg5 and LC3 while down-regulating mTORC1 and inducing lysosomal cathepsin B. These actions of cilostazol

involved recruitment of Sirtuin-1 as well as upstream Tyr-172 phosphorylation of AMPK[91]. Cilostazol improved cognition and reduced levels of A42 and hyperphosphorylated tau following intracerebroventricular injection of Aβ(25-35) into mice[145,146].

Protein deacetylation, as effected by inducers of Sirtuin-1, is of broader relevance to the ALN as reflected in activation of Atg gene transcription[18,147]. Further, acetyl transferases like p300 are druggable[18,148] and their inhibition (by garnicol) protected against autophagic deficits in a rodent model of PD[149]. Another p300 inhibitor, spermidine, has attracted attention by virtue of its autophagy-related increase in longevity[147] and spermidine inhibited the acetylation of Atg proteins 7, 11 and 15 as well as Histone 3, while indirectly inducing Beclin 1 by blocking its cleavage by caspase-3[150]. Spermidine also, by analogy to rapamycin, decreased disease progression in a mouse model of FTD[151] and reduced α-synuclein toxicity in *C. elegans*[152]. Depletion of acetyl coenzyme-A would also be worth exploring in models of NDAs[153]. Underpinning interest in inhibitors of acetyl transferase, p300 expression is increased in AD brain and involved in the aberrant acetylation of tau[148,154].

Inducers of autophagosome formation

As outlined in **Box 3**, the cell-permeable peptide, **Tat-Beclin**, increases autophagy by competitive inhibition of the Beclin 1 binding protein, "GAPR-1"[156]. 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[157]. 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[17,148,158]. 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[159]. 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, albeit with an uncertain mode of action[160]. Other potential approaches to Beclin 1 activation include inhibitors of (tau-phosphorylating) cyclin-dependent kinase-5[161].

The multi-modal agent, resveratrol, induced the formation of Atg4 and promoted autophagosome formation: this led to accelerated degradation of polyQ-Htt aggregates and protected SH-SY5Y cells from toxicity[162]. An unusual approach to augmenting autophagosome formation is represented by brain-penetrant "Autophagy Enhancer-99" (Auten-99) which blocks "Jumpy", a phosphatase that inhibits phosphotidyl-inositol-3-kinase-mediated generation of the autophagosome membrane (**Figure 3**). Auten-99 augmented autophagic flux in isolated neurones,

increased markers of autophagy in mouse brain and slowed neurodegeneration in *Drosophila* models of PD and HD[163].

Promoters of autophagosome transport and lysosomal fusion

Disruption of cytoskeletal networks and loss of axonal microtubule function, which occurs upon dissociation of tau, compromises transport of autophagosomes and late endosomes to lysosomes and hence impedes degration of neurotoxic proteins: **axonal transport of retromers (and protease-deficient lysosomes) is also decreased in AD 164 Maday, 2016; Tammineni et al, 2017**. Accumulation of autophagosomes and lysosomes in axonal swellings is linked to local APP processing into Aβ42 and plaque formation[14,164]. The microtubule stabilizer, paclitaxel, countered Aβ42-induced microtubule disruption, restored autophagosomal transport and promoted autophagy in neurones[165] while epothilone D countered microtubule disruption and cognitive deficits in aged P301S/P19 AD mice[166]. Nonetheless, a risk of excessive cytoskeletal rigidity should not be neglected, so mechanisms that promote microtubule/actin *dynamics* and cytoskeletal shuttling of autophagosomes/endosomes to lysosomes present alternative strategies for evaluation[167]. Several other, potentially-targettable mechanisms might also aid autophagosome delivery to (and fusion with) lysosomes[168]. These include Rab and Rab-effector proteins which facilitate the assembly of Synataxin17-SNARE complexes critical for fusion[169]. **Interestingly, genetic or pharmacological activation of Rab5 countered neurodegeneration in mouse C9orf72 models of ALS and FTD (Shi et al, 2018).** 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[31,170]. 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

After fusion of autophagosomes with lysosomes, neurotoxic proteins are degraded and amino acids released for re-utilization. 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 PS1 mutation, cAMP acidified lysosomes and augmented the availability of cathepsins[171]. *Second*, the TFEB inducer, 2-hydroxypropyl-β-cyclodextrin promoted the acidity of lysosomes in neurones[172]. *Third*, acidic nanoparticles like polylactic acid and poly(lactide)co-

glycolide increase acidification (**Box 3**). *Finally*, 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, justifying future studies in models of NDAs[173,174]. Nevertheless, the above strategies need better linking to improved neurotoxic protein clearance in NDAs. Further, it remains a challenge to act on the *causes* of poor lysosomal acidification, such as v-ATPase activity and insertion into lysosomes as well as deficiencies in progranulin[33,51,73,175].

Dysfunction of PARK9/ATP13a2 leads to an imbalance in the handling of zinc, a disruption of lysosomal activity and accumulation of α-synuclein[66]. 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[66,177]. Indeed, clioquinol countered disruption of autophagy by chloroquine in retinal cells, reduced Aβ42 accumulation in CHO cells expressing APP and mutant PS1, and diminished amyloid-misfolding and aggregation in Tg2576 AD mice[177,178]. Cathepsins are an important class of lysosomal hydrolase. Cystatin B and C are endogenous antagonists of the cysteine-active site on cathepsins and their genetic down-regulation ameliorated deficits in lysosomal proteolysis, synaptic plasticity and amyloid clearance in TgCNRD8 AD mice[179]. Pharmacological mimics of cystatins are currently being sought. **In addition, upregulation of retromer complex stimulates provision of hydrolases to the lysosome (Tammineni et al, 2017).**

Lysosomal enzyme replacement is a staple treatment for primary LSDs: for example, β-glucosidase supplementation for Gaucher's Disease (**Suppl Box 1)**[36]. 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[181]. Another glycosphindolipid synthesis blocker, miglustat[36] showed activity in cellular and *in vivo* models of PD[64], 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[36]. It increased expression of β-glucocerebrosidase, normalised autophagy and accelerated degradation of α-synuclein in a stem-cell model of dopaminergic neurones derived from PD patients bearing mutations for β-glucocerebrosidase[182]. Ambroxol, which also decreased ER stress in *Drosophila*[183], reduced α-synuclein levels in overexpressing, transgenic mice[184]: it is being evaluated for use in idiopathic PD. A downside of ambroxol is that it occludes the catalytic site of β-glucosidase, but novel agents like NCGC607 avoid this untoward effect[185]. 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**)[77].

Finally, a more global approach for harnessing lysosomal activity would be the induction of TFEB[18,20]. Harnessing TFEB by 2-hydroxypropyl-β-cyclodextrin promoted the clearance of proteolipid aggregates and α-synuclein in a cellular model of PD[176,186]. Reflecting increased transporter-driven clearance, it also augmented the elimination of Aβ in a Tg19959/CRND8 mouse model of AD[155]. 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[134]. Modulation of DNA methylation and histone marking offer further prospects for transcriptional control of lysosomal activity, while miRNAs could intervene at the level of translation **(Box 3)**[18,148].

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 shown for any drug (Suppl Table 1). Nonetheless, potential long-term effects remain under study and no medication that specifically and exclusively induces the ALN has as yet been therapeutically characterized. Further proof of target and mechanistic engagement in human brain remains challenging. Hence, it is premature to conclude as regards their efficacy (see also Perspectives below)**

In fact, the anti-oxidant, edavarone, which *decreased* autophagy in ischaemic brain and macrophages[187], was recently authorized for use in a subset of ALS patients(**Suppl Box 3**)[188]. **This appears paradoxical, but fits with the suggestion that** *high* ALN flux is *detrimental* under conditions of severe cellular stress in ALS[77] (Suppl Box 3). Whether decreased ALN flux is genuinely implicated in its clinical actions remains to be confirmed[3,188].

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[147,189]. 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[23,147]. Nonetheless, efforts to find autophagy-inducing mimetics that respect cerebral energy requirements are continuing[147] and clinical trials of caloric restriction and nutraceuticals should prove instructive[23,147]. 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[19,189].

Strategies for enhancing neurotoxic protein clearance by CMA and the UPS

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[2,56,62,67]. 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, recognition and elimination of neurotoxic proteins[2,56,190]. Of particular interest is Hsp70 which interacts with the E3 ubiquitin ligase "CHIP" to aid ubiquitination of proteins destined for proteasomal destruction[190]. Hsp70 binds to **Heat Shock Factor** 1 (HSF1) and, under neurotoxic protein stress, dissociation leads to their mutual activation with HSF1 driving transcriptional generation of Hsp70 and other chaperones that facilitate proteostasis[190,191]. Hsp70 also exerts a more general role in the refolding and disassociation of aggregated proteins[2,3,43]. One promising agent is the hydroxylamine derivative, arimoclomol, which increases the activity of Hsp70 by augmenting transcriptional activity of HSF1[192]. Arimoclomol rescued cultured motoneurones from oxidative stress and from the pro-apoptotic actions of staurosporine[193]. It also mediated the removal of mutant SOD1 aggregates and improved motor function in a mouse model of ALS[194]. Supporting interest in arimocomol, it mimicked recombinant Hsp70 to reverse 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 proteins: these findings suggest potential utility in HD[195]. Further, Hsp70 has been pharmacologically co-administered with inhibitors of the deubiquitinating enzyme, USP14, like IU1 and its more potent derivative IU1-47**,** to enhance proteasomal degradation of tau[196-198]. **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 proteosomal

subunits **(Harrigan et al, 2018). I**nterestingly, USP14 inhibitors promote the ubiquitination activation of Beclin 1 to recruit the ALN[198]

Hsp90 counters the effects of Hsp70 by forming a complex with it to impede substrate ubiquitination: it likewise exerts a suppressive influence on HSF1[192,199]. Amongst compounds that inhibit Hsp90, geldanamycin promoted elimination of both hyperphosphorylated tau and oligomeric α-synuclein in cell lines[200,201]. Moreover, geldanamycin reduced Lewy-like bodies[202] and Htt aggregates in *Drosophila* neurites[203] and reduced tau in AD mice[200]. The less cytotoxic analogue of geldanamycin, 17-AAG, has improved brain penetrance. It decreased Aβ levels,[204] improved memory[205] and lowered tau in transgenic AD mice[205]. 17-AAG also reduced α-syn oligomers in H4 cells[201] Another Hsp90 inhibitor, HSP990, has shown promise in that it lowered Htt aggregates and improved motor performance in two mouse models of HD[206].

Modulation of the phosphorylation status of the proteasome

Numerous targetable classes of kinase phosphorylate the proteasome[56,208]. **(Verplank and Goldberg et al, 2017)** Phosphodiesterase inhibitors protect cAMP from degradation to recruit protein kinase A and boost UPS activity. Correspondingly, rolipram relieved rat cortical neurones of AD pathology[209]. 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[210,211]. Rpn6 activation may also be involved in the anti-ageing effects of caloric restriction[62]. Interestingly, resveratrol inhibits Phosphodiesterase-4, suggesting that proteasomal recruitment may be yet another component of its global impact on neurotoxic protein clearance[96]. One concern with phosphodiesterase inhibitors/protein kinase A inducers is their huge range of targets (including AMPK), as well as side-effects, but it may be possible to target proteasome-specific isoforms. Further, acting upstream of cAMP may improve specificity. Chronic administration of CGS21680, a selective agonist at AC-coupled Adenosine-2A receptors, restored proteasomal activity in cellular and murine models for HD *via* protein kinase A-enforced **Ser-120** phosphorylation of the Rtp6 component of the 19S subunit[212].

Another kinase that activates the proteasome (Rpt6 subunit) - and directs it to dendritic spines - is Calmodulin-dependent kinase II **(VerPlank and Goldberg, 2017):** its recruitment may account for proteasomal activation by the $GABA_A$ receptor antagonist, bicuculline[42,213]. Protein kinase G similarly activates the proteasome and sildenafil's inhibition of cGMP breakdown-reduced neurotoxic protein aggregation in cardiomyocytes, encouraging studies in NDAs[56,208]. P38 mitogen-activated protein kinase, which accumulates in NDAs, *indirectly* influences the

phosphorylation status of the proteasome, likely *via* cAMP signalling[3,56]. P38 depletion, or its blockade by PD169316, accelerated the degradation of ubiquinated proteins, specifically promoting α-synuclein clearance and improving cell survival[214].

Phosphorylation is a dynamic process, and small molecule inhibitors of the nuclear proteasome phosphatase, "UBLCP1" suggest that calcineurin and other cytosolic phosphatases represent hitherto-unexploited targets for boosting UPS-driven clearance of neurotoxic proteins (VerPlank and Goldberg, 2017).

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 immunomodulatory drugs like pomalidomide, binding of which changes ligase specificity to encourage degradation of discrete classes of protein[215,216]. *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 permit polyubiquitination and UPS-driven removal (**Box 3**)[215,217]. Certain agents amplified PROTAC-mediated breakdown of α-synuclein[214], while other classes of bifunctional ligand bind a target protein plus Hsp70 which directs UPS degradation[216]. *Third*, target proteins can be bound by agents bearing bulky, hydrophobic adamantyl tags which provoke conformational instability and encourage proteasomal elimination[215]. *Fourth*, the cytosolic antibody receptor, "Tripartite Motif Protein 21" binds to the Fc domain of 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[218]. *Finally*, "Cellular Inhibitor of Apoptosis Protein" specifically binds mutant SOD1, thereby driving it to proteasomal degradation. This provides another potential path to discrete elimination of unwanted proteins in NDAs[219].

Control of transcription factors generating UPS components

The transcription factors NRf1 and Nrf2 are both substrates of proteosomal degradation, as well as inducers of proteosomal synthesis, and the latter has been specifically linked to neurodegenerative diseases (Vangala et al, 2016; Pajares et al, 2017) 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[132]. Interestingly then, translocation of Nrf2 to the nucleus is promoted by triterpenoid derivatives which

counter the ageing-related diminution of UPS activity[220]. In addition, sulforaphane elevates proteasome levels *in vivo* by inducing Nrf2*,* protects neurones against oxidative stress and has been proposed for the treatment of HD[221]. Several other agents promote the proteolytic competence of proteasomes and facilitate clearance of Aβ and/or tau in cellular models, including betulinic acid. Although enhanced transcription has been implicated in their actions, this remains to be clarified[221]. **Finally, mirroring the inhibitory influence of mTORC1 on the ALN, it also suppresses the formation and assembly of proteosomal subunits, so its suppression may promote UPS degradation of neurotoxic proteins in parallel (Rousseau and Bertolotti, 2016).**

Modulation of CMA-mediated clearance

Some mechanisms outlined above for the UPS, like increasing chaperone-driven delivery of client proteins to degradative machinery, are also relevant to the CMA, **and an approach along these lines has been proposed for selectively removing mutant htt (Bauer et al, 2010).** In fact, *specific* induction of CMA has received little attention, possibly since the rate-limiting element LAMP2A has proven intractable for small molecule chemistry. **Nonetheless, over-expression of LAMP2A accelerated CMA clearance of α--synuclein - and reduced its disruption of CMA while affording protection of dopaminergic neurons (Xilouri and Stefanis, 2015). Several routes to potential therapeutic exploitation may be evoked. *First*, Cathepsin A cleaves (mainly monomeric) LAMP2A resulting in its lysosomal degradation, so selective inhibitors should reinforce CMA 39 (Kaminskyy and Zhitovsky et al, 2012). *Second*, LAMP2A is stored in cholesterol-rich membrane regions: hence, cholesterol depletion might enhance transfer to regions where it becomes functionally active (Catarino et al, 2017). *Third*, the dynamics of LAMPA2A/translocation complex assembly are (oppositely) controlled by mTORC2 and the phosphatase "PHLPP1" which offer potential targets for boosting CMA (Arias et al, 2015).** *Fourth*, CMA is under the negative control of Retinoic Acid Receptorα and blockade by synthetic all-trans retinoic acid derivatives resulted in upregulation of CMA, including the activity of LAMP2A - despite its lack of a relevant promoter[222]. Mouse fibroblasts treated with these agents showed improved resistance to combined over-expression of α-synuclein and oxidative stress[222].

Importance of early intervention

There are, then, emerging opportunities for intensifying the elimination of neurotoxic proteins by the UPS and the CMA, both at the level of ubiquitination/folding and of degradation[56,208]. By analogy to the ALN, it is important that CMA and UPS effected elimination is

homeostatically regulated because *excess* activity is potentially dangerous[220]. Since the UPS and CMA are disrupted by neurotoxic proteins like Aβ42 and tau, early and preventative reinforcement of UPS and CMA elimination may be critical. This might be particularly efficacious when enacted in dendritic sites of neurotoxic protein spreading, where UPS reinforcement of clearance would also counteract NDA-related deficits in synaptic plasticity and learning[39,42,56].

Interplay between the UPS and ALN: therapeutic relevance

As pointed out above, there is evidence of coordinated regulation of the ALN and UPS *via* mTORC1 (Rousseau and Bertolotti, 2016). 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[223]. This finding underscores the reciprocal interplay between these clearance systems[3]. Indeed, the ALN can "sense" UPS failure to compensatorily upregulate its own activity. For example, proteosomal failure exacerbates ER stress and leads *via* the UPR to the expression of Sestrin-2 which recruits AMPK to down-regulate mTORC1: Nrf2 is also upregulated[224]. Supporting the potential therapeutic relevance of Sestrin-2, it protects dopaminergic neurones from the neurotoxin, rotenone, *via* AMPK-transduced autophagy[225]. Sestrin-2 overexpression also prompted mTORC1-dependent autophagy in cortical neurones in a presenilin-knockout model of AD[226]. Proteosomal 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 39, 67.**

Extracellular elimination of neurotoxic proteins and its impairment in NDAs

Exosomal liberation of neurotoxic proteins from neurones

When intracellular pathways of protection prove insufficient, neurones 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[69,227].

Exosomes are involved in the release of tau, APP/Aβ-42 and α-synuclein. Accordingly, they are linked to the progression of NDAs[66,69,228,229]. Intriguingly, when the ALN is overwhelmed and cargo accumulates, a specialised process of "*autophagic*" exocytosis participates in the neuronal liberation of neurotoxic proteins, accelerating transmission of pathology to

interconnected neurons. This discharge of neurotoxic proteins adds to the extracellular burden from dying cells, and underpins the importance of clearance mechanisms *extrinsic* to neurones[230]. **228** In this light, recuperation and digestion of extracellular proteins by glial cells is primordial[7]**.** However, there exist several other, therapeutically-pertinent mechanisms for ridding the brain of extracellular pools of neurotoxic proteins.

Clearance by proteases in the extracellular space

Neurones and glia contain many classes of protease, localized in all compartments where neurotoxic proteins accumulate - cytosol, mitochondria and even the nucleus[231-234] **Kaminskyy and Zhivotovsky, 2012).** However, certain intracellular proteases generate toxic fragments, notably of tau (calpains and caspases) and Htt (matrix metalloproteinases (MMPs)[235]. **(Kaminskyy and Zhivotovsky, 2012). Accordingly, their *inhibition* is of potential interest in the treatment of disorders like AD and Huntington's disease. Nonetheless, in addition to LAMP2A-degrading Cathepsin A (above), the proteases most relevant to promoting clearance** are those actively secreted, located on exosomes and/or presented on plasma membranes and which degrade extracellular pools of neurotoxic protein (**Figure 1**)[232]. They include several classes of MMP, neprilysin, insulin-degrading enzyme (IDE) and plasmin, all implicated in NDAs[231,234,236,237].

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/UPS elimination[237]. Cerebral levels of IDE are reduced in early AD and in mouse models of AD while, mirroring AD, Aβ42 accumulates in mice genetically depleted of IDE: in a vicious circle, Aβ42 itself decreases IDE expression[232,237]. IDE also degrades and prevents the formation of α-synuclein fibrils[237]. By analogy to IDE, neprilysin catabolizes Aβ42 and its loss in mouse models of AD and patients alike also contributes to levels Aβ42 accumulation[231,234,238].

Another Aβ42-degrading protease, plasmin, is derived from inactive plasminogen by actions of tissue-type plasminogen activator (used to treat stroke) or "Urokinase": it is secreted by neurones (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[232,234,239]. Plasmin also degrades α-synuclein to retard intercellular spreading[240].

Interestingly certain isoforms of MMPs cleave *fibrillar* as well as monomeric Aβ42[232] and extracellular α-synuclein is also a substrate for MMP-3[234,236]. Another protease with

pharmacotherapeutic potential is angiotensin-converting enzyme which contributes, albeit less prominently, to the degradation of neurotoxic proteins in NDAs[241]. Finally, the extracellular and intracellular (neuronal and glial) 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[242].

Clearance via the blood-brain barrier and the glymphatic system

In AD 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**)[243-251].

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**)[243-248,251]. In addition, proteins are degraded by vascular smooth muscle and endothelial cells of the BBB itself[249,250]. In ageing, AD and PD, a diminution of BBB-localized P-glycoprotein efflux transporters compromises elimination of neurotoxic proteins[246,252]. 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[248,250,251]. An ApoE4 genotype in AD exacerbates poor Aβ42 clearance by reducing its transport to the BBB and diminishing efflux[250].

Arterial pulsing aids CSF/ISF flow in flushing out interstitial extraneuronal proteins *via* the complementary glymphatic system (**Figure 1**)[243,245,249]. Its regulation is not well understood, but roles for Aquaporin-4 water channels, other astrocytic mechanisms and noradrenaline have been documented[253,254]. Deletion of Aquaporin-4 in astrocytes markedly reduced glymphatic flow and aggravated Aβ42 accumulation in a genetic mouse model of AD[254,255] while Aquaporin-4 expression is altered in the ageing, AD and PD brain[254,256]. Loss of sleep has been linked to an impairment of glymphatic clearance[253]. This is important since "rapid eye movement sleep-behavior disorder" is the most robust predictor of PD and, together with insomnia and anomolous sleep patterns, also occurs in other NDAs like early-onset AD, where disrupted sleep is correlated with alterations in Aβ levels[257].

Transfer of neurotoxic proteins into the circulation reduces their propensity to exert

detrimental effects on neurones and to trigger spreading[245]. Augmenting extracellular clearance is, therefore, an attractive goal for therapeutics.

Strategies for promoting extracellular clearance of neurotoxic proteins

Increasing protease-driven clearance

Overexpression of neprilysin or IDE reduces levels of Aβ42 and amyloid plaque burden in senescence-accelerated mice[234]. Suggesting feasibility of their exploitation, epigallocatechin, somatostatin and several other classes of compound promote the expression, secretion and (allosterically) catalytic activity of IDE and neprilysin in parallel with increased degradation of Aβ peptides[237,258]. Further, expresson of progranulin in the hippocampus of AD mice reduces the density of amyloid plaques by enhancing the activity of neprilysin[259]. Epigenetic regulation of neprilysin at the level of histones, as exemplified by valproate, offers another potential approach to potentiation[231]. As regards other proteases, augmentation of plasmin clearance by blockade of the endogenous plasminogen inhibitor "PAI-1" (the expression of which increases with ageing and in mouse models of AD) reduced Aβ levels and restored memory deficits in mouse models of AD[239,260].

These observations underscore the interest of proteases as targets for degradation of neurotoxic proteins[231]. Further, several drugs evoked above like resveratrol and curcumin induce IDE and/or neprilysin, suggesting a contribution to their actions[231]. Nonetheless, structure-activity relationships for small molecules that directly enhance the catalytic activity (or production) of proteases are not well-characterised[231,261]. 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[237]. Neprilysin targets a range of substrates like atrial natriuretic peptides and substance P, and *inhibitors* are employed in the therapy of heart failure,[231] while MMP activators exert deleterious as well as beneficial effects reflecting their influence on microglia and the BBB[236,262]. 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[231,237].

Immunotherapy for neurotoxic protein sequestration

Immunotherapies for neurotoxic protein clearance in NDAs have been pursued for over a decade, and include intravenous immunoglobulin which held promise in Phase II

(NCT00299988) but failed in Phase III trials. As reviewed elsewhere[69,263], the most advanced approach is currently antibodies for sequestering extracellular pools of Aβ and tau (AD) or α–synuclein (PD) and enhancing Fc receptor-facilitated uptake and destruction by microglia[7,264]. BBB penetration is limited, but they may generate a "peripheral sink" in addition to central actions. 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[265].

Furthermore, there are at least 5 antibodies under investigation for tau, including a Phase II trial (NCT02880956) for C2N8E12 in AD[266]. Another trial (NCT02985879) in post-cerebral palsy is employing a single-chain, passive antibody targeting extracellular tau. This is the second tau-based Phase II trial after AADvac-1 (NCT02579252) to use an *active* immunotherapy approach. Passive immunity approaches are also being tested using the PHF1 (Ser396/Thr404) epitope (ACI-35; ISRCTN13033912) and Ser409 epitope (RG1600; NCT03289143)[69]. Targeting extracellular tau to block intercellular spreading[227] should preclude the need for high antibody inclusion into cells for efficacy. Antibodies like PRX002[267] have also shown promise for reducing extracellular α-synuclein and propagation of pathology, and Phase I testing has been completed (NCT02157714 and NCT02095171)[263].

Potential problems should not be neglected, like deposition of immune-complexes in vascular tissue, inaccessibility of tau in exosomes, and antibody-driven *import* of Aβ into the brain. Nonetheless, employing biomarkers for identification of subjects with early-phase disease, surrogate/functional biomarkers of efficacy, and more effective antibodies, there are still reasonable prospects for achieving course-alteration with immunotherapy.

Improving BBB-mediated and glymphatic transfer to the circulation

As mentioned above, the BBB is equipped with potentially-targetable transporter proteins, channels and receptors (**Figure 1**)[246,248,251]. Inhibition of the α-secretase, "ADAM10" was found to drive LRP1-mediated extrusion of Aβ42 into the circulation[268]. In addition, LRP1 might be indirectly modulated by Aquaporin-4 channels[254] and epigenetically *via* miRNAs[148]. Further, the hydroxymethylglutaryl-coenzyme-A inhibitor, fluvastatin, upregulated LRP1 in the BBB to reduce Aβ42 load and provoke extrusion[269]. The antibiotic, rifampicin, likewise promoted Aβ42 clearance by inducing BBB-localised LRP1 as well P-glycoproteins[252,270]. Whether LRP1-driven uptake of Aβ42 by microglia (and hepatocytes) is involved in the beneficial effects of LRP1 up-regulation

remains to be clarified[250]. Interestingly, both fuvastatin and rifampicin have additional actions - including a probable induction of the ALN - that contribute to beneficial actions in models of AD[269,271]. As for RAGE receptors, their blockade should temper re-entry of Aβ into the brain - and exert anti-inflammatory properties[272,273]. Phase III studies are underway with azeliragon (TTP488) in AD (NCT02080364; 02916056) following promising improvement in cognition in a Phase II trial[274]. Interestingly, resveratrol downregulated RAGE as well as MMP-9, actions related to decreased hippocampal load of Aβ42[275]. 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[276]. 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[277,278]. 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[279].

Activation of Aquaporin-4 channels on perivascular astrocytes to aid the glymphatic system elimination of cerebral Aβ and other toxic proteins is a potential strategy for stimulating clearance. Antagonists have been identified as well as positive modulators, so this seems "chemically" feasible[249,251,254,255]. Reflecting a contrasting strategy, dobutamine stimulates arterial pulsation and the perivascular/glymphatic CSF flushing of neurotoxic proteins from the ISF *via* lymphatic conduits into the blood[245]. Deposition of Aβ42 in cerebral vessels impairs vascular function-flexibility and is accompanied by an upregulation of Phosphodiesterase-3 in smooth muscle cells[280]. 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[280]. Intriguingly, a retrospective clinical analysis suggested that cilostazol abrogates cognitive decline in patients with mild cognitive impairment and modest dementia[281]. Adrenergic mechanisms influence ISF volume and hence neurotoxic protein clearance[253], and additional pharmacological opportunities for promoting glymphatic efflux will likely emerge from an improved understanding of its regulation by astrocytic, neurotransmitter and other mechanisms[249,251,253].

Disruption of sleep impedes glymphatic clearance of neurotoxic proteins, so encouraging sleep hygiene should promote CSF/ISF transfer to the periphery. The atypical antidepressant and sleep-promoting agent, trazodone, is of particular interest since it normalized an over-protracted Unfolded Protein Response and reversed pathology in animal models of tauopathies (**Suppl Box 2**)[83]. Therapies that favour sleep or specifically counter sleep syndromes in NDAs may prove beneficial for improving glymphatic clearance of proteotoxic substrates and abating disease progression[243,249,257]. **Interestingly, alcohol displays a J-shaped curve, with low and high consumption respectively enhancing/reducing glymphatic function – and moderating/aggravating the risk of dementia (Lundgaard et al, 2018).**

Finally, a recent study in human subjects revealed that 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[282]. If confirmed, these observations may open a new avenue of research for clearing extracellular neurotoxic proteins in NDAs.

Therapetic 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[283]. Moreover, hub proteins like AMPK, mTORC1 and Sirtuin-1 impact both the ALN and manifold other processes implicated in NDAs, such as energy homeostasis[19,283,284]. Hence, drugs that modulate their activity will have beneficial and/or deleterious actions *beyond* their influence on clearance. Indeed, potential side-effects hould 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[19,69,284].

Third, numerous mechanisms remain to be pharmacologically manipulated. These include receptor tyrosine kinases for the ALN and "upstream" GPCRs *potentially* for all modes of elimination[24,26] **(Fraser et al, 2017).** For the ALN, additional targets include the Vps34 complex, Histone Deacetylase-6, Rab proteins implicated in autophagosome-lysosome fusion[169] and v-ATPase, crucial for lysosomal acidification[33]. There has been much recent progress towards manipulation of the UPS, whereas exploitation of the CMA remains a major challenge[2,39,56,68]**.** 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 **(Jansen et al, 2014; Xilouri and Stefanis, 2015) .** Further research is needed to confirm, clarify and potentially exploit the role of glymphatic clearance in the elimination of neurotoxic proteins in NDAs[285]. Another line of research could focus on the comparatively-neglected 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 role in clearance of Aβ42 is impaired in AD[251,286,287].

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)**[1,3,4,9,21]. 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 and measures should facilitate the development of translational readouts for facilitating clinical trials. **This is important since they are onerous and costly. Studies of the multi-functional ALN promoter and aggregation inhibitor, Methylene Blue exemplify the challenges faced in terms of patient selection, trial design, dose-response relationships, structural and functional 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[69,288]. 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 the 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[288]**.** In addition, retinal

imaging offers a window on cerebral clearance of tau[289] while biomarkers of neurovascular flow from the brain to the circulation are under development[249,250].

Sixth, the therapeutic strategies evoked herein are likewise 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[81]. Accordingly, studies in transgenic mice and fibroblasts from patients suggest that reinforcing Beclin 1 dependent ALN flux would be a beneficial intervention[290,291]. Blockade of mTOR1 to induce autophagy may likewise be useful[122]

Finally, reinforcing clearance might best be undertaken in association with other strategies like suppression of protein misfolding, restoration of cerebral energetics or moderation of neuroinflammation[7,23,147,163,284]. 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 and neurovascular/glymphatic 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 diseases 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.

Amyloid β42: The major neurotoxic product of APP processing that deposits into extracellular amyloid 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 system for flushing ISF-located neurotoxic proteins into the circulation that 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 act in detoxification. They can be generated by budding-off the endoplasmic reticulum and replicate *via* fission. Pexophagy refers to autophagy of peroxisomes.

Lysosomes: An acidic compartment for the degradation of proteins and other cellular constituents. Their breakdown yields products like amino acids and lipids which are recycled.

Autophagy-regulating genes: Autophagy was originally characterised in yeast by Y. Ohsumi (Nobel prize in Physiology or Medicine, 2016). The associated genes, identified using mutants, were termed Apg1-15. In view of conservation across species, the essentially same terminology is used for genes/proteins that regulate autophagy in humans.

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 and mitophagy: 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. (As part of a complex mTORC2, mTOR functions as a tyrosein protein kinase exerting other roles).

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; 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 autophagosomes with lysosomes.

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

Phospholipase D: Enzyme involved in the transformation of various lipids and involved in the fusion of autophagosomes and lysosomes.

Lysosomal storage disorders: Diseases resulting from genetic mutations that lead to failure of lysosomal digestion and cellular 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's Type C disease: Disease triggered by a defect in the NPC1 gene responsible for cholesterol transport. NPC patients often display Aβ42 and tau pathology, underpinning parallels to Alzheimer's disease in which cholesterol transport is also disrupted.

Hsc70: Hsc70 (Heat shock cognate 71kDa 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.

KFERQ: The KFERQ motive on a protein is the principal criterion for CMA capture. 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). Post-translational modification can, however, modify susceptibility of proteins with a KFERQ signal for CMA.

Lipofuscin: Pigmented cellular inclusions composed of undigested lysosomal content, 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 neurones 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 debated.

Apolipoprotein Epsilon 4 (ApoE4): Robust genetic risk factor for Alzheimer's disease as compared to ApoE2 and E3 alleles. ApoE is secreted by astrocytes and binds lipids like cholesterol which are carried to neurones. 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 (PS1): Catalytic unit of the γ-secretase complex that processes APP into β-amyloid. Mutations associated with familial AD, but altered APP cleavage unlikely to be the sole explanation. Rather, a role for reduced lysosomal acidification has been proposed based on deficits in maturation and translocation of vATPase subunits to the lysosome. Deficient mitophagy may also be implicated.

Amyloid precursor protein: Transmembrane protein highly expressed in neurones and involved in maintaining cell-cell contact. Successive cleavage by β- and γ-secretases results in the formation of 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 among affected carriers. The activity of β-glucocerebrosidase is impaired in a sub-population of idiopathic 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 diseases like Huntington's disease, Spinocerebellar Ataxia 3/Joseph-Machado disease (Ataxin-3), and Spinal and Bulbar Muscular Atrophy (Androgen Receptor).

TAT-Beclin: Synthetic peptide comprising 11 amino acids of the Human Immunodeficiency Virus Tat protein transduction domain, a diglycine linker and 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 to aid protein-folding: Heat Shock Factor 1 then trimerizes and increases transcription of Hsp70 and other neuroprotective proteins.

Exosome: Small (30-150nm), ceramide-rich, intraluminal vesicles formed from cytosolic endosomes, multivesicular bodies and lysosomes. Released with contents (proteins, lipids, 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 mainly act extrinsically to neurones.

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*[9,21]. 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 enhanced ALN flux[21].

Since LC3-II (membrane-bound) 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 preferred method for measuring cellular autophagosome levels by immunoblot since decreased amounts of LC3-I (cytoplasmic) occur in certain cell types upon conditions of activation[15]. Green fluorescent protein (GFP)-tagged LC3 has proven instrumental for quantifying autophagosomes but self-aggregation of cytosolic GFP-LC3 and the quenching of GFP fluorescence in acidic lysosomes complicates interpretation in cytological assays[21]. 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, a useful approach is to use compounds like bafilomycin or chloroquine which neutralise lysosomal pH and produce an additive elevation in LC3-II under conditions where 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 (e.g., in response to oxidative stress), and reduced protein synthesis in degenerating cells[292]. Therefore, parallel monitoring of p62 mRNA and UPS status is recommended[210]. Phospho-specific antibodies that detect activation states of key autophagy-regulatory kinases like AMPK, mTORC1 and Ulk1 are also useful 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[293]. 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[86]. For example, stimulating autophagy and TFEB nuclear translocation by trifluoperazine prevented neuronal loss in PINK1-deficient zebrafish[294]. 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[295]. In addition, genetic tools are available for manipulating each step of ALN disruption, while somatic mutant clones in subsets of *specific* neurones permit evaluation of ALN in impacted cells surrounded by wild-type tissue[296]. *Drosophila* have been used to validate the effects of drugs regulating the ALN: for example, rapamycin in a polyglutamine model of HD[116]. Nonetheless, mice remain the most common *in vivo* pre-clinical model for modulation of the ALN in NDAs[21] and a broad range of pharmacological agents has been studied, as summarized in **Table 2**. Apart from the brain, retinal tissue has proven instructive: for example, in evaluating axonal transport of acidic vesicles to lysosomes[297].

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 and diminished energy supply, should also be borne in mind[23,44,46,83,298].

Box 2. Defective mitophagy and restoration in NDAs

Neuronal mitochondria support the high energetic costs of a complex and dynamic 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 mitochondrial[19,299]. 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 mitophagic UPR that preserves mitochondrial proteostasis[45,233,300].

Mitophagy refers to a selective type of macroautophagy that leads to degradation of mitochondria (**Figure 2**)[58,300]. 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 mutated in familial PD: the mitochondrial kinase, PINK1 and the E3 ubiquitin ligase, Parkin[57,58]. 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 role in neurones[301]. 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[302].

Whether driven by the PINK1/Parkin system or ubiquitin-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[8,46,300]. 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[224]. This may reflect an adaptive mechanism, using mitochondria to clear aggregates[233]. However, in the long run, aggregation-prone proteins provoke mitochondrial dysfunction and block mitochondrial protein import. Stimulating mitophagy may, thus, improve both mitochondrial function and as well as cytosolic proteostasis[46].

As for pharmacological approaches for promoting mitophagy in NDAs[303], 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[304]. Small-molecule transcriptional activators of Parkin have also been proposed[305]. 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[306]. **(Harrigan et al, 2018). Interference with two other deubiquitinases, USP8 (delays Parkin binding to damaged mitochondria) and USP15 (suppresses Parkin-driven mitophagy) is also under scrunity as targets for promoting mitophagy in NDAs (Harrigan et al, 2018).**

The inner mitochondrial protein, prohibitin-2, directly binds LC3-II to target ruptured mitochondria for degradation and is depleted in human PD brain[10]. Since Prohibitin-2 overexpression is protective in cellular models of PD, it is an interesting target for potential therapy[307]. Compounds that stabilise Nrf2 are also of interest, since Nrf2 triggers Parkin-independent mitophagy by a mechanism involving activation of p62[308]. Replenishment of nicotinamide, which declines with age, may promote mitochondrial clearance by activating Sirtuin-1 driven mitophagy[309]. Further, in promoting mitochondrial proteostasis, nicotinamide derivatives opposed the deposition of Aβ in cellular and mouse models of AD[46]. 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[310]. Other natural compounds, such as urolithin A, promote mitophagy by mechanisms that remain to be determined[311]. Finally, lifestyle factors, like exercise and intermittent fasting, favour mitochondrial and neuronal health by a combination of mechanisms that include the stimulation of mitophagy[8,23,147].

Box 3: Novel, non-small molecule strategies for enhancing intracellular 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* and in mice without engendering cytotoxicity[156].

Aptamers are small oligonucleotides that recognise specific proteins. They offer another chemically-distinctive strategy for modulating clearance. Using this technology, the de-ubiquitinase, USP14[40] could be inhibited to facilitate tau clearance[196]. Inhibiting ubiquitin carboxyl-terminal hydrolase37, another proteosome-linked de-ubiquitinase, may also facilitate proteasomal clearance of neurotoxic proteins[312]. Similarly, aptamers moderated the ALN burden by blocking the misfolding and oligomerisation of tau[313] and α-synuclein[314].

Numerous classes of miRNA are deregulated in NDAs[148], including an increase of miR-34a in AD which neutralizes mRNAs encoding Sirtuin-1 and TREM2[148]. Conversely, miR-132, which likewise interacts with Sirtuin-1, is down-regulated in AD[148]. Another example is the loss of miR-124 in a lesion model of PD[315]. Selective targeting of miRNAs in NDAs is becoming possible using modified oligonucleotides like antagomiRs, locked nucleic acids and miRNA sponges[148]. 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[316].

“PROTACs” (see main text) permit *selective* proteosomal elimination of unwanted proteins. They are composed of two motifs joined by a linker: one recognises a specific protein like tau[217], whereas the other encodes an E3-ligase binding site[215]. 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[93,215]. In the 3XTgAD mouse model, PROTACs moderated levels of tau in the cortex and hippocampus suggesting target engagement in key pathological regions[215]. 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*[214]. Smaller PROTAC variants offer improved stability, higher potency and better structure-activity relationships[317].

Restoring lysosomal acidification using poly(DL-lactide-co-glycolide) acidic nanoparticles proved neuroprotective in preclinical models of PD.[318] Though they are poorly brain-penetrant, nanoparticles with improved pharmacokinetic profiles are being developed. Encouragingly, intranasal delivery reduced 6-hydroxydopamine-induced neurotoxicity in rats[319]. 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[320].

One strategy for *locally* enhancing intracellular clearance is virally-produced gene delivery to the pathological site, avoiding autophagic induction in "healthy" areas[321]. A target protein might be expressed in restricted areas using neuronal-type-specific promoters, like the dopamine transporter in dopaminergic neurones[322]. 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[323]. The latter approach enhanced access of siRNA to the striatum for knocking down mutant Htt[278]. 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[324]. 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[325].

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[326].

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

Neurotoxic proteins are eliminated by a broad suite of specific and non-specific mechanisms expressed in neurones, glial cells and endothelial/vascular smooth muscle cells of vessels. The three major modes of intracellular clearance are shown for neurones, but they are also active in other cells like microglia ("clearance"). **Under conditions of inflammation, proteosomal β-subunits in glia are switched and substrate specificity changes: the precise role of these "immunoproteosomes" - specialized in peptide production for antigen presentation - for neurotoxic protein elimination in NDAs is debated (Jansen et al, 2014**). Clearance also occurs in the extracellular space, the interstitial fluid (ISF) of the brain parenchyma that surrounds neurones, and the CSF with which the ISF exchanges. **Intraneuronal mechanisms of clearance are illustrated by both Aβ42 and tau, but only Aβ42 is shown for extracellular clearance since it has yielded the vast majority of available data.** Extracellular pools of neurotoxic protein are derived from release by terminals, extrusion by exocytosis and diffusion following cell death. They disrupt neuronal and synaptic function and are taken up by other neurones and glial cells ("spreading"). Therapeutically-relevant proteases degrading neurotoxic proteins include endothelin-converting enzyme and insulin degrading enzyme (IDE) (mainly cytosolic), neprilysin and matrix metalloproteinases (MMP) (intracellular and extracellular), and plasmin (mainly extracellular). Neurotoxic proteins 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 neurotoxic proteins 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 neurotoxic proteins *via* glial cells and the peri-venous space back into the CSF. Glymphatic-cleared, CSF-derived neurotoxic proteins 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). Neurotoxic proteins are ultimately eliminated in the kidneys and liver. Abbreviation not in main text: s, soluble.

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

Within neurones 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 "Rpn11" once committed to entering the proteosome pore: other deubiquitinases like USP14 may rescue them before entry[40]. 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 transportd the protein for the LAMP2A receptor on lysosomes where is translocated 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 endosomes, of which the "late" variety is a site for APP transformation into Aβ. The resultant amphisomes then likewise fuse with lysosomes. See also Figure 3.

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. **Thereafter, Phosphatidylinositol 3-Kinase (PI3KC3) generates phosphatidylinositol-3-phosphate (Ptsnins3P), a signal recognised by "WIPI proteins" (WD-repeat-protein-interacting-with-phospholnositides) that induce autophagosome elongation in association with 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. The Figure also

depicts exosomal release of neurotoxic proteins which may occur as a consequence of reduced ALN flux and accumulation of autophagosomes. For details, see main text. Abbreviations not in main text: FIP, Family interacting protein; PE, Phosphoethanolamine and PLD, Phospholipase.

Figure 4: Major molecular sites of action of agents that enhance 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 can be promoted by agents that enhance the activity of proteases like neprilysin, by immunotherapies targeting specific neurotoxic proteins, and by increasing BBB and glymphatic extrusion of neurotoxic proteins 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 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 they eventually become dysfunctional and/or inadequate to cope with the neurotoxic burden. Not all alterations in clearance and other features of specific NDAs are shown in view of space limitations, and essentially all are associated with neuroinflammation/immune deregulation, glial anomalies, disruption of cerebral bioenergetics, mitochondrial dysfunction and ER/oxidative stress. Several variants of frontotemporal dementia (FTD) are recognized, including behavioural, progressive non-fluent aphasia and semantic forms. While a distinct disease, 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 (corresponding protein) are indicated in column one. Examples of genes/proteins incriminated in pathological processes are given in columns 3-6. Genes (proteins) as follows: 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); PSE/2 (Presenilin 1/2); 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); UBQN2 (Ubiquilin 2); UCH-L1, Ubiquitin carboxy-terminal hydrolase L1 (deubiquitinase) and VCP (Valosin-containing protein). Aβ refers to Aβ42 and similar neurotoxic fragments of APP. See main text and following citations for further **information**[1-3,62 243,249,251] **Check ref link to text** Abbreviations not above nor in text: DA, Dopaminergic; GI, Gastrointestinal; MSN, Medium Spiny Neurone; 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 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, aggravates 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 and fusion with lysosomes (MAPT); ↓APP loading (PICALM); APP and CTF 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 behaviour 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. 5-10% *SNCA, PINK1, GBA, PARK2, LRRK2, PARK9, UCH-L1*	Motor impairment (poor gait, tremor, rigidity, bradykinesia); ↓olfaction; GI problems; cognitive deficits; pain; depression; prodromal RBD. Neuronal loss (DA cells in SNPC etc).	α–syn inclusions and Lewy Bodies (contain lipids, α–syn, Tau, other neurotoxic proteins, ubiquitin); ↑α-syn release; spreading in brain and, possibly earlier, 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 α-syn/LRRK2 block);** Slow α-syn dissociation from LAMP2A.**↓ UPS clearance (α-syn aggregates and mutant forms 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 cortex, 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 and spinal cord; inclusions may contain ubiquitin and ubiquitin-ligases.	Mainly ↓ALN, but may be ↑ and/or detrimental if cellular stress severe; ↓Autophagosome maturation (C9ORF72); ↓Cargo loading (SQSTM1, UBQN2, OPTN, TBK1); ↓Autophagosome retrograde transport (DCTN, C9ORF72); ↓Lysosomal function (CHMP2B/GRN); ↓Glial ALN flux (TREM2).	Aggregated proteins including poly GA block proteasome; ↓Hsp70 and Hsp40; ↓ Provision SOD1 and other proteins for UPS degradation (VCP); **↓ CMA clearance of TDP-43.**	BBB disruption; ↓glymphatic flow.

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 MSNs; disruption of corticostriatal pathway; failure of axonal transport.	Aggregates of mutant (excess CAG repeat number) Htt; mutant Htt inclusions with ubiquitin, beclin1, mTOR1, p62 and other cargo-loading proteins; Mutant Htt and Htt fragments 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 becomes *less* efficient in later 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.

The Table is representative of drug classes that exert effects in brain-related cell and animal models of clearance/neurotoxicity. ↓indicates reduced levels of a specific neurotoxic protein etc. For *in vitro* and *in vivo* models, cell line/species is given followed by drug action in the procedure/model indicated. SK-N-SH and its sub-line SH-SY5Y as well as M17 are immortalized, 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) or lipopolysaccharide (pro-inflammatory). In addition, prostaglandin J2 is neurotoxic to cells, but note that in this study IU1 itself induced tau cleavage at "protective" concentrations. Mutant protein variants are given as superscripts: e.g., Syn^{A53T}. YFP signifies yellow-fluorescent protein tagged, pro-aggregating proteins that fluoresce when they oligomerise. For *in vivo* models, overexpression of mutant forms of neurotoxic protein has commonly been used, in certain cases tagged with Green Fluorescent Protein (GFP) for improved visualization. Specific models employing transgenes and/or mutations (^{superscript}) are listed as, for example, R6/2-Htt^{150}. Transgenic models for HD and other polyglutamine disorders express pro-aggregant proteins bearing multiple CAG repeats. Thus, the R6/2 HD mouse expresses exon 1 of the human HTT gene containing 144-150 CAG repeats, while other HD models employ different numbers of CAG repeats. In a model of Joseph-Machado disease, mice overexpressed Ataxin 3(Q^{70})) with 70 CAG repeats. In a model of spinal and bulbar muscle atrophy, mice overexpressed a PolyQ mutant form of the Androgen receptor. 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 the Swedish plus Indiana (V717F) mutations; APP/PS1 mice bear the APP-Swedish mutation plus the PS1-L166P mutation; 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 comprise overexpression of wild-type or mutant (A53T, A30P) human α–synuclein, including on a α-syn knockout background (*$SNCAKO^{tm1Nbm}$*). R275W is a mitophagy-linked Parkin (PARK2 gene) 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. Certain drugs like resveratrol interact at several nodes in the ALN. For more information, see main text and citations. Abbreviations not above or in text: CaMKK2, Calmodulin Kinase Kinase 2; DA, dopaminergic; icv, intracerebroventricular; MAP Kinase, Mitogen Activated Protein Kinase; PE, Phosphotidylethanolamine; PrP, Prion protein; PS, Presenilin; and PtdnIns, 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: 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})[86]	Mice: ↓Htt, ↑motor function (Htt^{82Q})[87]
Calpastatin, Calpeptin	Investigational compounds (endogenous peptides)	Calpain inhibitors: ↓Cdk5 activation, ↑AMP/AMPK induction, ↓ cleavage Atg proteins	SK-N-SH: ↓Htt(Htt^{Q74})[86]	*Drosophila*: ↓Htt, ↓ neurodegeneration (Htt^{Q46})[44] Mice: ↓Htt aggregates, ↑motor function (Htt$^{171\text{-}82Q}$)[44]; ↓motoneuron loss (SOD1^{G93A})[90], ↓tauopathy(JNPL3-MAPT^{P301L})[89]
AICAR	Experimental agent. Potential treatment for myocardial ischaemia	AMP analogue - allosteric inducer of AMPK	N2a: ↑AMPK[91]; Glia: ↓toxicity(Aβ/LPS)[92]; SH-SY5Y: ↓α-syn (wild-type protein)[93]	-
A-769662	Experimental agent	Allosteric AMPK inducer	Striatal neurones/mouse fibroblasts: ↑LC3 and p62, ↓mHtt and ↑cell viability[94]	-
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[91]**;** ↓Aβ(APP695)[97]; Cortical neurones: ↓Aβ(J20)[97]	*C. elegans*: ↓polyglutamine(Htt^{Q128})[98]; Mice: ↓Aβ (APP/PS1)[97]
Metformin	Antidiabetic. Clinical evaluation for MCI	AMPK activator	SH-SY5Y: ↓α–syn[93]; ↓tau phosphorylation[100], ↓Aβ toxicity[101]	Mice: ↓TH neuronal loss, ↑motor function (MPTP)[102]
Trehalose	Disaccharide. Abiotic stress protectant. Food-additive	Glucose transporter inhibitor, ↑AMP/AMPK activation	PC12 : ↓α–syn(A30P/A53T) / Htt(Q74)[104]; Cortical neurones: ↓tau (Tau_{RD}ΔK280)[105]	Mice: SOD1(SOD1^{G93A})[103] ; ↓Htt (R6/2-Htt^{150Q})[107], ↓tauopathy (PS19-MAPT^{P301S})[108], ↓Aβ (APP/PS1)[106]
Lithium	Mood stabiliser, anti-epileptic Evaluated in FTD and ALS	↓Inositol monophosphate/IP3 AMPK activator?	SK-N-SH: ↓Htt (Htt^{Q74})[109]	Mice: ↑Survival(SOD1^{G93A})[111]; ↓tau/filaments, ↑motor function, ↑autophagy(JNPL3)[110]
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)[85]; Organoypic Hippocampal Slice/Neurones: ↓tau(JNPL3, MAPT^{P301L})[84]	Mice: ↓tau(JNPL3)[84]

Calcitriol (Vitamin D metabolite)	Treatment of Ca^{2++} deficiency.	CaMKK2 potentiator upstream of AMPK	–	Mice: ↓neurodegeneration (C57BL/6/MPTP)[112]
mTOR1 Inhibition	Macrolide. Immunosuppressant (organ transplants). Potential chemotherapy	mTOR1 inhibitor	PC12: ↓α–syn (MPTP)[113], ↓Htt(Htt^{Q74})[114] Cortical neurones: ↓FUS stress granule(FUS^{R521C})[115]	Drosophila: ↓Htt,↓neurodegeneration (Htt^{Q74})[116]; Mice: ↓Aβ/tau(3XTgAD)[119], ↓TDP43/p62 (FTLD-U/TDP43)[117] and neuronal loss (MPTP)[118]
Rapamycin				
Temsirolimus	Renal cell carcinoma	mTOR1/2 inhibitor	SH-SY5Y: ↓hyperphosphorylated tau (okadaic acid)[120]	Mice : ↓tau($MAPT^{P301S}$)[120], ↓α-syn/ neuroprotection(MPTP)[121], ↓Ataxin3 ($Ataxin3^{Q70}$)[122];↓Htt/ ↑motor skills (R6/2)[116]
Curcumin	Tumeric extract. Food colour. Dietary supplement. Clinically evaluated in MCI	Indirect mTOR1 repressor, p300 inhibition causing Atg deactylation	SH-SY5Y: ↓α-syn aggregation(Syn^{A53T})[125,126]; DA neurones: ↑neuroprotection (rotenone)[124]	Mice: ↓Aβ aggregation(Tg2576)[129], ↓tau dimers(hTau)[128], ↓α-syn(GFP-Syn)[127]
Fisetin	Plant polyphenol. Anti-oxidant	mTOR1-dependent activator of TFEB	Cortical Neurones: ↓phospho-tau[132]	Mice: ↓Aβ(APP/PS1)[133]
Nilotinib	Resistant chronic myelogenous leukemia. Clinically evaluated in PD	C-Abl kinase inhibitor, upstream recruitment of mTOR1	M17: ↓TDP43(GFP-TDP43)[137]	Mice: ↓α–syn, ↑motor function(Syn^{A53T})[136], ↓TDP43(TDP43)[137]
Sirtuin1 facilitation	Vitaminin in food. Treatment of niacin deficiency. Clinically evaluated in AD	NAD^+ precursor/Sirtuin1 promoter, Atg deacetylation, FOXO activation	Cortical Neurones: ↓Aβ toxicity (Aβ25-35/1-42)[141]	Mice: ↓Aβ and tau (3XTgAD)[142]
Nicotinamide				
Cilostazol	Treatment of intermittent claudication. Platelet aggregation inhibitor.	Phosphodiesterase 3 inhibitor, Upstream recruiter of Sirtuin-1	N2a: ↓Aβ(APP_{SWE})**[146]**; N2a: ↑AMPK, ↓mTOR1, ↑Autophagosomes, ↑cathepsin B[91]	Mice: ↓Aβ, ↓phospho and acetylated-tau (icv Aβ25-35)**[145]**
Spermidine	Natural polyamine. Potential promoter of longevity	p300 HAT Inhibitor, Atg and Histone H3 deacetylator, ↑Beclin 1	Cortical Neurones/PC12: ↑survival, ↓toxicity(staurosporine)[150]	*Drosophila:* ↑motor function (α-syn) [152]; *C. elegans*: ↓α–syn toxicity (UAS-GAL4-α-syn)[152]; Mice: ↓Aβ(Tg19959)[155], ↓TDP-43(FTLD-U)[151]

Autophagy activators: Enhanced autophagosome formation				
Isorhynchophylline	Plant alkaloid. Investigational compound	↑beclin 1	DA Neurones/N2a: ↓α–syn(Syn^{WT}, Syn^{A53T}, Syn^{A30P})[157]	-
Auten-99	Investigational compound	↑ PtdnIns3P activity (*via* Jumpy phosphatase inhibition)	SH-SY5Y: ↑survival(H_2O_2)[163]	*Drosophila:* ↓neurodegeneration, ↓p62($Parkin^{R275W}$)[163]
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)[165]	Mice: ↓tau (PS19, Tau^{P301S})[166]
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)[176]; N2a: ↓Aβ (APP_{SWE})[155]	Mice: ↓tau, ↓Aβ plaques, ↑memory (Tg19959/CRND8)[155]
Clioquinol	Anti-fungal, anti-protozoal drug	Zinc (and iron) chelator; Increased lysosomal acidification.	Fibroblasts: ↓α–syn(ATP13a2/PARK9 knockdown)[180]	Mice: ↓Aβ(Tg2576)[178]
GZ/667161, GZ/SAR402671	Investigational compounds, Clinically evaluated in PD	Inhibitors of glucosylceramide synthesis, substrate reducers	-	Mice: ↓α-syn/ubiquitin/tau, ↑memory(GBA^{D409V})[181]
Miglustat	Gaucher's disease, Niemann-Pick Type C1 disease	Inhibitor of glucosylceramide synthesis	Mesencephalic Neurones: ↓lipid accumulation in lysosome	Mice: ↓substrate storage, ↑longevity(MPTP)[64]
Ambroxol	Secretolytic for respiratory diseases. Clinically evaluated in PD and Gaucher's disease	Chaperone: aids GBA transport to lysosome	DA Neurones: ↓α-syn(GBA^{N370S})[182]	*Drosophila*: ↓ER stress($GBA^{N370S,L444P}$)[183]; Mice:↓α-syn (*SNCAXSNCAKO*^{tm1Nbm})[184]

NCGC607	Salicyclic acid derivative. Investigational compound	Chaperone: aids transport of GBA to lysosome - no catalytic inhibition	DA neurones from GD patients: ↓glycolipids, ↓α-syn ($GBA^{N370S+/+}$, $GBA^{N370S/c.84dupG}$)[185]	-
HEP14	Investigational compound	PKC-mediated TFEB activation and possibly ZKSCAN3 inhibition	-	Mice: ↓Aβ(APP/PS1)[134]
Facilitators of UPS and/or CMA degradation				
Arimoclomol	Niemann-Pick Type C1 disease. Clinical evaluation for ALS	HSF1 stabiliser, ↑HSP70 chaperone production	Motor Neurones: ↑survival(staurosporine, H_2O_2)[193]	Mice: ↓SOD1, ↓motor loss, ↑longevity($SOD1^{G93A}$)[194]
IU1/IU1-47	Investigational compounds	USP14 (deubiquitinase) inhibitors	Cortical Neurones: ↓tau, Ub-proteins (toxic prostaglandin J2)[197]; ↑tau degradation and ↑ALN flux[198]	-
Geldanamycin	Antibiotic. Potential anti-tumorigenic	Hsp90 inhibitor ↑HSP70 chaperone activity	M17: ↓tau(tau transfected)[200]; H4: ↓α-syn(α-syn-YFP complementation)[201]	Drosophila: ↓ α-syn (α-synA306/504) [202] Drosophila: ↓insoluble (Htt^{Q93})[203]; Mice: ↓tau (JNPL3)[200]
17-AAG	Investigational compound. Potential anti-tumerogenic	Hsp90 inhibitor (improved brain entry), ↑HSP70 chaperone activity	H4: ↓α-syn oligomers (α-syn-YFP complementation)[201]	Drosophila: ↓TDP43(androgen receptor/↑CAG repeats)[207]; Mice: ↓ Aβ and ↓synaptic toxicity/memory impairment (Tg2576)[204,205], ↓tau(JNP3L)[205]
HSP990	Investigational compound	Hsp90 inhibitor, HSF1 promoter, ↑HSP70 chaperone activity	-	Mice: ↓Htt aggregates, ↑motor performance (R6/2)[206]
Rolipram	Investigational compound. Potential use in auto-immune disorders	PDE inhibitor, ↑PKA-mediated proteasome phosphorylation	Cortical Neurones: ↓Aβ/α-syn synaptic damage (human brain extract)[209]	Mice: ↓tau, ↓ubiquitin, ↑improved cognition(rTg4510, JNPL3)[210]
PD169316	Investigational compound	p38 MAPK inhibitor, ↓p38 MAPK proteasome phosphorylation	Cortical Neurones: ↓α-syn (wild-type protein)[214]	-

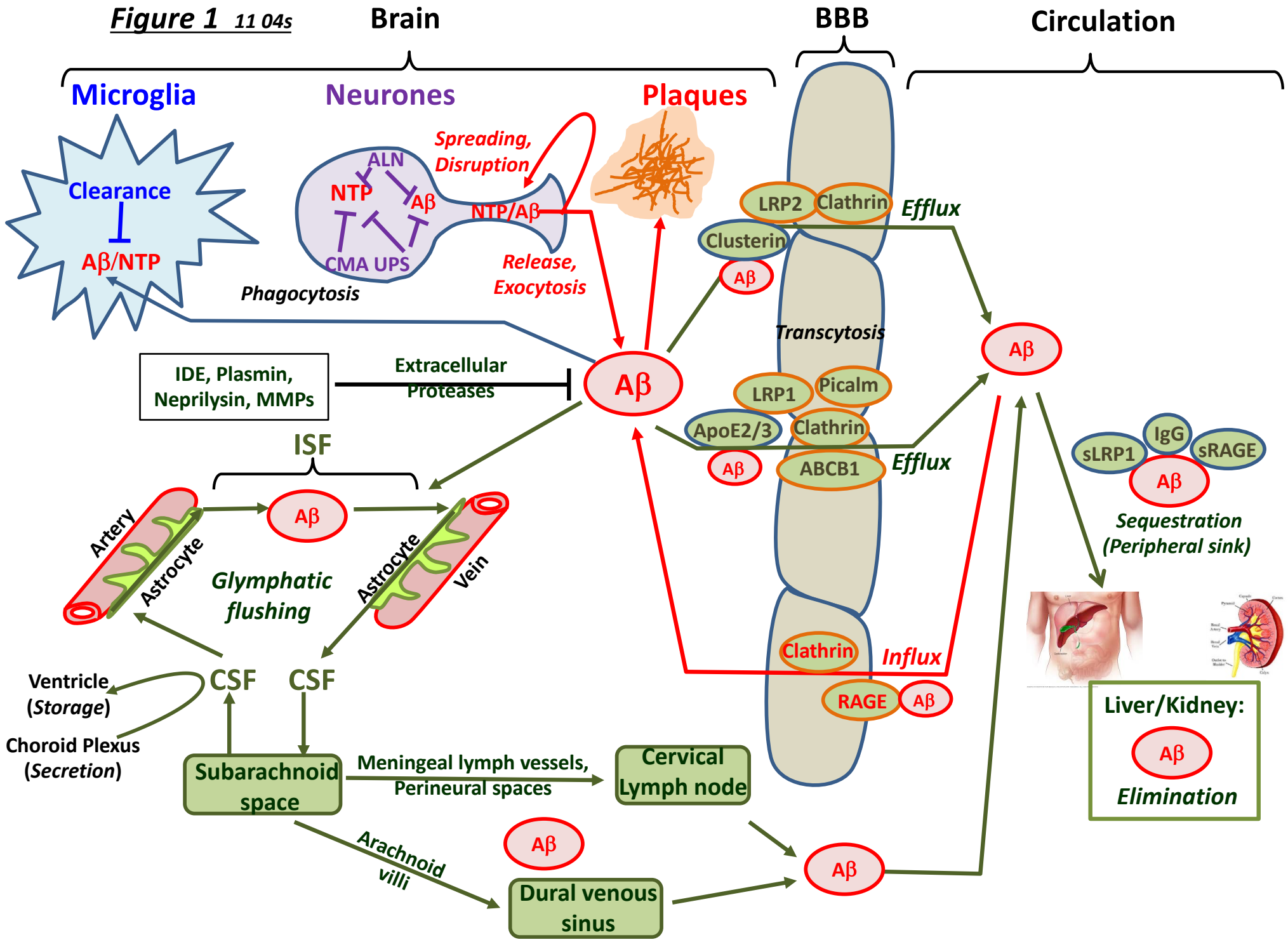


Figure 1 11 04s
Brain
BBB
Circulation
Microglia
Neurones
Plaques
Clearance
Aβ/NTP
Phagocytosis
ALN
NTP
Aβ
CMA UPS
NTP/Aβ
Spreading, Disruption
Release, Exocytosis
IDE, Plasmin, Neprilysin, MMPs
Extracellular Proteases
Aβ
LRP2
Clathrin
Efflux
Clusterin
Aβ
Transcytosis
LRP1
Picalm
ApoE2/3
Clathrin
Aβ
ABCB1
Efflux
Aβ
sLRP1
IgG
sRAGE
Aβ
Sequestration (Peripheral sink)
ISF
Artery
Astrocyte
Aβ
Astrocyte
Vein
Glymphatic flushing
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

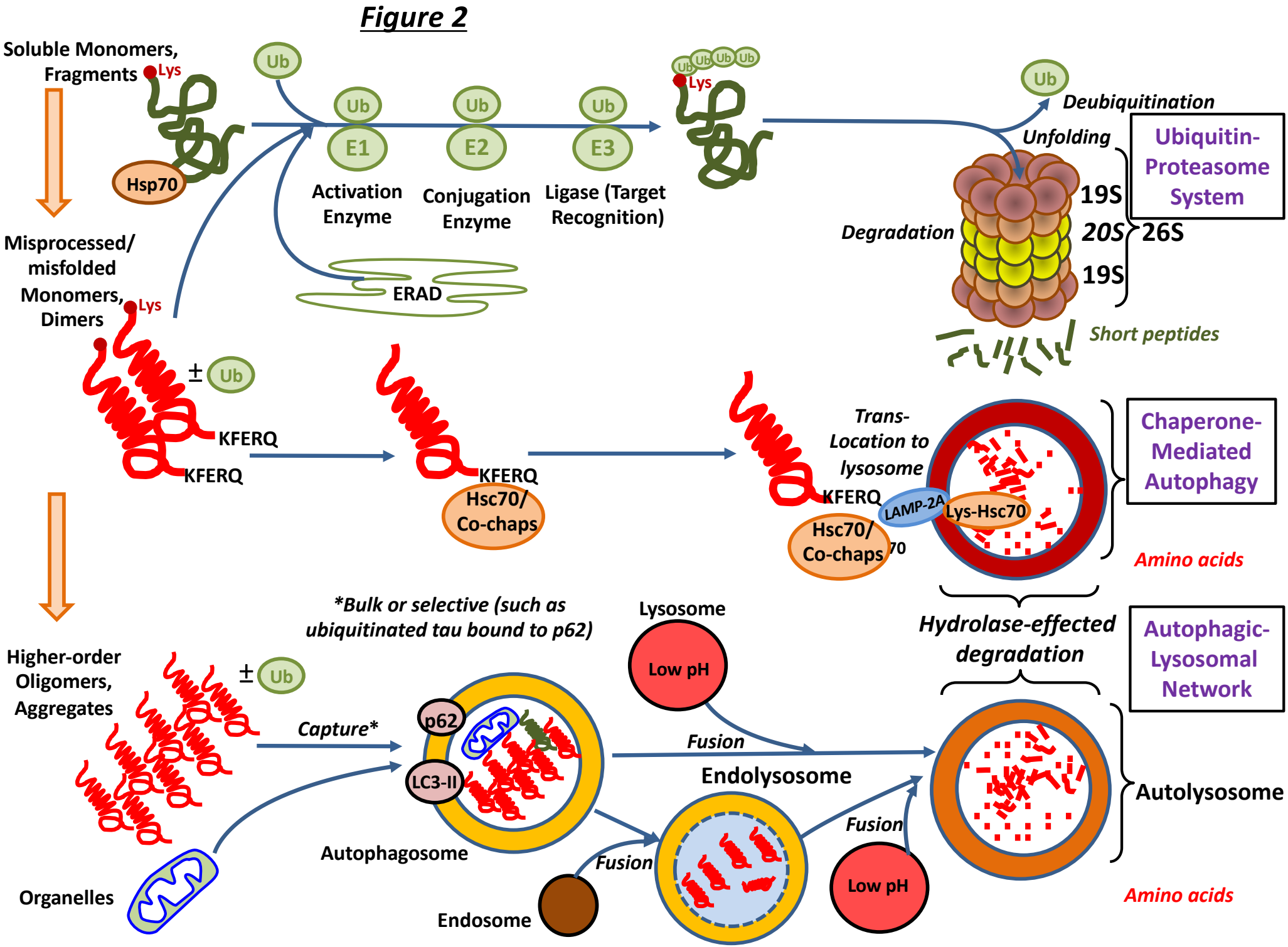
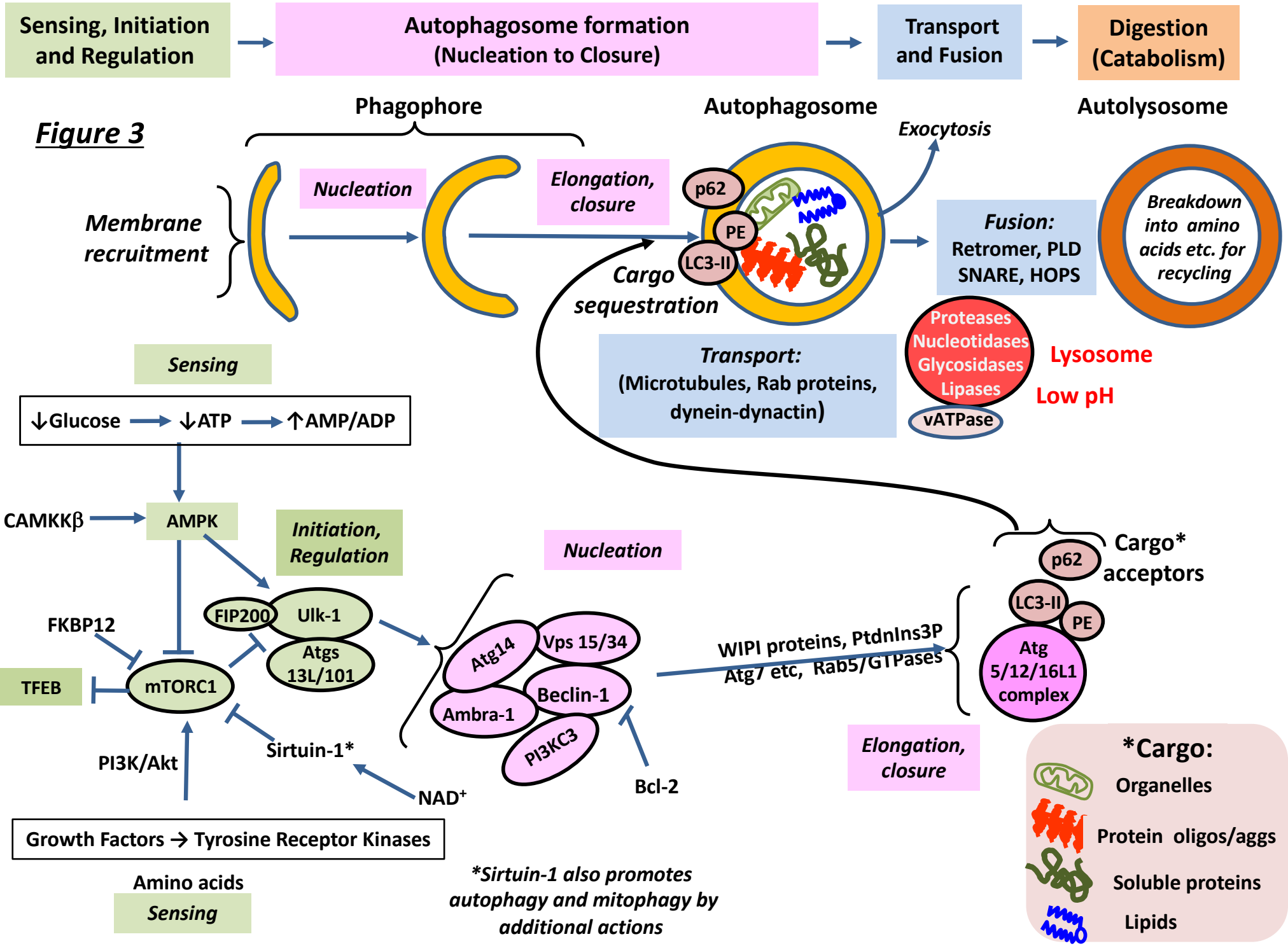
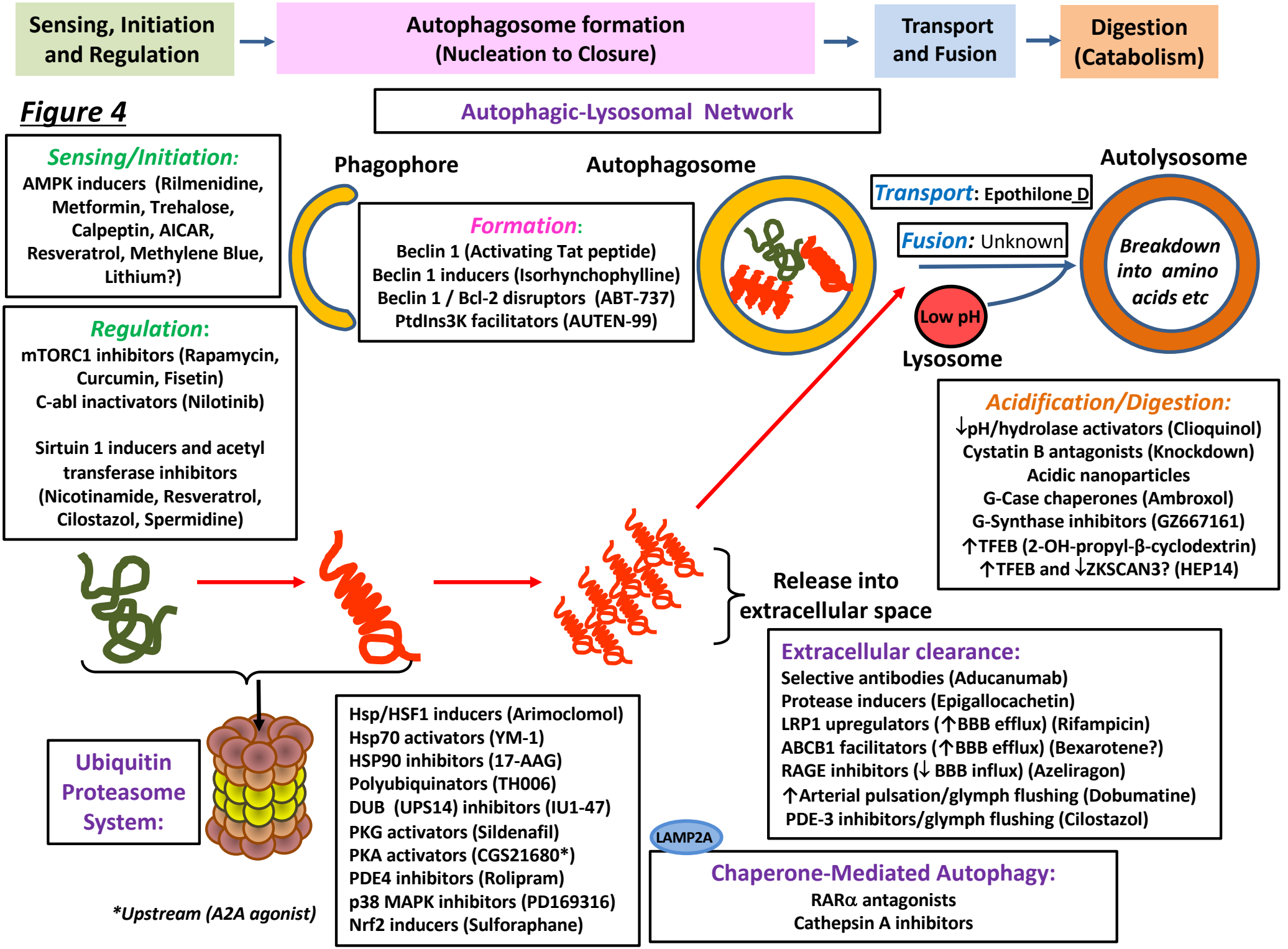


Figure 2
Soluble Monomers, Fragments
Lys
Ub
Hsp70
E1
E2
E3
Activation Enzyme
Conjugation Enzyme
Ligase (Target Recognition)
Deubiquitination
Unfolding
Ubiquitin-Proteasome System
19S
20S
26S
19S
Degradation
Short peptides
ERAD
Misprocessed/ misfolded Monomers, Dimers
± Ub
KFERQ
KFERQ
Hsc70/ Co-chaps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


Sensing, Initiation and Regulation
Autophagosome formation (Nucleation to Closure)
Transport and Fusion
Digestion (Catabolism)
Figure 3
Phagophore
Autophagosome
Autolysosome
Membrane recruitment
Nucleation
Elongation, closure
p62
PE
LC3-II
Exocytosis
Fusion:
Retromer, PLD
SNARE, HOPS
Breakdown into amino acids etc. for recycling
Cargo sequestration
Transport:
(Microtubules, Rab proteins, dynein-dynactin)
Proteases
Nucleotidases
Glycosidases
Lipases
vATPase
Lysosome
Low pH
Sensing
↓Glucose → ↓ATP → ↑AMP/ADP
CAMKKβ
AMPK
Initiation, Regulation
FKBP12
FIP200
Ulk-1
Atgs 13L/101
TFEB
mTORC1
PI3K/Akt
Sirtuin-1*
NAD+
Nucleation
Atg14
Vps 15/34
Ambra-1
Beclin-1
PI3KC3
Bcl-2
WIPI proteins, PtdnIns3P
Atg7 etc, Rab5/GTPases
Cargo* acceptors
p62
LC3-II
PE
Atg 5/12/16L1 complex
Elongation, closure
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
Autophagic-Lysosomal Network
Sensing/Initiation:
AMPK inducers (Rilmenidine, Metformin, Trehalose, Calpeptin, AICAR, Resveratrol, Methylene Blue, Lithium?)
Regulation:
mTORC1 inhibitors (Rapamycin, Curcumin, Fisetin)
C-abl inactivators (Nilotinib)
Sirtuin 1 inducers and acetyl transferase inhibitors (Nicotinamide, Resveratrol, Cilostazol, Spermidine)
Phagophore
Autophagosome
Formation:
Beclin 1 (Activating Tat peptide)
Beclin 1 inducers (Isorhynchophylline)
Beclin 1 / Bcl-2 disruptors (ABT-737)
PtdIns3K facilitators (AUTEN-99)
Transport: Epothilone D
Fusion: Unknown
Low pH
Lysosome
Autolysosome
Breakdown into amino acids etc
Acidification/Digestion:
↓pH/hydrolase activators (Clioquinol)
Cystatin B antagonists (Knockdown)
Acidic nanoparticles
G-Case 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 (UPS14) 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-proteosomal clearance that have been, or are being, clinically evaluated for the treatment of Neurodegenerative Disorders of Aging. The clinical trial identifier is shown together with the phase of testing, doses under study (oral) and primary measures/readouts used. These drugs were not specifically developed as modulators of neurotoxic protein clearance but, based on experimental data, are known to modulate it. 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 A□1-42 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, see main text), 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 (RemberTM). Further analysis of the AD trial suggested that it may indeed have beneficial effects, notably on brain atrophy[1], though 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. For all drugs, with the exception of edaravone, drugs promote ALN activity in experimental models. Ironically, then, the only drug to have received FDA authorization is edaravone. As discussed in Supplementary Box 3, edaravone may *reduce* ALN activity, but this remains controversial and it has other therapeutically-useful actions like anti-oxidant properties. In addition to studies indicated in the Table, an open label study with rilmenidine was recently undertaken with a view of evaluating its efficacy in the treatment of Huntington's disease[2]. 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; MOCA, 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	Aging	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[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
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; MOCA; 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	Tolerated; low adverse effects; possible increased survival; slower ALSFRS-R decline[7]
Arimoclomol	ALS	NCT00244244	II	75-300mg/3x/d	Safety, tolerability, pharmacokinetics; rate of decline on ALSFRS-R	Tolerated, low adverse effects; slower ALSFRS-R decline with Arimoclomol[8]
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[9]
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)[10]; FDA approved

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Add Seripa et al new ref for Methylene bleu –se Suppl Ref list

Revision Ref Suppl Main Text

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Add to Supplementary Table 1 in response to Referee 2 on methylene bleu

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Add to Supplementary Box 1

McDonald, JM and Kraine, D. Lysosomal proteins as a therapeutic target in neurodegeneration. Ann Rev Med 68, 445-458, 2017. ***CANT FIND A PMID ENTERED BY HAND, IF PBM THE DROP IT***