

REVIEW ARTICLE

Crucial role of protein oligomerization in the pathogenesis of Alzheimer's and Parkinson's diseases

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Misfolding and aggregation of the proteins amyloid- β , tau and alpha-synuclein is the predominant pathology underlying the neurodegenerative disorders, Alzheimer's and Parkinson's disease. While end stage insoluble products of aggregation have been well characterised in human and animal models of disease, accumulating evidence from biophysical, cellular and *in vivo* studies has shown that soluble intermediates of aggregation, or oligomers, may be the key species that mediate toxicity and underlie seeding and spreading in disease. Here, we review the process of protein misfolding, and the intrinsic and extrinsic processes that cause the native states of the key aggregating proteins to undergo conformational change to form oligomers and ultimately fibrils. We discuss the structural features of the key toxic intermediate, and describe the putative mechanisms by which oligomers may cause cell toxicity. Finally, we explore the potential therapeutic approaches raised by the oligomer hypothesis in neurodegenerative disease.

Introduction

Protein misfolding and aggregation are molecular processes known to occur in all neurodegenerative disorders, including the commonest diseases, Alzheimer's disease (AD) and Parkinson's disease (PD). During misfolding, intrinsically disordered monomeric proteins (such as A β and tau in AD, and α -syn in PD), undergo conformational changes from their native states into highly ordered β -sheet rich fibrillar amyloid structures [1]. The detection of these amyloid structures in post-mortem brain tissue has allowed accurate post-mortem diagnosis of subtypes of neurodegenerative diseases. As the presence of intracellular and extracellular fibril

deposition is seen predominantly in disease, it is this insoluble end-stage species of protein aggregation that has traditionally defined disease. However, soluble transient intermediate species called oligomers are generated during this process of aggregation. There is accumulating evidence that the soluble oligomeric forms of such proteins may be the key toxic species involved in disease, whilst the role of the end-stage fibrillar aggregate, or inclusion body, is less clear. Post-mortem sporadic PD brain exhibits an increase in oligomeric α -syn [2]. Direct application of *In vitro* formed α -syn oligomers, or overexpression of α -syn mutants induces a range of

Abbreviations

α -syn, alpha-synuclein; AD, Alzheimer's disease; ApoE, apolipoprotein E; ApoJ, apolipoprotein J; APP, amyloid precursor protein; A β , amyloid- β peptide; CSF, cerebrospinal fluid; FRET, foster resonance energy transfer; GBA, glucocerebrosidase; hFPD, human prefoldin; IDP, intrinsically disordered protein; IL-1 β , interleukin 1 β ; MAPK, mitogen-activated protein kinase; mPTP, mitochondrial permeability transition pore; NAC, non-amyloid-component; NFT, neurofibrillary tangle; NFTs, neurofibrillary tangle; NMDA-R, *N*-methyl-D-aspartate receptor; NSAIDS, non-steroidal anti-inflammatory drugs; PD, Parkinson's disease; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD2, manganese superoxide dismutase; TFEB, transcription factor EB; Tg, transgenic; TIRF, total internal reflection fluorescence; TLR, toll-like receptor.

cellular damage affecting membrane integrity, mitochondria, ER, autophagy, and synaptic transmission, and ultimately leading to cell toxicity [3–5]. *In vivo* studies, expression of α -syn mutants that promote oligomer formation (but inhibit fibril formation) results in dopaminergic neuronal death, [3,6] while conversely α -syn mutants that form fibrils did not lead to dopaminergic neuronal loss. Comparable with the literature on PD, an increase in soluble oligomeric forms of both A β and tau, key aggregating proteins, has also been reported in the brain of AD patients, and there is a correlation between the soluble A β and tau concentrations and cognitive decline in both AD patients and animal models [7]. In addition, functional studies have shown that toxicity can be induced by accumulation of soluble oligomers of either A β or tau rather than the monomers or fibril [8–11].

Taken together, multiple lines of evidence now suggest that oligomeric species of aggregating proteins are key pathological modulators involved in disease initiation and progression. In this review, we discuss major questions to understand the role of the oligomer in neurodegenerative diseases;

- 1 What are the native states and conformational evolution as proteins misfold from monomers into variable aggregating species (e.g. soluble β -rich oligomers and amyloid fibrils)?
- 2 What are the environmental triggers (intrinsic or extrinsic) of conformational changes from monomeric to oligomeric species?
- 3 What are the possible mechanisms underlying the toxicity by oligomers of aggregating proteins in neurodegenerative diseases?
- 4 What are the potential therapeutic strategies to target oligomeric species?

Native states and the role of oligomeric species in neurodegenerative diseases

Natively, the proteins of the major components in neurodegenerative diseases such as α -syn, A β and tau are classified as intrinsically disordered proteins (IDP) that lack a fixed or stable three-dimensional structure. Instead, they retain conformational freedom in association with other molecules, and can adopt a range of different structures, a property that is key to their wide biological functions (Box 1). Consistent with the structure of IDPs, α -syn consists of a positively charged N-terminal repeat region that allows an interaction between α -syn and lipids (in which α -syn adopts an α -helical structure); a hydrophobic central region that

Box 1

Native structure of key protein responsible for aggregation in PD and AD

α -syn: A small acidic protein of 14.5 kDa and 140 amino acids. There are three distinct domains consisting of N-terminal lipid-binding α -helix, amyloid-binding central domain (non-amyloid-component, NAC) and C-terminal acidic tail. (a) The amino-terminal sequence (residues 1–60) that contains highly conserved hexamer motif is the site of mutations A53T, A30P and E46K found in familial PD cases. (b) The central region (residues 61–95) is termed the NAC and this region can undergo conformational changes from a random coil to β -sheet structure to form amyloid β -like fibrils. (c) The carboxy-terminal of α -syn (residue 96–120), acidic rich residues, is responsible for regulating aggregation and residue serine 129 is one of the well-known phosphorylation sites. Dopamine also interacts with those residues for α -syn [119,120].

A β : The peptides are produced by a series of enzymatic cleavages of the amyloid precursor protein. Most A β peptides in human brain tissue are either 40 or 42 residues in length [121].

Tau: The longest tau isoform is 441 amino acid residues. N1 and N2 are the polypeptide sequences, P1 and P2 are proline-rich regions and R1-R4 are the microtubule-binding domains. In monomeric tau, β structure sequences are present in R2 and R3 regions allowing self-assembly into filaments [122].

allows aggregation into β -sheet structure (the NAC, or ‘non-A β -component’), and a negatively charged acidic C-terminal region that does not associate with membranes. In cells, α -syn exists as primarily monomeric with high conformational flexibility, and adopts either an unfolded state in the cytosol, or an α -helical structure when membrane bound [12]. Some studies have suggested that α -syn may exist as a folded tetramer, but the true native conformer state(s) remains unresolved [13,14]. While the exact physiological function of the native conformations of α -syn are not known, α -syn has been reported to play a major role in SNARE complex assembly in neurotransmitter release, synaptic vesicle recycling, dopamine metabolism, and regulation of mitochondrial ATP synthase efficiency [15].

During pathology, protein misfolding and self-assembly leads to the native monomeric state

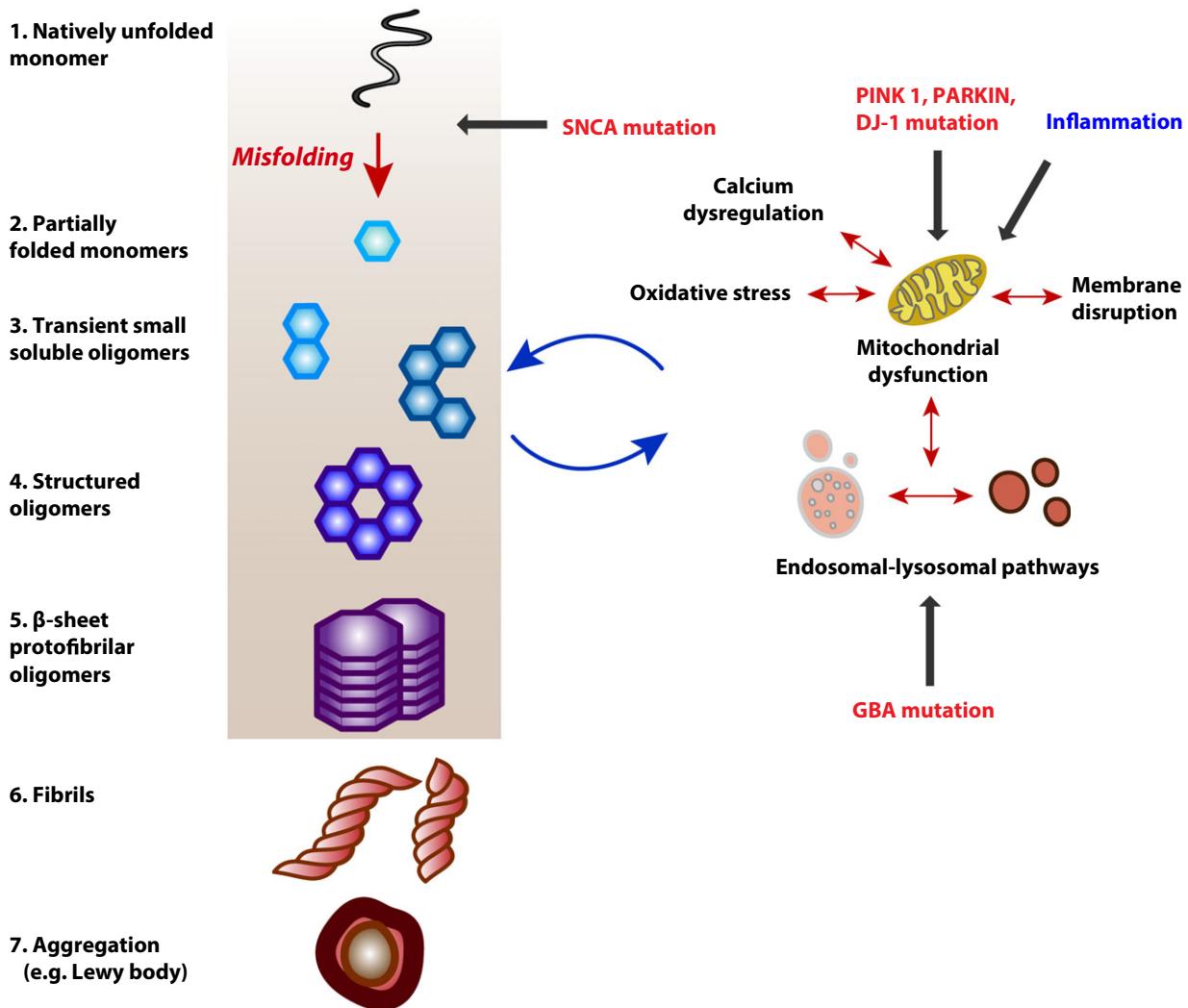


Fig. 1. Structural evolution during aggregation. Intrinsically disordered proteins such as α -syn, A β , phosphorylated tau that mostly exist as (1) unfolded monomers can be transformed into (2) partially folded conformation by pathological stimuli. Internal stimuli such as genetic mutations in SNCA, or environmental stress such as inflammation can trigger misfolding. The partially folded monomers can initiate aggregation and transform into (3) transient small soluble oligomers in the earlier stages and (4) later β -sheet structures. In turn, accumulation of oligomeric species facilitates the pathological cascade. The β -sheet structure protofibrillar oligomers (5) can transform intracellularly into (6) fibrils and finally accumulate as (7) fibrillary aggregates.

undergoing a structural change to first form small soluble amorphous oligomers, then later oligomers with high β -sheet content, protofibrils, and finally insoluble amyloid fibrils (Fig. 1). Transformation from an unfolded protein to a partially folded protein, that can undergo self assembly, may occur in conditions such as lowered pH, or increased temperature that alter the hydrophobicity and net charge resulting in the acquisition of ordered structure [16]. Oligomerisation is simply promoted by agitation but high initial concentrations, temperature, lipids, the presence of fibrillar seeds or iron accelerate the oligomerisation

process [17–19]. While ‘oligomer’ is a widely used term, it is defined as an assembly of misfolded proteins that maintain their solubility in a range of sizes from dimers to protofibrils. For example, it is reported that the dimeric form of α -syn is unstable and transient with discrete structural conformations [20], but there is little study of the trimeric form of α -syn. A metastable a-helical tetramer is generally believed to be a non-toxic species [21]. Although a heterogeneous range of size and structures are generated during the oligomerisation process, the pathological properties of the ‘toxic oligomer’ are proposed to be β -sheet rich, and have

high numbers of exposed hydrophobic residues, which allows the species to integrate into membranes and induce calcium fluxes through channel or pore formation [22].

Once misfolding occurs, aggregates may be generated in cells by the initial formation of small soluble oligomers from monomers, followed by sequential monomeric addition eventually forming mature fibrils, a process termed primary nucleation. Alternatively, disassembly or fragmentation of fibrils can generate aggregate intermediates. Finally, seeding or templating in which monomers are added onto a seed, or template, of cross β -sheet structure can also lead to the generation of oligomeric intermediates [23]. Importantly, α -syn fibrils propagate to neighbouring neurons, where the seeding/templating property of the cross β -sheet structure of fibrils, may underlie disease spread and progression [24]. Perhaps both the complexity of the different aggregation processes (primary nucleation, secondary nucleation or fragmentation), coupled with the diversity of experimental conditions used to aggregate proteins, has led to a large range of aggregation intermediates reported in the literature, and used in different experimental paradigms. It is increasingly clear that these different structures, or strains, of proteins may possess different properties in relation to inducing toxicity, or seeding and spread [25,26].

Intrinsic and extrinsic triggers for oligomerisation

Key aggregating proteins are known to misfold and oligomerise following exposure to various extrinsic (e.g. oxidative stress, dopamine) or intrinsic challenges (e.g. high concentration, overexpression, presence of mutants) [27]. As transition from monomers to oligomers is a crucial step to generating the neurotoxic intermediate species that then drives pathogenesis, an understanding of why oligomerisation occurs in pathological states is important. Although it remains unclear exactly how the transition is triggered, it has been suggested that a critical concentration of the monomers of the aggregating proteins is required. Genetic evidence demonstrates that patients with duplication and triplification of the SNCA gene exhibit elevated levels of α -syn protein, associated with widespread early onset aggregation and neuronal death [28,29]. Furthermore, variants in SNCA are a common genetic risk factor for developing sporadic PD, and risk variants in the SNCA genomic region and intergenic sequences are reported to act by regulating expression of α -syn, which may drive aggregation [30]. Interestingly normal

ageing is also associated with increased levels of monomers [31].

Extrinsic factors, or biological processes, that accelerate or trigger oligomerisation interestingly overlap with the mechanisms underlying the oligomer-induced toxicity, highlighting the bi-directional effects of protein aggregation and oxidative stress, protein aggregation and inflammation, and protein aggregation and impaired clearance. Therefore, once both processes have been triggered, they act synergistically such that the cellular stress increases the accumulation of protein aggregates, which in turn exacerbates the cellular stress. Here, we address two potential triggers for intracellular oligomerisation, the redox balance of the cell, and post-translational modifications of the aggregating proteins themselves.

Under healthy conditions, the generation of reactive oxygen species (ROS) is balanced by the antioxidant system while in neurodegenerative states, an imbalance allows excess accumulation of ROS that can accelerate oligomerisation [32]. Interestingly, it is reported that ROS induced by hydrogen peroxide accelerates the formation of dimers and soluble β -sheet oligomers, and thus promotes α -syn aggregation under oxidative stress [33–35]. Oxidised unfolded monomers of α -syn accelerate fibril formation and oxidised α -syn is also toxic to dopaminergic neurons [36,37]. In addition, treatment of α -syn with oxidising agents is reported to accelerate crosslinking of tyrosines, which results in triggering oligomerisation of the protein [20,38]. Oxidation of Met35 promotes the formation of A β protofibrils from monomers and a study has reported that the Met35-oxidised A β interacts with lipid membranes which can disrupt ion-channel functions [39–41]. With consistency, oxidative stress has also been reported to lead to an increase in tau phosphorylation which facilitates aggregation [42].

Phosphorylation is the most widely studied post-translational modification that is known to promote the propensity of aggregating proteins to form oligomers. In PD, Lewy bodies found in post-mortem brain with PD contain phosphorylated α -syn at residue S129 [43], and the soluble non-fibrillar fractions from the brain with PD were also found to be phosphorylated at S129 [44]. Tau has been long reported to be a phosphoprotein and is known to be mostly phosphorylated at the site of Ser-Pro and Thr-Pro motifs. Phosphorylation negatively regulates microtubule assembly [45]. Tau oligomers are known to exist in a variety of states which are either hyper-phosphorylated or unphosphorylated forms. Under physiological conditions, phosphorylation is crucially required for tau to regulate its binding to microtubules which determines their

stability in neurons, while hyper-phosphorylated tau results in abnormal protein aggregates in brain [46]. For A β , serine residues 8, 26 and tyrosine residue 10 have been proposed for potential phosphorylation sites but the most studied is the serine 8 site, and the phospho-serine-8-specific antibodies have shown the presence of phosphorylated A β in both patients with AD and animal models [47]. A β phosphorylated at serine 8 has shown to be localised to amyloid plaques under pathological conditions and *in vitro* studies have shown that phosphorylation enhances the formation of oligomeric species [48].

Mechanisms of toxicity induced by oligomers

Since the earliest reports of a potential role of oligomers in disease, there has been an increase in studies that compare the different species of aggregating proteins *in vitro* and *in vivo*, in an attempt to elucidate the specific ‘toxic’ species and its potential mechanism of action. It is worth highlighting that different studies employ different methods to generate oligomeric intermediates, and different methods to characterise them, and it remains a challenge to compare and validate findings across studies. Broadly however, soluble oligomeric species show high surface to volume ratio and hydrophobic like properties [49] and are prone to bind to membranes which leads to pore formation and membrane permeability [3]. As well as exhibiting structural differences to the monomeric state, oligomers may initiate aberrant cellular processes through specific and non-specific interactions with receptors, mitochondria, synaptic vesicles, and membranes leading to aberrant signalling or cellular dysfunction. In the next section, we will discuss major mechanisms underlying the oligomer-induced toxicity reported to date.

Mitochondrial dysfunction

Mitochondrial dysfunction is a key feature in the pathogenesis of Parkinson’s disease, and has been described in other neurodegenerative diseases. Mitochondria play a major role in neurons in the generation of ROS and redox signalling, cellular respiration and ATP production, calcium buffering, and cell death [50]. We, and others, have shown that α -syn oligomers induce mitochondrial depolarisation and impair respiration through an interaction with complex I [51,52]. Oligomeric α -syn also promotes calcium-induced mitochondrial depolarisation and swelling, and cytochrome c release, demonstrating α -syn-induced mitochondrial

dysfunction is dependent upon both complex I as well as mitochondrial uptake of exogenous calcium [53]. α -syn oligomers have been demonstrated to interact with the outer mitochondrial membrane Tom20 and impair mitochondrial protein import. Early opening of mitochondrial permeability transition pore (mPTP) is considered to be one of the key modulators involved in programmed cell death which occurs during neuronal cell loss through neurodegeneration [54,55]. We recently demonstrated that α -syn oligomers localise to the inner mitochondrial membrane and come into close proximity to the ATP synthase, and are able to directly induce classical PTP opening in isolated brain mitochondria, and in intact mitochondria in human neurons [56].

Similarly, application of oligomeric species of tau is also reported to cause mitochondrial dysfunction, such as a decrease in complex I activity [57,58]. Lasagna *et al.* [58], showed decreased complex I level in tau oligomer injected hemisphere compared to the side with either with injected fibrils or monomers. In line with the study, it has been reported that there is a reduction in levels of the 24- and 75-kDa subunits of complex I and modifications of mitochondrial encoded complex I subunit mRNA in response to tau accumulation but not formation of neurofibrillary tangles (NFT) [57,59]. Tau oligomers co-localise with porin, a mitochondrial protein, and suggest that tau oligomers could disrupt microtubule stability and trafficking [58].

Membrane disruption and calcium dysregulation

The protein α -syn binds to synthetic and biological lipids, and its interaction with membrane surfaces can initiate and accelerate its aggregation. This process may be dependent on the high local concentration of α -syn at the membrane, exposure of hydrophobic residues leading to self-assembly, or the physicochemical properties of the surrounding lipids [60,61]. Furthermore, once oligomers are formed, they are able to disrupt membranes and this is due to their structural composition, with a high lipophilic component promoting membrane interaction, and the cross β -sheet structure leading to integration into the lipid bilayer [62]. The consequence of such membrane disruption is the ability to induce ion fluxes across the membrane, although whether this mechanism is through pore formation [26] or membrane destabilising is not clear. Calcium is the most pleiotropic ion that is capable of triggering intracellular pathways in response to external stimuli [5,63,64]. In the brain, calcium dysregulation due to aberrant calcium signalling or disrupted calcium homeostasis has been reported in

neurodegenerative diseases [64]. We have reported that α -syn oligomers induce cytosolic calcium influx in neurons, and this results in an increase in cytosolic calcium before inducing cell death; cell toxicity is rescued by exclusion of extracellular calcium [5], confirming the importance of oligomer-induced membrane disruption in inducing neuronal toxicity. In other *in vitro* studies, oligomeric α -syn was able to induce calcium flux across both artificial membrane and neuronal membrane, using a pore-forming mechanism [26]. The significance of oligomers generated during the disease course (rather than synthetic oligomers) generating calcium influx has been investigated by Drews *et al.* [65], showing that A β oligomers from human cerebrospinal fluid (CSF) can permeabilise membranes and induce calcium influx in both control and patients with AD. However, A β oligomer is also reported to induce calcium influx into neuronal cells and interestingly even low picomolar concentration of the oligomers comparable to the concentration of species detected in CSF, induces calcium influx [65]. The same group has investigated how A β oligomers initiate the pathway of damage, using picomolar concentrations of both A β (1–40) and A β (1–42) [66]. Notably, α -syn oligomers and A β oligomers have also been reported to trigger calcium deregulation through receptor-mediated mechanisms, involving an interaction between the oligomer complex and PrP^c [67,68]. Therefore, oligomer-induced calcium signalling may be a common phenomenon in neurodegeneration, and calcium-mediated toxicity a unifying event by oligomers in all neurodegenerative diseases.

Calcium dysregulation is not only a primary mechanism mediating the oligomeric toxicity, but also a key stimulator to initiate or accelerate oligomerisation. For instance, high levels of intracellular calcium induced by thapsigargin depletes endoplasmic reticulum calcium stores, promotes oligomerisation of α -syn, which in turn exacerbates calcium dysregulation [69].

Endosomal–lysosomal pathways

Failure of the endosomal–lysosomal pathways that remove aggregated protein is another mechanism that underlies oligomer-mediated toxicity. The presence of oligomeric species of amyloidogenic proteins leads to incomplete clearance, which promotes further aggregation. It was reported by Lee and colleagues that α -syn oligomers are cleared through the lysosome and that inhibition of lysosomal activity accelerates its aggregation and toxicity [70]. Elevated activity of autophagy conversely reduces the accumulation of oligomeric α -syn in neuronal cell lines [71]. An *In vivo* study has shown that the overexpression

of α -syn induces the accumulation of aggregates, and overexpression of transcription factor EB (TFEB), a master regulator of lysosomal biogenesis, was able to reverse the lysosomal defects and accumulation of aggregates [72]. Interestingly, mutations in the GBA gene are an important genetic risk factor for PD. The glucocerebrosidase (GBA) gene encodes a lysosomal enzyme that has shown to be linked to α -syn oligomerisation [73]. Among patients carrying GBA mutation, brain samples of insoluble fractions from patients with parkinsonism contained oligomeric α -syn, but only monomeric forms were detected in the non-parkinsonism group [74]. A decrease in lysosomal glucocerebrosidase activity has also been suggested to promote the production of stable α -syn oligomers [75].

Structurally oligomers are naturally resistant to cytosolic proteases but interestingly there is a report that granulovacuolar degeneration found in early tau aggregation is derived from the endosomal–lysosomal system, and these deposits are the most likely substrate for initial seeding or nucleation of tau aggregation [76]. Another hypothesis is that nucleation of tau generates oligomeric tau aggregates by capturing normal tau (or mutant tau) in the process. Tau oligomers can only be cleared via the endosomal–lysosomal processing pathway and these oligomers contribute to further congestion and dysfunction in lysosomal processing. In addition, tau aggregation propagates itself by autocatalytic binding of tau and ultimate formation of tau fibrils. In the case of amyloid precursor protein and presenilin mutations, it has been reported that a congested endosomal–lysosomal pathway delays the critical time point to remove proteins, in particular membrane bound proteins from mitochondria, and this can accelerate aggregation and neuronal dysfunction in AD [77].

Oxidative stress

Oxidative stress is caused by the imbalance between ROS such as O₂[−], H₂O₂ and O, and antioxidants such as glutathione or enzymes such as manganese superoxide dismutase (SOD2) [78,79]. Overproduction of ROS has been well established in neurodegenerative diseases [80]. In terms of oligomeric-mediated toxicity, there has been accumulating data showing oligomeric species of aggregating proteins produce high levels of ROS [65,81]. Acute application of recombinant β -sheet-rich oligomers dramatically induced higher production of ROS in neuron and astrocytes co-cultures, while monomers, unstructured oligomers, and insoluble fibrils did not [4,23,82,83]. We have demonstrated that

the oligomer-induced ROS is dependent on metal ions, and that deferoxamine (an iron chelator), penicillamine (a copper chelator), and clioquinol (a highly lipophilic copper and zinc chelator) applied directly to the oligomers before application to cells, reduced the ability of the α -syn oligomers to induce ROS both rat primary and iPSC-derived cortical neurons [83]. The same ‘toxic’ α -syn oligomer is also able to induce an increase in lipid peroxidation, and both oligomer-induced lipid peroxidation and oligomer-induced cell death, could be prevented by pre-incubation of cells with isotope-reinforced polyunsaturated fatty acids (D-PUFAs) that prevent oxidation of lipids [84].

It seems also evident that ROS-mediated injury is a key pathological mechanism in AD. For instance, AD brains display an increase in levels of malondialdehyde and 4-hydroxynonenal, and lipid peroxidation markers. De Felice *et al.* [85], has demonstrated the mechanism underlying oligomer-induced ROS. According to this study, A β oligomer stimulates excessive formation of ROS through a mechanism dependent on *N*-methyl-D-aspartate receptor (NMDA-R) activation. Interestingly, in the AD brain A β -mediated mitochondrial oxidative stress causes hyperphosphorylation of tau, which may trigger the cascade pathways leading to neuronal dysfunction and eventually cell death [86].

Inflammation

Inflammation is considered a critical component in neurodegenerative disease pathogenesis, since microglia can be activated in response to misfolded proteins. Therefore, one hypothesis is that release of protein aggregates from neurons activates microglia which consequently initiates an inflammation response [87]. Activation of microglia results in elevation of the level of cytokines, chemokines, ROS and interleukine that are crucially involved in neuronal death [82]. It has been demonstrated that α -syn aggregates released from neurons are taken up by astroglial cells where they trigger an inflammatory response [70]. Furthermore, specific structures of α -syn oligomers released by neurons can activate inflammatory responses in microglia and astrocytes through the Toll-like receptor (TLR) pathways [88–90]. Similar to α -syn pathology, in the case of tau, there are reports showing that formation of neurofibrillary tangle (NFTs) is caused by local microglial cell-driven neuroinflammation even without activation of peripheral immune activation. Recent studies have shown that bacterial LPS-induced systemic inflammation increased tau pathology through cyclin-dependent kinase 5 activation [91]. Microglia-induced tau phosphorylation may be initiated by

IL-1 β receptor activation and p38 mitogen-activated protein kinase (MAPK)-mediated signal transduction, as it has been shown that aged mice acutely expressing IL-1 β display increased tau pathology [92]. In addition, a study with tau transgenic (Tg) mouse model showed that early immunosuppression helped delay the progression of tau pathology [93].

Therapeutic approaches

Based on the evidence reviewed here, it is clear that intracellular oligomerisation generates aggregation intermediates that are responsible for driving toxicity, and that furthermore spread to the extracellular space and seed pathology in neighbouring cells in neurodegenerative diseases [94,95]. Therefore, the main strategies of the therapeutic approaches have focused on either targeting or inhibiting the aggregation process *per se*, or modulating the environmental stresses that accelerate/exacerbate aggregation.

Antibody therapeutics; extracellular clearance by immunisation

There has been a number of trials based on antibodies targeting the aggregating protein. It should be recognised that targeting antibodies to the CNS need to address the challenge of crossing the blood–brain barrier which results in limited accessibility of most macromolecules to the brain. Antibodies engineered to incorporate sequences to allow permeability of blood–brain barrier naturally, or through using active transport can be employed. Recently, developments in single chain antibodies, such as camelid raised antibodies called nanobodies, allow increased stability and solubility as well as easy production, and may cross the blood–brain barrier [96]. In fact, immunotherapy has been tried in AD and PD, however, there is no data to show a significant improvement in clinical symptoms or mortality. For example, clinical trials such as Bapineuzumab and solanezumab did not show any beneficial effect in patients with both moderate and mild forms of AD. There is a number of possible explanations for these failures, including robust antibody fragment binding and the selection of the protein species to target. Bapineuzumab is a murine monoclonal antibody targeting the N-terminal region of A β and has been shown to bind to amyloid plaques. Solanezumab binds to the mid-domain of the A β peptide recognising soluble monomers but not oligomers, and failure in these trials may indicate the importance of careful targeting of the soluble oligomeric species. Nonetheless, it will be technically challenging to develop an antibody that can bind

selectively and stabilise and remove the oligomeric species. Additionally, immunisation approaches need to target early disease, at the point at which monomers are forming the toxic oligomeric species, and not the later fibrils. Therefore, another explanation given for the failure of the clinical trials has been the time point selected to treat is too late, while recognising that it is difficult to accurately diagnose patients in the prodromal or pre-clinical phases of disease. In order to capture early disease time points, such trials of antibodies need to be combined with more advanced biomarker or diagnostic tools to detect early, or prodromal, disease. Finally, a direct measurement of oligomer levels would be important to demonstrate the change in oligomer load by the intervention used.

Chaperone therapy (increasing degradation through chaperones, lysosomal up-regulators)

A molecular chaperone is a protein that selectively recognises and binds to exposed hydrophobic surfaces of non-native proteins to mediate the folding of protein [97]. It is also defined as a class of proteins that interact with proteins to stabilize their native structures and subsequently involve them in the pathways of protein degradation, which are capable of removing misfolding proteins such as the ubiquitin–proteasome system for degradation of α -syn misfolding [98,99]. Based on the initial studies proving that chaperones protect neurons against protein aggregation-induced toxicity in AD and PD, there is accumulating evidence of their specific interaction with oligomeric species to interfere with the process of aggregation. For instance, α -syn oligomer formation is significantly reduced in Heat shock protein 70 (Hsp70) chaperone-stimulated conditions. Complexes of α -syn are reported to be neutralised by Hsp90 chaperone in an ATP-dependent manner [100,101]. In addition to their role in counter-balancing aggregation, binding of the molecular chaperone to oligomer can directly inhibit the oligomer-induced cell toxicity. More specifically molecular chaperones can convert the small size oligomers into larger nontoxic aggregates with a decrease in the surface-to-volume ratio which can then enable autophagy to perform its clearance [102]. For example, the Hsp27 chaperone has been shown to increase the size of preformed A β 43 oligomers so that they are unable to exert their toxicity in mouse neuroblastoma cell culture [103]. Biochemical studies have also shown stabilisation of oligomeric A β 42 in the presence of the human prefoldin (hFPD) chaperone [104].

Extracellular chaperones support the specific internalisation of aggregating proteins, particularly in AD.

Apolipoprotein J (ApoJ, commonly known as clusterin) and E (ApoE) are two major apolipoproteins in the brain that facilitate the *in vivo* clearance of misfolded proteins [105]. Clusterin, in complex with A β , interacts with the cell surface receptor megalin on mouse teratocarcinoma F9 cells and promotes internalisation and the subsequent degradation of A β [106]. Similarly, ApoE and A β complexes are internalised in smooth muscle primary cultures by endocytosis-mediated by the lipoprotein receptor [107]. However, A β also complexes with α 2M, another extracellular chaperone also known to be internalised by lipoprotein receptor-related protein (LRP)-mediated endocytosis to degrade A β aggregation [108]. *In vivo* studies further support the clearance of A β by these extracellular chaperones [109–111]. Taken together, it is suggested that extracellular chaperones and endocytosis mechanisms are important for the control of A β turnover, which may be a key therapeutic target.

Antioxidant therapy

Although it still remains unclear whether ROS is a primary driver of pathogenesis or whether it is a secondary consequence of disease, it appears evident that overproduction of ROS is a key pathological mechanisms contributing to neuronal cell death in both AD and PD. Antioxidants may be exogenous or endogenous compounds, and both of them can work by neutralising free radicals through a number of different mechanisms, including scavenging free radicals, reducing metal ions, and preventing lipids from being oxidised, and increasing electron transfer in the respiratory chain of mitochondria [112]. However, despite significant benefits reported in animal studies, there has been little clinical success using antioxidants in both AD and PD. Several reasons may underlie the poor translation of such therapies to clinical benefit. For example, as with the immunotherapy studies, it may be that oxidative stress is an early phenomenon intricately linked to protein aggregation and therefore the trials in late stage disease may be ineffective. The compounds themselves may have limited bioavailability at the correct intracellular target. Finally, scavenging ROS may not be as effective as targeted inhibition of the production of ROS from its source.

Concluding remarks

We provide a range of evidence to support the hypothesis that oligomeric species are crucial in mediating cell toxicity in neurodegenerative diseases. The discovery

of the role of the oligomer has raised with it a number of challenges: the ‘toxic’ species is likely to be a rare event, among a pool of monomeric protein and aggregation intermediates of differing structures and sizes. Furthermore, it is heterogeneous and transient, and these properties make it difficult to understand the key disease-causing species either in nature or human diseases. Such challenges have complicated the oligomer field, and led to many different biochemical and optical methods used to generate intermediates, and to characterise them. Therefore, it is difficult to standardise the ‘oligomer’ used in experimental studies, and even more challenging relating the synthetic oligomers to the endogenous intermediates generated during disease. However, the recognition of the importance of the oligomer has spurred progress in methods to enable the detection of rare protein species to address this challenge. Single molecule-based fluorescent approaches are now able to visualise and quantify individual aggregates using single molecule confocal microscopy for labelled aggregates (which relies on the Förster resonance energy transfer (FRET) between two different colour labelled monomers in close proximity during the formation of oligomers [4,19,113], or sm Thioflavin T-based total internal reflection fluorescence (TIRF) microscopy for unlabelled aggregates [114,115]. Proximity ligation assay (PLA) is another method to detect oligomers both *in vitro* and *in vivo* based on closely located monomers during the formation of oligomers. Roberts *et al.* has provided the first visual evidence of elevated α -syn oligomers in PD by generating PLA probes from antibodies raised against α -syn. Commercially available oligomer detection ELISA kits for α -syn, tau and A β which utilise conformational or antibodies have been used particularly for biomarker studies in biological fluids such as CSF and blood [116,117]. Lastly, immunohistochemistry with antibodies specifically raised against oligomeric forms has been reported, although such antibodies may also detect other aggregating proteins rather than one specific oligomer [7,118]. It is hoped that such advances, once applied to relevant disease cell models and tissue, will be able to address the outstanding questions in the field.

These include:

- 1 What is the critical monomeric concentration and required to form intracellular oligomers?
- 2 Where is the exact location that monomers form oligomers within cells, and when and why does this occur in disease?
- 3 Which of the oligomeric states are causally involved in neurodegeneration?

- 4 How do we apply technical advances and detection tools to visualise the oligomeric process in cells and tissue?

Ultimately, we hope to further our understanding of how, where, when and why the oligomerisation process takes place in cells to generate toxic intermediate species. We hope to discover structure–function relationship and structure-toxicity mechanisms within human neurons. Understanding these relationships will allow the field to fully exploit the oligomer hypothesis for improved diagnostics, as a biomarker for disease, and as potential disease targets for therapy.

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Author contributions

M-LC and SG conceived of the ideas and wrote the manuscript.

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