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Pathogenic mechanisms of prion protein, amyloid- β and α -synuclein misfolding: the prion concept and neurotoxicity of protein oligomers

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Abstract

Proteinopathies represent a group of diseases characterized by the unregulated misfolding and aggregation of proteins. Accumulation of misfolded protein in the central nervous system (CNS) is associated with neurodegenerative diseases such as the transmissible spongiform encephalopathies (or prion diseases), Alzheimer's disease and the synucleinopathies (the most common of which is Parkinson's disease). Of these, the pathogenic mechanisms of prion diseases are particularly striking where the transmissible, causative agent of disease is the prion, or *proteinacious infectious particle*. Prions are composed almost exclusively of PrP^{Sc}; a misfolded isoform of the normal cellular protein, PrP^C, which is found accumulated in the CNS in disease.

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Today, mounting evidence suggests other aggregating proteins such as amyloid- β ($A\beta$) and α -synuclein (α syn), proteins associated with Alzheimer's disease and synucleinopathies respectively, share similar biophysical and biochemical properties with PrP^{Sc} that influences how they misfold, aggregate and propagate in disease. In this regard, the definition of a 'prion' may ultimately expand to include other pathogenic proteins. Unifying knowledge of folded proteins may also reveal common mechanisms associated with other features of disease that are less understood, such as neurotoxicity. This review discusses the common features $A\beta$ and α syn share with PrP and neurotoxic mechanisms associated with these misfolded proteins.

Abbreviations:

α syn – alpha synuclein

APP - amyloid- β protein precursor

$A\beta$ – amyloid beta

ApoE – apolipoprotein E

AD – Alzheimer's Disease

ALS – amyotrophic lateral sclerosis

CAA – cerebral amyloid angiopathy

CNS – central nervous system

CSF – cerebrospinal fluid

CJD – Creutzfeldt-Jakob disease

DLB - dementia with Lewy bodies

FTD – frontotemporal dementia

GPI - glycosylphosphatidylinositol

LB – Lewy body

LN – Lewy neurite

LTP - long term potentiation

LTD - long term depression

MSA - multiple system atrophy

PD – Parkinson's Disease

PrP^C – cellular prion protein

PrP^{Sc} – disease associated prion protein

PK – proteinase K

PMCA – protein misfolding cyclic amplification

TSE – transmissible spongiform encephalopathy

Introduction

Neurodegenerative proteinopathies are a group of disorders distinguished by the intra- or extracellular accumulation of specific proteins as β -sheet rich aggregates in the central nervous system (CNS) and associated neuronal vulnerability. They include Alzheimer's disease (AD),

synucleinopathies (the most common of which is Parkinson's disease, PD), transmissible spongiform encephalopathies (TSEs; also known as prion diseases), Amyotrophic Lateral Sclerosis (ALS) and frontotemporal dementia (FTD), among others. Although distinguished by disease-specific pathology and clinical presentations, these disorders share conspicuous similarities. They are largely age-related disorders with most cases being of sporadic origin. Currently they are incurable with therapeutic strategies only aimed at alleviating symptoms and despite extensive efforts, the normal physiological function of the proteins that aggregate in disease remains unknown. These proteins are, however, all intimately associated with their respective disorder, as mutations in the gene encoding the protein, or its precursor forms cause early-onset familial disease.

Of the proteinopathies, the unusual mechanisms governing the infectious nature of prion diseases are particularly striking. They represent a group of neurodegenerative diseases where the causative agent of disease is the prion or *proteinaceous infectious particle* (Prusiner 1982) that is composed almost exclusively of PrP^{Sc}; a misfolded isoform of the normal cellular prion protein, PrP^C. The prion hypothesis dictates that, in disease, aberrant PrP^{Sc} molecules aggregate together and induce conformational misfolding in susceptible PrP^C causing nucleation-dependent, autocatalytic amplification of transmissible prions. This continuous cycle of seeding allows prions to propagate within the host resulting in accumulation of β -sheet rich PrP^{Sc} in the CNS and widespread damage to neurons. During prion propagation, extension of the growing PrP^{Sc} aggregate can cause the aggregate to break, thus creating more nucleation seeds to amplify propagation (s 1). Prion propagation occurs during a long silent incubation period that can span decades, which is followed by the rapid onset of clinical disease and death.

Today, accumulating evidence suggests misfolded proteins associated with other proteinopathies share conserved molecular and biochemical properties with PrP^{Sc} that influences how they misfold, aggregate and propagate in disease. Some of the strongest evidence for prion-like behaviour exists for amyloid- β (A β) and α -synuclein (α syn), proteins associated with AD and synucleinopathies (e.g. Multiple System Atrophy (MSA), Dementia with Lewy bodies (DLB), PD), respectively. Indeed, detailing of the stereotypical spread of these proteins in the CNS over the course of disease provided the first evidence for propagation of pathogenic α syn and A β seeds (Braak *et al.* 2003, Braak & Braak 1991, Thal *et al.* 2002). The accumulating evidence of prion-like characteristics of A β and α syn has led to suggestions of the expansion of the prion concept. This review discusses the characteristics A β and α syn share with PrP^{Sc}, and explores how these common characteristics may also inform our understanding of neurotoxicity. Although outside the scope of this review, it is important to note that in addition to A β and α syn, many other proteins associated with neurodegenerative proteinopathies have been shown to share similarities with

prions, including superoxide dismutase 1 (SOD1), huntingtin protein, and tau (Lee & Kim 2015, Holmes & Diamond 2014, Costanzo & Zurzolo 2013).

PrP^{Sc}: the conventional prion

Prion diseases represent a group of infectious, invariably lethal neurodegenerative diseases that affect both humans and animals. In humans, prion disease can be caused by mutations in the gene encoding PrP^C, *PRNP*, resulting in familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome or fatal familial insomnia (FFI). These diseases can also be contracted following exposure to prions and include variant CJD (vCJD), kuru and iatrogenic CJD (iCJD), or they may arise from an unknown origin (sporadic CJD). Non-human prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in deer and elk. The pathology of prion disease is characterized by extracellular deposition of aggregated PrP^{Sc} (Figure 2), along with astrogliosis and vacuolation including associated neuropathological spongiform change in the CNS.

The infectious nature of prions is well established to transmit disease both within and across species. Early studies showed brain homogenate from scrapie-infected sheep could successfully transmit disease to goats (Cuillé & Chelle 1936) and mice (Chandler 1961). Likewise, numerous studies demonstrated human prion disease could be transmitted to non-human primates (Gajdusek *et al.* 1966, Gibbs *et al.* 1968, Masters *et al.* 1981). In humans, acquired prion disease has been associated with several serious epidemics. Kuru, one of the most prominent forms of prion disease, afflicted the native Fore tribes of Papua New Guinea. In these populations, ritualistic cannibalism was identified as the route of transmission and cessation of this practice has virtually eradicated new cases of disease (Collinge & Alpers 2008). The finding that consumption of BSE-infected beef was responsible for vCJD (Will *et al.* 1996, Hill *et al.* 1997) led to one of the largest health crises of the 20th century, causing widespread economic damage to countries found to have a contaminated beef-industry and concern of large-scale public exposure to the pathogen due to the long incubation period of disease. Additionally various iCJD cases have been documented with routes of unintentional human-to-human transmission including the use of inappropriately sterilised surgical equipment (Bernoulli *et al.* 1977, El Hachimi *et al.* 1997), dura mater grafts (Miyashita *et al.* 1991, Willison *et al.* 1991), corneal grafting (Duffy *et al.* 1974, Heckmann *et al.* 1997), blood transfusion (Llewelyn *et al.* 2004, Peden *et al.* 2004) and use of human cadaveric pituitary-derived growth hormone or gonadotrophin (Cochius *et al.* 1990, Powell-Jackson *et al.* 1985) (for an extensive list of references on human-to-human transmission of prion disease, refer to: (Brown *et al.* 2006)). Of these, the highest number of iCJD cases are associated with dura mater grafts and hormone administration (Brown *et al.* 2012). These cases of acquired disease have led to major reform in the handling of biological samples in the food and medical industry to reduce contamination and prevent future outbreaks of prion disease.

The conversion of PrP^C to PrP^{Sc} is concomitant with substantial structural and biophysical alterations to the molecule. Upon misfolding, the α -helix rich PrP^C that is usually attached to the cellular membrane via its glycosylphosphatidylinositol (GPI) anchor, switches to one primarily composed of β -sheet (Borchelt *et al.* 1990, Pan *et al.* 1993). These changes lead to an increased resistance to degradation by heat and proteases; the latter feature of which is often exploited for its detection by incubation with proteinase K (PK). In addition, it is well accepted not all PrP^{Sc} are identical and rather, PrP^{Sc} encompass a range of various sized PrP^{Sc} quasi-species (Li *et al.* 2010) that harbour unique properties; for example, the most infectious prion particles appear to be those of small masses consisting of 14-28 molecules (Silveira *et al.* 2005, Simoneau *et al.* 2007).

Numerous data demonstrate that propagation of PrP^{Sc} via conformational misfolding of PrP^C is an essential component of disease pathogenesis. Transgenic PrP^C knockout mice are resistant to infection by prions (Bueler *et al.* 1993) and ablating PrP^C expression in prion-infected mice after the onset of clinical symptoms halts disease progression and rescues early neuropathological changes (Mallucci *et al.* 2003). The development of assays that study the conversion of PrP^C to PrP^{Sc} in cell free systems provides additional insight into the misfolding of the protein. In the cell free conversion assay, radio-labelled recombinant PrP^C adopts PrP^{Sc}-like features such as resistance to PK when incubated in the presence of partially denatured PrP^{Sc} derived from prion-infected hamster (Kocisko *et al.* 1994). Another cell free assay, the Protein Misfolding Cyclic Amplification (PMCA) assay, shows amplification of PK-resistant PrP occurs when excess PrP^C is subjected to repeated rounds of sonication and incubation in the presence of a small PrP^{Sc} seed (Saborio *et al.* 2001). When subjected to PMCA, normal brain homogenate seeded with a small amount of prion-infected brain homogenate amplifies PK-resistant, insoluble PrP^{Sc}-like species, that transmits disease when inoculated into wild-type mice (Castilla *et al.* 2005). Collectively these studies demonstrate the requirement of PrP^C in the generation of PrP^{Sc} and in the propagation and transmission of prions *in vivo*.

An important component to proving the prion hypothesis required demonstration that PrP^{Sc} alone was a transmissible element that could induce the pathology of prion disease. Studies showed recombinant PrP^C may be misfolded to form a PrP^{Sc}-like species that causes disease when inoculated into PrP-overexpressing mice (Legname *et al.* 2004) or in wild-type mice when the protein is misfolded in the presence of normal brain homogenate (Makarava *et al.* 2010), however variations existed in the pathology and long incubation period required for presentation of disease indicating PrP^C alone may not be the sole pathogenic agent in traditional prion disease. An explanation for this variation was suggested by the observation that additional factors, or co-factors, could contribute to the misfolding of PrP^C. Using PMCA, PrP^{Sc}-like species may be formed from normal brain homogenate or purified brain-derived PrP^C combined with RNA

species (Deleault *et al.* 2007, Barria *et al.* 2009), both that cause disease in wild-type rodents. The latter finding, along with others, show polyanionic and lipid species are required for efficient misfolding of PrP^C and infectivity (Deleault *et al.* 2005, Deleault *et al.* 2007, Deleault *et al.* 2003, Geoghegan *et al.* 2007, Wang *et al.* 2007). By the addition of co-factors, misfolding recombinant PrP in the presence of RNA and lipids produces *de novo* prions that are highly infectious *in vivo* and manifest disease in wild-type mice which faithfully replicates conventional prions (Wang *et al.* 2010). These studies confirm an altered conformation of PrP is the major component responsible for infectivity of prions, however the requirement of co-factors suggests other molecules may form a natural component of a transmissible prion.

An unusual feature of prion disease is strain variability, whereby unique patterns of clinical presentation, incubation period, PrP^{Sc} deposition and neuronal vulnerability are displayed amongst prions (Morales *et al.* 2007). Unlike conventional pathogens where strain variability is a result of genomic alteration, it is unusual for multiple disease phenotypes to be enciphered by a polypeptide chain. Important knowledge into strain variability came from elegant studies on two hamster-adapted prion strains Hyper (HY) and Drowsy (DY). These two strains arose from the same original animal and were propagated through hamsters with identical *PRNP* sequence, however while hamsters inoculated with HY prions develop ataxia and hyperaesthesia, hamsters infected with DY prions show lethargy and a longer incubation period (Bessen & Marsh 1992a). These divergent clinical features are coupled with unique pathological profiles of protein aggregation between the two strains (Bessen & Marsh 1992a) and biochemical analysis of the brains of animals infected with HY or DY reveal differences in electrophoretic mobility of PK-resistant PrP (Bessen & Marsh 1992b), suggesting the structure of PrP^{Sc} is altered between the two strains. This observation is further supported by the finding that these strains also exhibit different infrared spectrum of absorption in the range that correlates to β -sheet content (Caughey *et al.* 1998), indicating the secondary structures of HY and DY are dissimilar. Structural variability of PrP^{Sc} is also likely to occur in the human condition, where PrP^{Sc} present in sporadic prion diseased brain are distinguished by changes in electrophoretic mobility which appears to correlate with pathological and clinical features of disease (Hill *et al.* 2003). Although the general consensus is that the conformer of the misfolded protein encodes its disease phenotype, the origin of unique strains and how these conformational changes incite the unique clinical and pathological profiles is still poorly understood.

Prion-like properties of A β

The accumulation of amyloid plaques primarily composed of A β in the extracellular space of the CNS is a common feature of Alzheimer's disease (Masters *et al.* 1985) (Figure 2). A naturally-occurring cleavage product of the integral membrane protein, amyloid- β protein precursor (APP) (Kang *et al.* 1987), A β is produced by the sequential cleavage by β - and γ - secretases that cleave

at various sites in APP, producing A β peptides which differ by several amino acid lengths. Along with A β deposition, AD is associated with the accumulation of neurofibrillary tangles consisting of hyperphosphorylated forms of the microtubule-associated protein, tau. Aside from age, the biggest known risk factor for AD is the expression of the ϵ 4 allele of the lipoprotein ApoE (ApoE4) (Corder *et al.* 1993) that worsens the trajectory of cognitive decline in association with A β burden in preclinical AD individuals (Mormino *et al.* 2014, Lim *et al.* 2015a, Lim *et al.* 2015b).

Some of the first indications of prion-like behaviour of A β came from primate studies, where A β deposition was found in the CNS of marmosets 6-7 years after intracerebral inoculation of human AD tissue (Baker *et al.* 1993), suggesting pathogenic A β seeds may propagate within its host similar to prions. Further support for this concept came following the generation of transgenic animals. Mice expressing APP containing mutations associated with human familial AD spontaneously develop A β pathology and are used to model disease, with two of the most widely used transgenic mice Tg2576 (Hsiao *et al.* 1996) and APP23 (Sturchler-Pierrat *et al.* 1997) expressing the Swedish familial mutation K670M/N671L. Unilateral inoculation of dilute brain homogenate from human AD brain into the hippocampus and neocortex of young Tg2576 mice accelerates the deposition of A β , with extensive A β -pathology found 5 months post injection whilst limited or no deposition occurred in Tg2576 counterparts injected with age-matched non-AD or young non-AD brain extract (Kane *et al.* 2000). Under the same experimental conditions, a similar finding was observed by aging the mice to 12 months, where although A β deposition was present bilaterally, protein deposition was significantly more extensive in the hemisphere inoculated with AD brain (Walker *et al.* 2002). Consistent with the ability of A β -rich brain homogenate to induce this pathology, a similar phenotype may be achieved by inoculating young pre-symptomatic mice with brain homogenate from aged counterparts that have developed extensive A β deposition (Meyer-Luehmann *et al.* 2006).

Since these findings, numerous studies have highlighted the relevance of A β in accelerating pathology in AD-model mice. Removal of A β from aged APP23 brain homogenate via immunodepletion or immunization against A β abrogates the ability of extracts to trigger A β pathology (Meyer-Luehmann *et al.* 2006) and pre-treating human AD brain homogenate with magnetic beads coupled to a compound that binds misfolded protein decreases A β levels in the homogenate and reduces its seeding potential *in vivo* (Duran-Aniotz *et al.* 2014). Consistent with these observations, the concentration of A β in the seed and its expression in the brain determines the time and severity of A β deposition, and is independent of the age of the mouse when inoculated (Hamaguchi *et al.* 2012, Meyer-Luehmann *et al.* 2006). In AD-model mice, A β pathology may also be triggered by A β seeds derived from human brain of patients with amyloid burden without dementia (Duran-Aniotz *et al.* 2013) and synthetic misfolded A β (Stöhr *et al.*

2012, Stöhr *et al.* 2014). The development of A β -pathology in transgenic APP-model mice has been studied where A β propagation follows a systematic spread with induction of pathology corresponding to the limbic connectome (Ye *et al.* 2015). Collectively these studies give strong support for A β being the agent driving the accelerated A β pathology seen in AD-model mice and supports the concept that A β may propagate similar to prions.

The transmissible nature of A β seeds share several similarities with prions including their resistance to inactivation by treatment with formaldehyde (Fritschi *et al.* 2014a) and the ability to be transmitted on steel wires (Eisele *et al.* 2009); a concept developed to replicate transmission of prions via surgical equipment (Flechsig *et al.* 2001, Zobeley *et al.* 1999). It has been reported small aggregates of A β are the most infectious (Langer *et al.* 2011). Although synthetic A β may induce AD pathology *in vivo* (Stöhr *et al.* 2012, Stöhr *et al.* 2014), it is markedly less effective than brain homogenate (Stöhr *et al.* 2012), which may indicate biological co-factors are required for efficient propagation of A β *in vivo*. The propagation of A β *in vivo* has also been confirmed following injection of A β seeds via the intraperitoneal route (Eisele *et al.* 2010, Eisele *et al.* 2014) consistent with propagation of pathogenic seeds from the periphery to the CNS.

Although compelling, there are several caveats to using AD-model mice to study A β propagation. Acceleration of an existing A β pathology in transgenic mice after exposure to an A β extract does not necessarily prove that A β is infectious; however the generation of A β pathology in transgenic mice expressing wild-type human APP or mutant human APP that would not develop spontaneous A β pathology in the experimental timeframe does support the transmissible properties of A β aggregates (Morales *et al.* 2012, Rosen *et al.* 2012, Hamaguchi *et al.* 2012, Stöhr *et al.* 2012). Cerebral spinal fluid (CSF) from AD patients that contain higher levels of A β compared to brain extracts does not induce A β pathology in young APP23 mice (Fritschi *et al.* 2014b), which may indicate co-factors in the brain but not CSF influences the ability of A β to propagate pathology. An additional consideration using these transgenic mice is that although A β deposition can be accelerated by the addition of A β seeds, neurological dysfunction is absent, and thus the model of A β propagation does not model all features of AD.

Evidence of transmission of A β in humans has been reported where unusual deposition of A β was found in the brains of iCJD patients who contracted disease following human cadaveric pituitary-derived growth hormone treatment (Jaunmuktane *et al.* 2015). Of a small sample group of eight, four contained extensive deposition of A β in the CNS parenchyma and varying degrees of cerebral amyloid angiopathy (CAA); the pathological deposition of A β in blood vessels that occurs in approximately 80% of AD patients (Serrano-Pozo *et al.* 2011). An additional three cases had sparse A β deposition or A β deposition in association with PrP plaques. Given the young age of the patients (age range: 36 – 51 years), it is unlikely the A β pathology arose sporadically. A possible

explanation for this observation is that A β seeds were present in the cadaver-derived growth hormone. As evidence that A β deposition occurred through a transmission event, the growth-hormone recipients did not have any known factors that may have predisposed them to A β pathology, including known AD-associated genetic mutations and positive ApoE4 status. It is possible that the A β deposition was a result of cross-seeding by PrP^{Sc}, however in the majority of cases co-localization was not found and post mortem pathological assessment of a larger number (35) of patients with genetic or sporadic prion disease failed to identify A β pathology in comparable age groups, with the exception of two cases that were positive for the ApoE4 allele (Jaunmuktane *et al.* 2015). As proof-of-principle for the presence of A β in pituitary-derived growth hormone, deposited A β has been found in the pituitary gland of AD patients (Irwin *et al.* 2013, Jaunmuktane *et al.* 2015). Another study has reported unusual A β pathology in iCJD patients who contracted disease after dura mater grafting (Frontzek *et al.* 2016). Five out of seven patients (age range: 28 - 63 years) reported A β pathology and CAA consistent with AD, which was significantly greater than the frequency of A β pathology seen in 21 age-matched controls. In both cases, although it is intriguing to postulate iatrogenic transmission of A β has occurred, the overarching manifestation of iCJD makes it impossible to ascertain whether this transmission would have resulted in clinical AD.

Strain variation may also be a feature of A β . Differences are found in the pathological and biochemical profiles of A β isolated from different transgenic mutant APP mice such as APP23 (containing the APP Swedish mutation, K670M/N671L) (Sturchler-Pierrat *et al.* 1997) and APP/PS1 mice; a transgenic mouse line that contains the Swedish mutation along with a mutation in the AD-associated gene *PSEN1* at position L166P (Radde *et al.* 2006). The large A β deposits formed in the brain of APP23 mice are pathologically dissimilar to the A β aggregates in APP/PS1 mice which present as small, compact plaques (Sturchler-Pierrat *et al.* 1997, Radde *et al.* 2006). A β species derived from these transgenic mouse lines also harbour divergent A β peptide ratios and spectre upon incubation with luminescent amyloid-binding dyes (Heilbronner *et al.* 2013). Consistent with the concept of prion strains, inoculation of APP/PS1 or APP23 brain homogenate into young APP23 mice induces the formation of A β aggregates that are similar in morphology, binding affinity to amyloid dye and peptide ratio to that of the inoculum (Heilbronner *et al.* 2013). Similarly, distinct clinical and pathological phenotypes are found in the human condition, an example of which is seen in AD-patients carrying the Arctic or Swedish mutation in APP. The Arctic mutation (E693G) occurs within the A β sequence of APP and in humans disease is characterized by increased protofibril formation and the deposition of a specific A β peptide (A β 38) preferentially surrounding blood vessels (Moro *et al.* 2012). Disease in patients carrying this mutation is associated with rapid cognitive decline and lower plasma levels of A β compared to what is typically seen in AD patients (Nilsberth *et al.* 2001). In contrast, the Swedish mutation (K670M/N671L) is located outside the A β sequence, causing disease associated with the

overproduction of wild-type A β and AD pathology that is similar to sporadic disease (Citron *et al.* 1992, Mullan *et al.* 1992). These mutations in APP confer unique biochemical profiles of A β species formed, where brain-derived Arctic A β species are more PK resistant than those isolated from Swedish or sporadic AD patients (Watts *et al.* 2014). Transgenic mice inoculated with human brain from patients carrying either the Arctic or Swedish mutation generate distinct profiles of A β species that reflect the human condition, where APP23 mice inoculated with human brain homogenate containing the Arctic mutation have a shorter incubation period and reduced ratio of A β peptides compared to mice inoculated with human homogenate from mutant Swedish or sporadic AD origin. In addition, Arctic-inoculated mice contain abundant A β 38-positive CAA deposits in the thalamus that appeared fuzzy by immunohistochemistry analysis, with A β aggregates extending outwards from the blood vessels (Watts *et al.* 2014). Strikingly, these apparently strain-specific features are conserved following serial passage in mice (Watts *et al.* 2014). These studies demonstrate that, similar to PrP^{Sc}, unique pathological and biochemical profiles of A β are found amongst cases of AD, which are conserved upon introduction in susceptible hosts.

Strain variability in prion disease is attributed to alterations in the conformation of PrP^{Sc}, and likewise, evidence suggests the conformation of A β may encipher unique pathological and clinical profiles in AD. Transmission electron microscopy and solid-state NMR has been used to model A β species where fibrillization of monomeric A β seeded with human brain from different AD patients produces discrete populations of fibrils with unique morphology (Lu *et al.* 2013). The ability of AD brain to seed fibrils that are not polymorphic suggests that a single nucleation event may be responsible for the spread of A β in disease and, given that the patients from which the A β seeds were derived exhibited different clinical and pathological profiles, the conformational properties of A β may translate the clinical presentation in human AD. Consistent with this, producing recombinant A β fibrils in the absence of presence of detergent produces distinct species of A β that induce differential plaque characteristics and A β peptide ratios in mouse brain (Stöhr *et al.* 2014). Hence the conformation of the A β species appears to be a contributing factor to the clinical and pathological development of disease.

Prion-like properties of α -synuclein

The deposition of misfolded α syn in the CNS is a pathological hallmark of synucleinopathies and includes PD, DLB and MSA, among others. Although overlapping clinical presentations can make correct diagnosis difficult, synucleinopathies are distinguished by unique profiles of cell types and brain regions susceptible to develop α syn pathology and the locality of neuronal vulnerability. For example, the pathology of PD and DLB is characterized by intraneuronal deposits of α syn called Lewy bodies (LB) or Lewy neurites (LN) whereas MSA-associated α syn is distinguished

predominantly by cytoplasmic inclusions called glial cytoplasmic inclusions (GCIs) which predominately occur in oligodendroglia (Figure 2). The expression of α syn protein is associated with disease as duplications or triplications in the gene encoding α syn, *SNCA*, and various point mutations (such as A53T, A30P, E46K) cause early onset familial PD. Similarly, transgenic animal models that express human wild-type α syn, or α syn containing PD-associated mutations, spontaneously develop α syn pathology and neurological illness and are used to model disease (Masliah *et al.* 2000, Kahle *et al.* 2000, Feany & Bender 2000, van der Putten *et al.* 2000). Transgenic animal models expressing mutant α syn are typically used to study disease associated with protein aggregation, however many other transgenic systems have been developed to study other features of disease such as mitochondrial dysfunction and motor impairment (Chesselet & Richter 2011).

Some of the earliest evidence that α syn may have the characteristics of a prion came from autopsy of PD patients who, as part of a clinical trial, had fetal nigral dopaminergic nerve cells grafted into their brain. The presence of α syn deposits in the transplanted tissue suggested in the 11-16 years post-surgery, α syn transmission occurred from the host to the grafted tissue (Kordower *et al.* 2008, Li *et al.* 2008). Contrasting reports on fetal transplants in PD patients have described grafted regions remain dopaminergic and absent of α syn pathology for up to 14 years post surgery (Mendez *et al.* 2008), however numerous *in vitro* data support propagation of α syn seeds. In immortalized cells overexpressing A53T α syn protein, exposure to misfolded α syn species leads to punctate staining of α syn suggestive of propagation of disease-associated inclusions (Danzer *et al.* 2009). Similarly, using cationic-liposomes to aid transfection, fluorescently-tagged fibrillar α syn has been shown to enter immortalized cells overexpressing α syn and induce changes in endogenous protein consistent with pathogenic α syn such as phosphorylation, ubiquitination and insolubility (Luk *et al.* 2009). A caveat to these studies is the requirement for α syn to be overexpressed in the cells, which does not model disease where the majority of synucleinopathies express normal levels of α syn protein, and the use of artificial vehicle reagents to aid the delivery of misfolded protein into the cell. However a similar finding has also been found following extracellular exposure of primary neuronal cultures from wild-type mice to fibrillar α syn species without the aid of vehicle reagents, which resulted in the recruitment of endogenous α syn into insoluble LB-like inclusions and associated changes to neuronal vulnerability including decreases in synaptic proteins and impairment in neuronal excitability (Volpicelli-Daley *et al.* 2011).

Several other studies support *in vitro* propagation of α syn. Co-culturing two cell lines expressing α syn containing different fluorescent tags results in the progressive emergence of double-labelled cells (Hansen *et al.* 2011) and treating tagged α syn cells with misfolded α syn tagged with a different probe leads to significant co-localization in cultured cells (Bousset *et al.* 2013). In immortalized cells expressing tagged- α syn, incubation with human brain derived from MSA

patients causes the tagged- α syn to aggregate (Prusiner *et al.* 2015, Woerman *et al.* 2015). In these cultures, α syn aggregates have been shown to transverse in both retrograde and anterograde direction and transfer to neighbouring neurons and glial cells (Hansen *et al.* 2011, Desplats *et al.* 2009, Danzer *et al.* 2009). Collectively these studies give strong evidence for prion-like transmission of α syn *in vitro*.

Prion-like propagation of α syn is further supported by *in vivo* studies inoculating α syn seeds into susceptible mice. In transgenic mice expressing homozygous mutant A53T α syn (M83^{+/+}), inoculation of fibrillar recombinant α syn or brain homogenate from aged, terminal mice, into the brain of young pre-symptomatic mice accelerates the development of α syn CNS pathology and clinical disease (Luk *et al.* 2012b, Mougenot *et al.* 2012, Peelaerts *et al.* 2015, Sacino *et al.* 2014c, Rutherford *et al.* 2015, Bétemps *et al.* 2014, Sacino *et al.* 2013, Sacino *et al.* 2014a). A similar observation is found following intramuscular or intravenous injection of α syn seeds (Sacino *et al.* 2014b, Peelaerts *et al.* 2015). In these studies, similar to the human condition, aggregated α syn is largely phosphorylated and other pathological features of disease present, including reactive astrogliosis and microgliosis. Consistent with the observations in human studies, grafting dopaminergic neurons into transgenic mice overexpressing human α syn leads to the identification of α syn aggregation in the grafted tissue (Hansen 2011).

Seeded propagation has also been reported after inoculation of misfolded recombinant α syn in wild-type rodents (Luk *et al.* 2012a, Paumier *et al.* 2015, Masuda-Suzukake *et al.* 2014, Masuda-Suzukake *et al.* 2013, Tran *et al.* 2014, Sacino *et al.* 2013) and transgenic mice overexpressing wild-type α syn (M20^{+/+}) that do not spontaneously develop inclusions over their lifetime (Sacino *et al.* 2013, Sacino *et al.* 2014a). In some studies, motor impairment has been reported to accompany propagation (Tran *et al.* 2014, Luk *et al.* 2012a), and accordingly, treating mice with antibodies targeting misfolded α syn via intraperitoneal injection reduces neuronal loss and delays the onset of clinical disease (Tran *et al.* 2014). Similarly, human MSA brain may induce propagation of α syn and clinical disease in hemizygous M83^{+/-} mice, which do not spontaneously develop inclusions or clinical disease in most experimental timeframes (Prusiner *et al.* 2015, Watts *et al.* 2013). Although propagation of α syn has been extensively reported, with α syn pathology shown to extend along white matter tracks to regions distant from the injection site, it has recently been shown well-used antibodies targeting phosphorylated α syn (pSer128/81A) cross-reacts with neurofilament subunit L that is abundant in white matter (Sacino *et al.* 2014c), hence previously reported models of propagation may include artefacts of phosphorylated α syn expression associated with using these antibodies. This observation may be a matter of dose threshold of the antibody, as others have found no cross-reactivity (Luk *et al.* 2012a, Paumier *et al.* 2015, Masuda-Suzukake *et al.* 2013, Tran *et al.* 2014, Guo *et al.* 2013), including minimal pSer128/81A positive staining in transgenic α syn knock-out mouse brain and primary neuronal cultures (Luk *et al.*

2012a, Volpicelli-Daley et al. 2011). Regardless, the specificity of these antibodies requires further investigation and is an important technical issue to clarify.

Although the evidence for prion-like propagation of α syn is extensive, variation exists in the incubation period, pattern of α syn pathology and mouse line used. For example, whilst M83^{+/+} mice develop extensive α syn pathology following injection with recombinant fibrillar α syn, in other transgenic mouse lines, such as M47^{+/+} (expressing human α syn E46K mutation), α syn deposition is largely restricted to the injection site (Sacino et al. 2014c). This may indicate artefacts associated with α syn aggregation with the expression of certain transgenes, or that the normal wild-type α syn cannot be patterned and amplified using the E46K mutated α syn. Furthermore, it has recently been reported injection of spinal cord homogenate from wild-type mice induces robust CNS α syn pathology in M83^{+/+} mice (Sacino *et al.* 2015), suggesting inherent characteristics of the transgenic M83 mouse may contribute to α syn pathology. Although propagation of α syn pathology has also been reported in wild-type animals (Luk et al. 2012a, Paumier et al. 2015, Masuda-Suzukake et al. 2014, Masuda-Suzukake et al. 2013, Tran et al. 2014, Sacino et al. 2013, Sacino et al. 2014a), similar results are unable to be achieved in other studies (Sacino et al. 2014c) which may reflect the type of seed injected being unable to interact with the native α syn. An important consideration is that mouse α syn contains a threonine at position 53 and given that most studies using transgenic α syn mice express the transgene on a wild-type background, species-specific contributions by endogenous α syn may be a confounding factor in these studies. However recently it has been shown transgenic mice expressing wild-type human α syn on a knockout α syn background are also conducive to CNS α syn propagation following injection of human brain homogenate from DLB and MSA patients (Bernis *et al.* 2015), giving evidence for α syn propagation being a conserved observation in various genetic backgrounds.

Similar to prions, lipids may represent a co-factor to enhance the fibrillization of α syn; lipid-rich exosomes accelerate the fibrillization of monomeric α syn (Grey *et al.* 2015) and appear to act by stimulating primary nucleation (Galvagnion *et al.* 2015). Misfolded α syn species may be produced using PMCA (Herva *et al.* 2014), however unlike PrP misfolding, aggregation of α syn may be induced easily from recombinant wild-type protein in the absence of co-factors or serial rounds of propagation. Prions do not exclusively form amyloid and may exist as various soluble and insoluble species, and similarly the capacity of α syn to trigger misfolding is conserved in both fibrillar and non-amyloidogenic seeds (Sacino et al. 2013). The ability to generate various α syn species from the same starting material *in vitro* is reminiscent of strain variation amongst prions. Two populations of α syn, ribbons and fibrils, exert differential abilities to cross-seed fibrillization of monomeric α syn or tau protein and induce toxicity in immortalized cells (Bousset et al. 2013, Guo et al. 2013). Following injection into rat brain, ribbons induce more abundant deposition of phosphorylated α syn, whilst fibrils were shown to represent the most toxic species, causing cell

death and associated motor impairment after injection (Peelaerts et al. 2015). Brain tissue from MSA patients exhibited differences in triggering aggregation of α syn *in vitro* (Woerman et al. 2015) and α syn isolated from human PD brain show differential PK-induced cleavage products (Guo et al. 2013). Intriguingly, recent evidence also demonstrates differences in transmissibility between α syn derived from various human conditions. In M83^{+/-} mice, inoculation with human MSA, but not PD brain homogenate induces disease consisting of deposited phosphorylated α syn, progressive neurologic dysfunction, astrogliosis and microgliosis. The aggregation of α syn in these mice following inoculation with MSA brain extract was predominantly observed as cytoplasmic inclusions located in neuronal populations, which is in contrast to its deposition in oligodendroglia characteristic of MSA (Prusiner et al. 2015). The pathological phenotype induced in these animals is independent of total α syn protein and instead correlates to the capacity of the brain homogenate to induce aggregation in immortalized cells expressing tagged-A53T α syn. This observation is in contrast to PD brain tissue, which was unable to induce aggregation in the same model of transgenic cells or transmit disease to M83^{+/-} mice. The specific biochemical and biophysical differences between MSA and PD-derived α syn that translate these pathogenic properties is unknown. Furthermore, the disease that develops in MSA-infected mice is transmissible, which causes disease when inoculated into young M83^{+/-} mice.

Mounting evidence appears to draw parallels between α syn, A β and PrP^{Sc}, with studies supporting prion-like seeding, propagation and transmissibility of pathogenic proteins causing disease, however caveats exist associated with the use of transgenic mice to adequately model disease. For example, the possibility of other agents aside from α syn to causes aggregation raises important considerations when evoking prion-like mechanisms and reflects hazards associated with using transgenic mice where aggregation of protein is an inherent characteristic of that model. Studies that use transgenic mice that remain asymptomatic may alleviate these issues, however it is not clear whether expressing lower levels of the transgene are entirely innocuous. In prion disease, transmission of disease into susceptible wild-type animals may readily be achieved, and represent *bona fide* models of disease. Therefore the gold-standard for investigating prion-like propagation and transmission of misfolded protein would require the use of non-transgenic animals. Numerous data support the ability of certain α syn species to propagate and transmit disease in wild-type mice (Luk et al. 2012a, Paumier et al. 2015, Masuda-Suzukake et al. 2014, Masuda-Suzukake et al. 2013, Tran et al. 2014, Sacino et al. 2013), however in the case of A β , equivalent studies require the use of transgenic APP animals (Figure 3).

If conclusive evidence arises showing other misfolded proteins constitute a prion, there would undoubtedly be substantial implications on the health and safety regulations of these diseases. However it is important to bear in mind that prions derived from different proteins will likely have different biochemical characteristics. Recently it has been shown misfolded α syn are markedly

easier to decontaminate from surfaces and do not require the stringent techniques required for decontamination of prions (Bousset *et al.* 2015), and as such the potential for untoward public health risks, such as unintentional transmission of misfolded α syn, seems unlikely. This observation reflects fundamental differences in the structure of these proteins and stability of the misfolded isoform. Although A β and α syn are largely unstructured proteins, the GPI-anchored PrP^C has a well-defined tertiary structure, and PrP^C and PrP^{Sc} are stable proteins; as such, the activation energy required for misfolding of PrP^C into PrP^{Sc} is high. This is in contrast to α syn and A β where the activation energy required is low and the prion-like ‘misfolding’ of protein reflects the aggregation of protein and its ability to form amyloid. In this regard, the size of the infectious unit of PrP may differ from α syn and A β .

Neurotoxicity of misfolded protein

A consistent feature that accompanies the accumulation of protein in the CNS is the progressive damage and loss of neurons. Although disease-specific differences are found in the tempo and localization of neurotoxicity between proteinopathies, similarities are found which may indicate conserved mechanisms underlie the generation of cell death. This may reflect conserved fibrillization properties, where fibril formation involves the misfolding of monomeric protein and extension into various species (oligomers, protofibrils) prior to its maturation into mature fibrils (Figure 1). Given the current attention on the prion concept in proteinopathies, extrapolation of knowledge of folded proteins may prove rewarding in deciphering general neurotoxic mechanisms.

Historically, the presence of misfolded proteins in the brain led to the belief that the sheer aggregation of protein was responsible for neurotoxicity in proteinopathies, however today this is considered an oversimplification. In prion disease, large variation exists in the protein deposition seen in the CNS at post mortem, where in some cases neurodegeneration occurs with minimal PrP^{Sc} load (Medori *et al.* 1992, Collinge *et al.* 1990). Transgenic mice expressing mutations associated with familial prion disease have less aggregated protein in their brain at terminal stage disease compared to prion-infected wild-type mice (Manson *et al.* 1999), or PrP^{Sc} is entirely absent (Hsiao *et al.* 1990). Prion infection in mice expressing PrP^C lacking a GPI-anchor causes disease characterized by a long incubation period prior to the development of clinical symptoms, despite large amounts of PrP^{Sc} accumulation in the CNS (Chesebro *et al.* 2010). In addition, under certain conditions, subclinical infection can occur where PrP^{Sc} propagation occurs without the development of clinical symptoms or neurodegeneration. Mice infected with hamster prions remain asymptomatic despite having prion titres in their brain equivalent to end-stage disease in conventional mice (Hill *et al.* 2000). Other states of subclinical infection are achieved when low inoculum or oral infection routes are used (Thackray *et al.* 2002, Thackray *et al.* 2003, Collins *et al.* 2005). The PERK arm of the unfolded protein response (UPR) has emerged as a pertinent

signalling pathway associated with neurodegeneration in prion disease (Moreno *et al.* 2012) and treating prion-infected mice with compounds targeting this pathway reverses cognitive deficits and improves synaptic markers independent of amyloid load (Moreno *et al.* 2013, Halliday *et al.* 2015). Kinetic studies also reveal a divergence between infectivity and toxicity, with the two occurring in separate phases in disease (Sandberg *et al.* 2011, Sandberg *et al.* 2014, Mays *et al.* 2015).

Disassociation between protein aggregation and neurotoxicity is similarly observed in AD and synucleinopathies. In human AD brain, cell loss is not associated with regions of protein deposition (Giannakopoulos *et al.* 2003, Bennett *et al.* 2004) and in certain transgenic animal models of AD, death can occur in the absence of amyloid formation (Carlson *et al.* 1997). Likewise, insoluble α syn does not correlate well with the degree of neurodegeneration in human brain (Hughes *et al.* 1992, Tompkins & Hill 1997, Parkkinen *et al.* 2005). This observation extends to animal models where in transgenic rodent and fly models of PD, cell loss can occur in the absence of protein aggregation (Auluck *et al.* 2002, Masliah *et al.* 2000). These studies suggest that the deposition of misfolded protein is unlikely to be the toxic agent and instead may be an unrelated endpoint to disease pathogenesis.

In prion disease, toxicity is it not considered associated with a loss of function of the protein because PrP knockout mice exhibit no neurodegenerative phenotype (Bueler *et al.* 1992, Mallucci *et al.* 2002). Instead neurotoxicity is linked to prion replication, in view of the finding that only cells that express PrP^C may be damaged by PrP^{Sc}. Prion-infected PrP knockout mice hosting grafted PrP-expressing tissue only develop neurodegeneration in the PrP-expressing tissue, whilst PrP deposition is widespread across the brain (Brandner *et al.* 1996). Similarly, ablation of PrP in mice with established prion infection terminates disease development and reverses clinical symptoms, despite these mice harbouring levels of PrP^{Sc} in the brain equivalent to end-stage disease (Mallucci *et al.* 2003). These studies give strong evidence for the conversion of PrP^C to PrP^{Sc}, and not the sheer aggregation of protein, being a major component to neurotoxicity. These findings may be relevant to neurotoxic mechanisms of α syn and A β misfolding, and thus unifying knowledge may help shed light on general toxic mechanisms of folded protein.

Toxic oligomers

Some of the strongest evidence exists for small soluble oligomers of misfolded protein being the neurotoxic agent in proteinopathies. These species are highly unstable compared to the organized β -sheet rich structures formed by mature fibrils and by virtue of size, smaller aggregates have a higher relative exposed surface area than larger mature aggregates (Chiti & Dobson 2006). Indeed, it has been suggested the recruitment of misfolded protein into aggregates is a mechanism

employed by the cell to sequester small toxic species away from vital cellular compartments where they cause substantial damage. This may represent a common mechanism shared amongst the various proteinopathies.

A β oligomers

In human AD brain, neurodegeneration has been correlated to the pool of soluble A β species (McLean *et al.* 1999, Mc Donald *et al.* 2010); a finding which is consistent with the fact the particularly aggressive AD-associated Arctic mutation produces larger quantities of prefibrillar species (Nilsberth *et al.* 2001). The toxicity of A β in cultured cells is enhanced in heterogeneous populations of A β species compared to discrete species, with the toxicity of protofibrils being linked to the expression of monomeric protein (Jan *et al.* 2011). This finding suggests ongoing fibril formation involving interactions between misfolded species and monomeric A β is pertinent to toxicity. In wild-type mice, oligomeric A β preparations, but not insoluble amyloid plaque cores, alters synaptic plasticity by blocking long term potentiation (LTP) and activating long term depression (LTD) (Walsh *et al.* 2002, Shankar *et al.* 2008). They also trigger dendritic spine retraction (Lacor *et al.* 2007, Shankar *et al.* 2007), reduce dendritic spine density (Shankar *et al.* 2008) and cause memory deficits (Shankar *et al.* 2008, Lesne *et al.* 2006). Collectively these studies give strong evidence for oligomeric A β being the neurotoxic species.

The mechanisms underlying A β oligomeric-induced toxicity has also been studied, where the N-Methyl D-Aspartate (NMDA) and metabotropic glutamate-5 (mGlu5) receptors have been associated with the development of LTP and LTD respectively (Shankar *et al.* 2007, Shankar *et al.* 2008). Interestingly, the modulation of the mGlu5 receptor appears to involve PrP^C, which acts as a receptor for A β oligomers and complexes with mGlu5 (Lauren *et al.* 2009). Accordingly, PrP-directed antibodies inhibit A β -oligomer induced toxicity (Klyubin *et al.* 2014). It has been reported PrP knockout mice are resistant to toxicity elicited by A β oligomers (Lauren *et al.* 2009), however follow up studies have been unable to observe such a striking effect (Balducci *et al.* 2010, Calella *et al.* 2010, Cisse *et al.* 2011, Kessels *et al.* 2010). Aside from acting on neuronal receptors, A β oligomers may also cause toxicity by inducing hyperphosphorylation of tau, leading to collapse of microtubule cytoskeleton and neuritic dystrophy (Jin *et al.* 2011).

The conformation of the toxic A β species is still unclear. Investigations into the toxicity of structurally defined oligomers demonstrate neurotoxicity in cultured cells increases with oligomer order for species larger than a monomer (Ono *et al.* 2009); a finding which is supported by studies showing the smallest toxic A β species is the dimer (Shankar *et al.* 2008, Jin *et al.* 2011). However others report dimers correlate poorly with toxicity in cultured neurons and instead trimers and tetramers may be the smallest toxic agents (Jana *et al.* 2015). Trimers are also believed to be the basic component of A β *56 (Lesne *et al.* 2006); a non-fibrillar 56-kDa A β oligomeric species that

has also been implicated in toxicity. This species is naturally present in human brain and CSF, where it correlates with soluble tau and negatively correlates with several synaptic proteins in cognitively intact individuals (Handoko *et al.* 2013, Lesne *et al.* 2013). In rats, A β *56 induces transient cognitive dysfunction independent of amyloid deposition and neuronal loss (Lesne *et al.* 2006) and as such A β *56 may contribute to the early deficits seen in AD, prior to the accumulation of protein indicative of disease. Given A β *56 is likely to be a dodecamer (based on its apparent molecular weight on SDS-PAGE), further support for it being the toxic agent comes from studies highlighting the role of large oligomeric structures of A β as potent toxic oligomers (Gong *et al.* 2003, Bitan *et al.* 2003, Bernstein *et al.* 2009). However given that there is no evidence A β *56 alone can induce persistent neurological dysfunction, its generation is not considered necessary for toxicity associated with A β accumulation.

asyn oligomers

In human DLB brain, the presence of *asyn* aggregates at presynaptic terminals, rather than LBs, correlates with decreases in presynaptic markers and dendritic spine loss (Kramer & Schulz-Schaeffer 2007). Similarly, soluble oligomers are found enriched in regions of PD brain found to have deregulated markers of protein synthesis (Garcia-Esparcia *et al.* 2015). In rodents, the expression of mutant *asyn* that preferentially forms oligomers is more toxic than mutations which more readily form fibrils (Winner *et al.* 2011). In primary neuronal cultures and transgenic invertebrate (*Caenorhabditis elegans* and *Drosophila melanogaster*) model systems, the expression of variants of *asyn* that increase its propensity to oligomerize and decrease the formation of fibrils are more toxic than wild-type *asyn* (Karpinar *et al.* 2009). Misfolded *asyn* species produced in the presence of a compound that promotes formation of large fibrillar aggregates and reduces oligomer species exhibits reduced seeding potential and toxicity to cultured cells compared to untreated fibrils (Lam *et al.* 2016). Various oligomeric species produced from synthetic wild-type *asyn* display varying degrees of toxicity to cultured cells (Danzer *et al.* 2007) and compared to preparations of fibrillar *asyn*, primary neuronal cultures treated with purified oligomeric species activate higher levels of reactive oxygen species (Chen *et al.* 2015). Collectively these studies give strong evidence for oligomers being the most toxic *asyn* species.

Studies have investigated the mechanism of toxicity by *asyn* species. Prefibrillar *asyn* oligomers have been shown to cause mitochondrial impairment (Luth *et al.* 2014) and golgi fragmentation and trafficking malfunction in association with reduced cell viability (Gosavi *et al.* 2002) *in vitro*. The identification of mitochondria as a target for *asyn* oligomers aligns with a plethora of data linking mitochondria dysfunction to synucleinopathies; in human PD brain deficits are found in mitochondrial complex I activity (Schapira *et al.* 1989, Parker *et al.* 1989, Janetzky *et al.* 1994) and several genes that cause familial PD are associated with mitochondria functioning, including

PINK1, *LRRK2*, *Parkin* and *DJ-1* (Camilleri & Vassallo 2014). Additionally chemicals that induce mitochondria dysfunction are capable of manifesting PD in humans and in animal models in the absence of α syn deposition (Langston & Ballard 1983, Burns *et al.* 1983, Betarbet *et al.* 2000). Therefore it is possible that mitochondria dysfunction is a central mechanism associated with toxicity in the various subsets of synucleinopathies.

Another potential site for α syn oligomer-induced damage is the cellular membrane. It is well known α syn has a high affinity to bind to lipid species (Ruiperez *et al.* 2010) and in cultured neurons exogenously added α syn oligomers can form clusters on the cell membrane and associate with the $\alpha 3$ subunit of Na^+/K^+ -ATPase, causing alterations to the distribution of the receptor and deficits in Na^+ gradient and pumping potential (Shrivastava *et al.* 2015). This finding aligns with other *in vitro* studies suggesting pore formation or leak channel as toxic mechanisms by α syn oligomer or protofibril species (Danzon *et al.* 2007, Volles *et al.* 2001). Given that mutations in the gene encoding the $\alpha 3$ subunit of Na^+/K^+ -ATPase are associated with rapid-onset dystonia Parkinsonism (de Carvalho Aguiar *et al.* 2004, Rodacker *et al.* 2006, Clapcote *et al.* 2009), an adverse interaction with α syn and the receptor is likely to be relevant to at least a subset of human synucleinopathy conditions.

Although α syn oligomers have been shown to be toxic to neurons, they may not represent the only toxic α syn species. Fibrils also form clusters on neuronal membranes and associate with the $\alpha 3$ subunit of Na^+/K^+ -ATPase with higher affinity than oligomers (Shrivastava *et al.* 2015). They are also reported to exhibit the highest degree of toxicity compared to oligomers and ribbons when injected into rat brain (Peelaerts *et al.* 2015); an observation that is supported by several *in vitro* studies (Bousset *et al.* 2013, Pieri *et al.* 2012). Therefore the relevance of oligomeric, and larger α syn species to toxicity needs further investigation.

PrP oligomers

Although difficulties in producing and isolating oligomeric PrP species have hampered studies on toxic PrP-oligomers, it is likely the structure of oligomers are conserved amongst amyloid-forming proteins. Support for this comes from the finding that synthetic antibodies are capable of binding oligomeric species of amyloid-forming protein irrespective of primary sequence including PrP, α syn, A β and islet amyloid polypeptide (iAPP) (Kayed *et al.* 2003). Similarly, compounds that bind to and inhibit oligomer formation have been reported efficacious to both prion and PD-animal models (Wagner *et al.* 2013).

Studies using synthetic PrP show strong support for small aggregates being the toxic species. Species reminiscent of oligomers have increased toxicity *in vitro* (Kazlauskaitė *et al.* 2005, Novitskaya *et al.* 2006, Simoneau *et al.* 2007) and *in vivo* (Simoneau *et al.* 2007) compared to

larger fibrillar species. During PrP^C misfolding, it is believed monomeric PrP adopts an α -helical conformation prior to its dimerization and extension into oligomers (Jansen *et al.* 2001).

Monomeric recombinant α -helical PrP is highly toxic to murine neuronal but not fibroblast cell cultures and causes toxicity in a dose-dependent manner when injected into mouse brain (Zhou *et al.* 2012). These studies are supported by kinetic studies showing oligomeric species increase with the development of clinical disease in prion-infected mice (Mays *et al.* 2015).

Although the evidence for oligomers being the toxic agent is compelling, no common mechanism of action has been identified. This may be a result of the differential endogenous localization of the proteins and/or different biochemical or morphological properties of the putative toxic species. The latter factor is particularly relevant given that 'oligomers' refers to essentially any species larger than a monomer and thus represents a wide range of various sized species. Given that these species exist in equilibrium with each other, distinguishing one species as toxic over another will be challenging. This was reflected by studies showing the reported putative toxic A β dimer rapidly aggregates into metastable protofibrils (O'Nuallain *et al.* 2010) that may be more toxic in nature. An additional consideration is the potential for artificial manipulation of fibrillization by laboratory processes or reagents. The popular laboratory solvent SDS is capable of inducing artificial aggregation of A β (Bitan *et al.* 2005) and α syn (Giehm *et al.* 2010); which in the case of α syn follows an atypical fibrillization pathway. Thus the *in vitro* generation of oligomers or preparations of tissue are important considerations when deciphering the toxic species.

Conclusion

A growing body of evidence is finding parallels between A β , α syn and the well-studied PrP^{Sc}. Studies show that similar to prions, seeds of A β and α syn are capable of inducing aggregation in normal protein that propagate within the CNS, that in some cases leads to disease in susceptible animals. The concept of strain variation may also be a conserved feature of these proteins, where differences in structure of misfolded species incite unique disease phenotypes. Current evidence suggests certain α syn species are more prion-like than others, where in contrast to α syn derived from PD brain, MSA-associated α syn is efficient at propagating *in vitro* and *in vivo*, causing disease in susceptible transgenic mice (Prusiner *et al.* 2015). This is an interesting finding given that, similar to prion disease, the clinical progression of MSA is rapid in onset, whereas AD and PD are slower progressing disorders. Although compelling, there are experimental considerations in current studies, such as the ability of transgenic animals to accurately model disease and assess prion-like characteristics of aberrant proteins. Therefore, important future studies should include the use wild-type animals and robust biochemical techniques that investigate the different species of pathogenic protein aggregates, to define those that are most proficient at propagating, seeding

and causing toxicity. Further examination will, no doubt, shed important light on these issues which may ultimately lead to the expansion of the prion concept.

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FIGURE LEGENDS

Figure 1. Nucleation-dependent model of prion replication. Misfolding of normal cellular prion protein, PrP^C converts it into its infectious, disease-associated isoform PrP^{Sc}. PrP^{Sc} induces nucleation-dependent misfolding in other PrP^C leading to amplification and propagation of transmissible prions. The growing aggregates extend into oligomers, protofibrils and then fibrils that form the protein aggregates characteristic of disease. A natural component of prion propagation is breakage of the growing PrP^{Sc} aggregate, which produces more nucleation sites for templated misfolding.

Figure 2. Disease-associated accumulation of misfolded protein in the central nervous system. The accumulation of PrP^{Sc}, A β and α syn in the central nervous system is associated with prion, Alzheimer's and synucleinopathy disease, respectively. These proteins can accumulate in both intra- and extracellular compartments in neurons or other glial cell types, such as oligodendroglia.

Figure 3. Propagation of misfolded protein in rodent models following intracerebral inoculation of a misfolded seed. Propagation of PrP^{Sc}, A β and α syn has been studied in various mouse models. Images represent the endogenous aggregation of protein in the brain of rodents that occurs over their lifetime (green line) and aggregation that occurs following the injection of a seed (red line) (A) Wild-type mouse models do not spontaneously develop protein aggregation over their lifespan and the induction of a seed induces aggregation of protein (B) Transgenic mouse models that spontaneously develop protein aggregation and the incorporation of the seed accelerates the deposition of protein. (C) Transgenic mouse models that do not spontaneously develop inclusions over the experimental timeframe, and the induction of a seed induces protein aggregation.

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