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Like prions: the propagation of aggregated tau and α-synuclein in neurodegeneration

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The abnormal aggregation of a small number of known proteins underlies the most common human neurodegenerative diseases. In tauopathies and synucleinopathies, the normally soluble intracellular proteins tau and α-synuclein become insoluble and filamentous. In recent years, non-cell autonomous mechanisms of aggregate formation have come to the fore, suggesting that nucleation-dependent aggregation may occur in a localized fashion in human tauopathies and synucleinopathies, followed by seed-dependent propagation. There is a long prodromal phase between the formation of protein aggregates and the appearance of the first clinical symptoms, which manifest only after extensive propagation, opening novel therapeutic avenues.

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Abbreviations: CBD = corticobasal degeneration; (v)CJD = (variant) Creutzfeldt-Jakob disease; MSA = multiple system atrophy

Introduction

Common human neurodegenerative diseases are characterized by the presence of abundant filamentous inclusions (Goedert, 2015; Walker and Jucker, 2015). Each type of inclusion has one protein as its major component, with amyloid-β, tau and α-synuclein being the most commonly affected (Fig. 1). These proteins undergo a transformation from a soluble to an insoluble filamentous state, with a number of intermediates. Most cases of disease are sporadic, but a small percentage are inherited, often in a dominant manner. The latter are caused by mutations in the genes encoding the proteins that make up the inclusions, or proteins that increase their production, underscoring the importance of inclusion formation for neurodegeneration. It constitutes the gain of toxic function that most probably causes human neurodegenerative diseases.

Mutations in *MAPT*, the tau gene, cause an inherited form of frontotemporal dementia and parkinsonism, with abundant filamentous tau inclusions in the brain. Mutations in *SNCA*, the α-synuclein gene, and multiplications thereof, give rise to familial forms of Parkinson's disease and dementia with Lewy bodies. Lewy pathology, which is made of α-synuclein filaments, defines Parkinson's disease and dementia with Lewy bodies at the neuropathological level.

Until recently, cell autonomous mechanisms were believed to account for human neurodegenerative diseases. These mechanisms imply that brain cells degenerate, as a result of the same aggregation events occurring independently. While it is easy to see how such mechanisms can lead to inherited forms of disease (mutant proteins are widely expressed), it is more difficult to understand how cell autonomous mechanisms could give rise to sporadic diseases. At death, protein inclusions are present in thousands of nerve cells. It appears unlikely that the same molecular events take place independently thousands of times in

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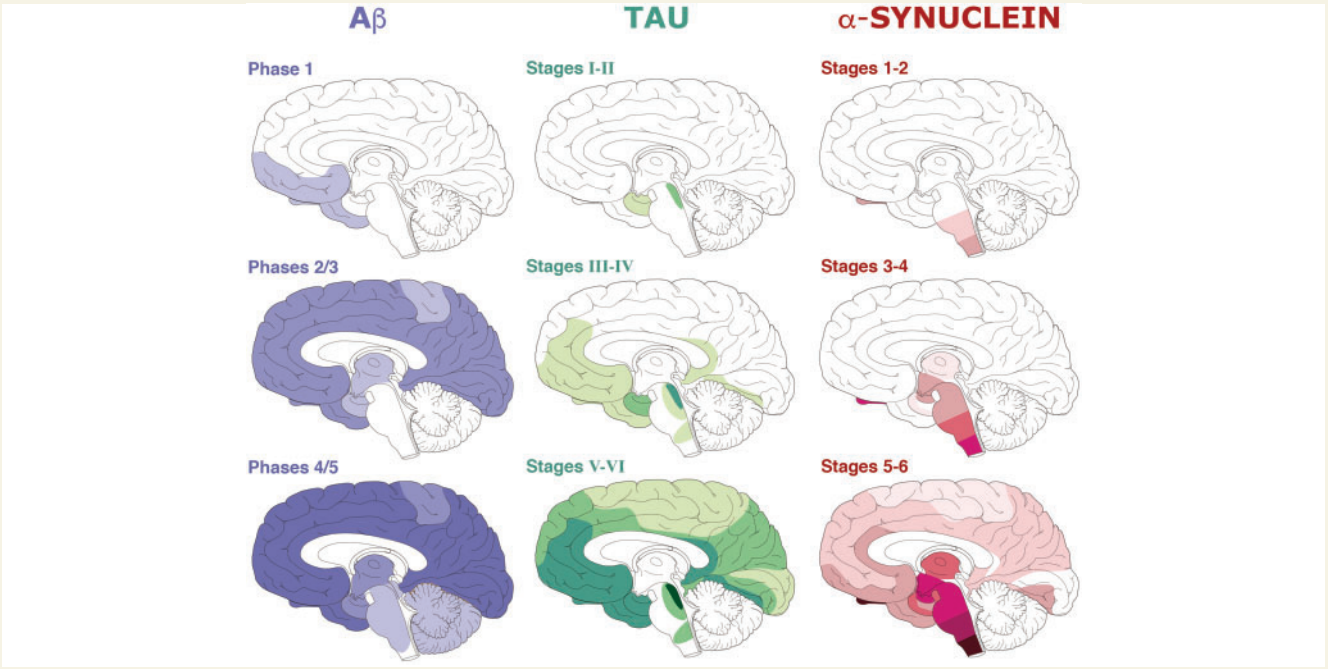

Figure 1 Distribution of amyloid-β, tau and α-synuclein inclusions in human brain. *Left*: Amyloid-β (Aβ) plaques develop first in basal temporal and orbitofrontal neocortex (Phase 1). They are observed later throughout the neocortex, hippocampal formation, amygdala, diencephalon and basal ganglia (Phases 2 and 3). In severe cases of Alzheimer's disease, amyloid-β plaques are also found in mesencephalon, lower brainstem and cerebellar cortex (Phases 4 and 5). *Middle*: Tau inclusions develop in the locus coeruleus, as well as in transentorhinal and entorhinal regions (Stages I and II). This is followed by their presence in the hippocampal formation and some parts of the neocortex (Stages III and IV), followed by large parts of the neocortex (Stages V and VI). *Right*: The first α-synuclein inclusions are present in the olfactory bulb and the dorsal motor nucleus of the vagal and glossopharyngeal nerves of the medulla oblongata (Stages 1 and 2). From the brainstem, the pathology ascends through the pons to midbrain and basal forebrain (Stages 3 and 4), followed by the neocortex (Stages 5 and 6). This figure is based on the work of Braak, Del Tredici, and collaborators. From Goedert (2015).

post-mitotic cells. The first inclusions may form in a localized fashion, from where they propagate to normal cells, resulting in degeneration (cell non-autonomous mechanisms). Even though cell non-autonomous mechanisms are not necessary for explaining inherited diseases, they may nonetheless operate. This appears to be the case of pathological huntingtin (Cicchetti *et al.*, 2014; Pecho-Vrieseling *et al.*, 2014). It has previously been suggested that neurodegenerative diseases may involve the transfer of substances between nerve cells (Saper *et al.*, 1987) and it has been known for many years that some proteins can move trans-synaptically (Schwab and Thoenen, 1976).

Spreading is consistent with staging schemes that have postulated a stereotypical progression of inclusions from a single site (transentorhinal cortex for the tau inclusions of Alzheimer's disease, ambient gyrus for the tau inclusions of argyrophilic grain disease, cortical sulci for the tau inclusions of chronic traumatic encephalopathy and dorsal motor nucleus of the vagus nerve, as well as olfactory bulb, for the α-synuclein inclusions of Parkinson's disease) (Braak and Braak, 1991; Braak *et al.*, 2003; Saito *et al.*, 2004; McKee *et al.*, 2013). However, staging cannot prove the prion-like spreading of protein aggregates, because it is also compatible with brain regions being sequentially affected by aggregate formation ('just one damned thing after another', Bell, 1909). Propagation of tau pathology is supported by its absence from a piece of disconnected cerebral cortex in an individual with Alzheimer's disease who had undergone an operation to remove a meningioma 27 years earlier (Duyckaerts *et al.*, 1997). The tumour and the operation disconnected a small piece of frontal cortex. While there was abundant tau pathology in limbic and isocortical regions, tau-positive neuritic plaques, neurofibrillary tangles and neuropil threads were not present in the disconnected frontal cortex.

Additional evidence for the existence of prion-like mechanisms in the human brain has come from the development of scattered Lewy pathology in foetal human midbrain neurons that were therapeutically implanted into the striata of patients with advanced Parkinson's disease (Fig. 2) (Kordower *et al.*, 2008; Li *et al.*, 2008). Lewy pathology was detected in 2–5% of grafted cells in patients who had survived for 10 or more years, approximately the same percentage as neurons with Lewy pathology in the pars compacta of the substantia nigra in Parkinson's disease. α-Synuclein immunoreactivity was not seen in nerve cell bodies 18 months after grafting, but it was present in a non-aggregated form after 4 years and in an aggregated,

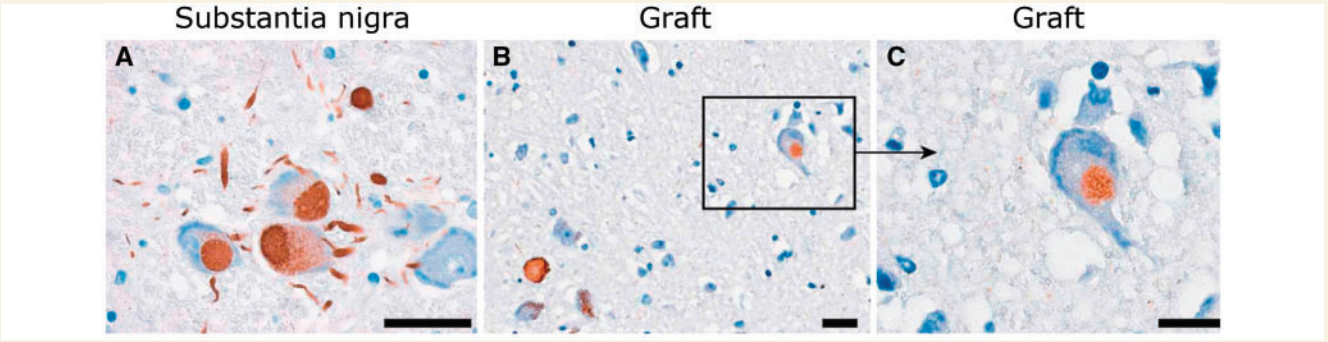

Figure 2 Possible host-to-graft spreading of Lewy pathology in Parkinson's disease. The patient received a transplant of foetal human mesencephalic dopaminergic nerve cells into the putamen 16 years previously. Immunohistochemistry for α-synuclein visualizes Lewy bodies and neurites in the host substantia nigra (**A**) and the transplant (**B** and **C**). Adapted from Li *et al.* (2008).

ubiquitinated state after 14 years (Chu and Kordower, 2010). After 24 years, 11–12% of grafted dopaminergic neurons exhibited α-synuclein and ubiquitin-positive inclusions (Li *et al.*, 2016). The clinical benefits of transplantation were gradually lost after 14 years. Although these findings are compatible with the prion-like spreading of Lewy pathology, it is also possible that the microenvironment of the nigrostriatal system in Parkinson's disease predisposes to the formation of Lewy pathology.

Over the past 7 years, experimental studies have shown that the injection of tau and α-synuclein inclusions into animals induces neurons to form intracellular inclusions at the injection sites, from where they can spread to distant brain regions (Clavaguera *et al.*, 2009; Desplats *et al.*, 2009; Hansen *et al.*, 2011; Goedert, 2015). Unless these findings are not related to what is happening in the human brain, cell non-autonomous mechanisms must also be at work in human neurodegenerative diseases.

These mechanisms are often called 'prion-like', referring to the intercellular spreading of protein aggregates, resulting in their propagation through the brain. The acronym 'prion' stands for 'proteinaceous infectious particle' (Prusiner, 1982), encompassing both intercellular propagation and inter-organismal transmission. There is no evidence to suggest that Alzheimer's disease and Parkinson's disease can transfer between individuals. Hence the use of 'prion-like'. Interneuronal spreading of tau and α-synuclein aggregates requires their release into the extracellular space, uptake by connected cells and seeded aggregation of soluble protein (Fig. 3). If the spreading of aggregates is linked to the development of clinical symptoms, it represents a therapeutic target through the inhibition of aggregate uptake or release, inhibition of seeding and activation of the clearance of protein aggregates.

Aggregated tau

Tau aggregates are characteristic of a number of human neurodegenerative diseases, the so-called tauopathies (Table 1). They form in a variety of brain regions, where they are largely neuronal. However, glial tau inclusions are also found in some diseases, such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). In white matter tauopathy with globular glial inclusions, oligodendroglial tau inclusions predominate (Kovacs *et al.*, 2008).

In adult human brain, six tau isoforms ranging from 352 to 441 amino acids are produced from a single gene through alternative mRNA splicing (Goedert *et al.*, 1989; Goedert, 2015). Three isoforms have three repeats each, and three isoforms have four repeats each. The repeats and some adjoining sequences constitute the microtubule-binding domains of tau. They also make up the core of tau filaments. In Alzheimer's disease, the most common human neurodegenerative disease, and in chronic traumatic encephalopathy, all six tau isoforms are present in the filaments. In other disease filaments—such as those of PSP, CBD, argyrophilic grain disease (AGD) and white matter tauopathy with globular glial inclusion—tau isoforms with four repeats are found. In Pick's disease, three-repeat tau isoforms predominate in the inclusions. Unlike Alzheimer's disease, these diseases lack amyloid-β pathology.

Several lines of investigation in animal models and cell culture have shown that assembled tau can behave like a prion. Injection of human mutant tau inclusions into the brains of mice transgenic for wild-type tau induced the formation of inclusions made of wild-type human tau (Fig. 4), followed by their spreading to distant brain regions (Clavaguera *et al.*, 2009). Subsequently, several groups studied the spreading of pathological tau along the entorhinal cortex/hippocampal pathway. They used mouse models that apparently expressed human P301L tau only in the entorhinal cortex (de Calignon *et al.*, 2012; Harris *et al.*, 2012; Liu *et al.*, 2012). Several months after the appearance of the first tau inclusions, hippocampal neurons developed filamentous tau pathology. Using this system, together with a mouse line transgenic for mutant amyloid precursor protein and presenilin-1, it has been shown that

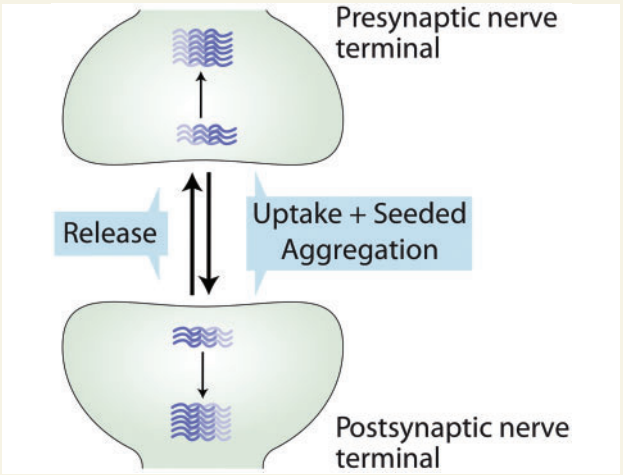

Figure 3 Hypothetical scheme showing interneuronal transfer of protein aggregates. Transfer requires release and uptake of protein aggregates, as well as seeded aggregation.

amyloid-β deposition in cerebral cortex can increase the spreading of aggregated tau (Pooler *et al.*, 2015).

The initial work showed that induction and propagation of tau aggregation were tau-dependent and almost entirely caused by the insoluble fraction of P301S tau brain extracts (Clavaguera *et al.*, 2009). Following on from this, synthetic filaments formed from aggregated recombinant tau were also shown to induce tau aggregation in transgenic mouse brain (Clavaguera *et al.*, 2013*a*; Iba *et al.*, 2013). More recently, synthetic tau filaments were injected into the locus coeruleus of mice transgenic for human P301S tau (Iba *et al.*, 2015). Mature inclusions formed at the sites of injection and in the contralateral locus coeruleus. However, the entorhinal cortex, which receives efferent projections from the locus coeruleus, failed to develop tau inclusions.

When presymptomatic P301S tau mice were intracerebrally injected with brain extracts from symptomatic animals (Ahmed *et al.*, 2014), tau inclusions formed at the injection sites after 2 weeks. Contralateral and caudo-rostral propagation was evident in nuclei with strong afferent and efferent connections to the injection sites, showing that the spread of pathology was dependent on connectivity, not proximity.

Virally-mediated expression of tau has also been used to investigate the propagation of pathology. Tau pathology was observed in the dentate gyrus following adeno-associated virus-mediated expression of P301L tau in layer II of the entorhinal cortex (Siman *et al.*, 2013; Asai *et al.*, 2015; Wegmann *et al.*, 2015). Expression of mouse tau was not necessary for the formation of tau pathology. Spreading of wild-type tau was also observed when lentiviral vectors were used (Dujardin *et al.*, 2014). It remains to be determined what the function of the spread of wild-type tau is and if it interacts with mutant tau.

The intraperitoneal injection of brain extracts from symptomatic P301S tau mice into presymptomatic mice promoted the formation of cerebral tau inclusions (Clavaguera *et al.*, 2014). Aggregated tau can thus promote inclusion formation in the CNS of transgenic mice following peripheral administration. Similar findings have been reported for prions, assembled amyloid-β and α-synuclein assemblies (Eisele *et al.*, 2010; Prusiner, 2013; Sacino *et al.*, 2014),

Distinct conformers of assembled tau appear to exist, reminiscent of prion strains (Clavaguera *et al.*, 2013*b*; Sanders *et al.*, 2014). They may explain the variety of human tauopathies. Inclusions formed after intracerebral inoculation of brain homogenates from all cases of Alzheimer's disease, tangle-only dementia, Pick's disease, AGD, PSP and CBD into the mouse line transgenic for an isoform of human wild-type four-repeat tau (Clavaguera *et al.*, 2013*b*). Tau assemblies reminiscent of human disorders were observed following the injection of brain homogenates from patients with the four-repeat tauopathies AGD, PSP and CBD. Similar inclusions also formed after the intracerebral injection of brain homogenates from human tauopathies into wild-type mice (Clavaguera *et al.*, 2013*b*).

Some of these findings were replicated when Alzheimer's disease and CBD brain homogenates were intracerebrally injected into mice transgenic for human P301S tau (Boluda *et al.*, 2015). Also, conformationally distinct tau assemblies made of four tau repeats formed in human embryonic kidney (HEK) cells. Inoculation of these assemblies into the hippocampus of young P301S tau transgenic mice induced pathologies that were stable through serial transmission. When HEK cells expressing four tau repeats were seeded with homogenates from these brains, inclusions formed that were identical to those present initially (Sanders *et al.*, 2014).

Although these observations are consistent with the existence of distinct tau aggregate strains, this issue deserves further investigation. Ultimately, the definition of a protein strain will be structural. Network connectivity studies using functional MRI have provided evidence that different tauopathies may be caused by distinct molecular conformers of assembled tau (Zhou *et al.*, 2012). This may explain (at least in part) selective neuronal vulnerability. Host factors may also play a role (Walsh and Selkoe, 2016).

Intercellular transfer of tau assemblies and the adoption of self-propagating conformations have been demonstrated in cell culture (Frost *et al.*, 2009; Nonaka *et al.*, 2010; Kfoury *et al.*, 2012; Santa-Maria *et al.*, 2012; Wu *et al.*, 2013). Inhibition of the spreading of pathology could lead to prevention of clinical symptoms. It is therefore essential to understand the molecular mechanisms involved in uptake, seeding and release of tau inclusions. Uptake of aggregated tau appears to rely on macropinocytosis and cell surface heparan sulphate proteoglycans (Kfoury *et al.*, 2012; Holmes *et al.*, 2013). Both monomeric and aggregated tau are taken up by cells, but only aggregated tau

Table 1 Diseases with tau inclusions

Alzheimer's disease
Amyotrophic lateral sclerosis/parkinsonism-dementia complex
Argyrophilic grain disease
Chronic traumatic encephalopathy
Corticobasal degeneration
Diffuse neurofibrillary tangles with calcification
Down's syndrome
Familial British dementia
Familial Danish dementia
Familial frontotemporal dementia and parkinsonism
Gerstmann-Sträussler-Scheinker disease
Guadeloupean parkinsonism
Huntington's disease
Meningio-angiomatosis
Myotonic dystrophy
Neurodegeneration with brain iron accumulation
Niemann-Pick disease, type C
Non-Guamanian motor neuron disease with neurofibrillary tangles
Pick's disease
Postencephalitic parkinsonism
Progressive supranuclear palsy
SLC9A6-related mental retardation
Subacute sclerosing panencephalitis
Tangle-only dementia
White matter tauopathy with globular glial inclusions

is able to seed the aggregation of soluble, monomeric tau (Falcon *et al.*, 2015). Most seeds are trafficked to lysosomes, but some endosomes rupture and seeds enter the cytoplasm, where they induce tau assembly.

Tau seeds made from aggregated recombinant tau are not phosphorylated, whereas seeded tau is hyperphosphorylated. Seeded tau is also p62-positive, like the tau inclusions in human brains and in mice transgenic for human P301S tau (Schaeffer *et al.*, 2012). Expressed tau can only be seeded when it is aggregation-competent (Falcon *et al.*, 2015). Aggregation inhibitors may thus be able to reduce tau-induced seeding and spreading.

Using a cell assay, we found that the seeding potency of recombinant P301S tau aggregated with heparin was lower than that of aggregated tau from the brains of mice transgenic for human mutant P301S tau (Fig. 5) (Falcon *et al.*, 2015). Similar discrepancies between recombinant and brain aggregates have been described for prions, amyloid-β, α-synuclein and reactive amyloid A (Meyer-Luehmann *et al.*, 2006; Zhang *et al.*, 2008; Luk *et al.*, 2012*a*; Stöhr *et al.*, 2012; Prusiner, 2013; Recasens *et al.*, 2014). Recombinant tau aggregates were more resistant to disaggregation by guanidine hydrochloride and digestion by proteinase K than tau aggregates from transgenic mouse brain, consistent with the view that more stable aggregates possess lower seeding activity. Recombinant aggregated tau was

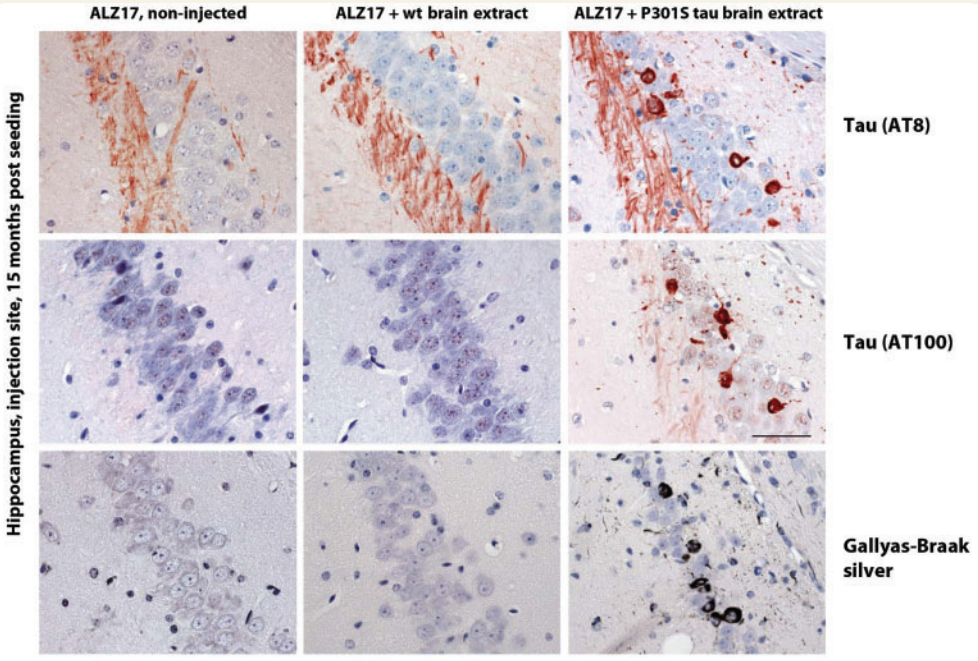

Figure 4 Induction of filamentous tau pathology in mice transgenic for one isoform of wild-type human tau (line ALZ17) following injection with brain extract from symptomatic mice transgenic for one isoform of human mutant P301S tau. Staining of the hippocampal CA3 region of 18-month-old ALZ17 mice with anti-tau antibodies AT8 and AT100 and Gallyas-Braak silver. Non-injected (*left*), 15 months after injection with brain extract from non-transgenic control mice (*middle*) and 15 months after injection with brain extract from 6-month-old mice transgenic for human P301S tau (*right*). The sections were counterstained with haematoxylin. Scale bar = 50 μm.

also found to be more stable than tau filaments extracted from Alzheimer's disease brain (Morozova *et al.*, 2013). Distinct conformations accounted for differences in seeding potency. Thus, tau filaments formed from recombinant P301S tau following seeding with aggregated tau from transgenic mouse brain showed resistance to guanidine hydrochloride similar to that of tau seeds from the brains of mice transgenic for human P301S tau (Falcon *et al.*, 2015) (Fig. 5). The seeding potency of tau filaments was like that of brain-derived aggregated P301S tau, consistent with the prion concept.

We dissected the molecular characteristics of seed-competent tau from the brains of symptomatic P301S tau transgenic mice and found that sucrose gradient fractions from brain lysates seeded tau aggregation in transfected cells only when tau aggregates (>10mers) were present (Jackson *et al.*, 2016) (Fig. 6). There was no detectable seeding by fractions containing small, oligomeric tau aggregates (<6mers), despite the presence of a 400 kDa form of tau on non-denaturing gels. Fractions containing large tau aggregates induced the formation and spreading of filamentous tau in presymptomatic transgenic mice, whereas fractions containing tau monomers and small aggregates were inactive. Seed-competent sucrose gradient fractions contained aggregated tau species ranging from ring-like structures to small filaments. These findings demonstrate that short fibrils are the major seed-competent tau species in the P301S tau transgenic mouse model.

Cells may interact with particles, rather than protein monomers. Because oligomeric species may be made of fewer than a hundred and fibrils of thousands of monomers, at equal concentrations of monomer, many more oligomers than fibrils were injected. It will be interesting to see if similar species of aggregated tau underlie seeding, spreading and neurodegeneration in Alzheimer's disease and other human tauopathies.

Less is understood about the mechanisms underlying the release of aggregated tau. One study concluded that microglial cells promote tau propagation through exosome-dependent mechanisms (Asai *et al.*, 2015). It has been suggested that exosome-mediated secretion of tau may play a role in disease (Saman *et al.*, 2012; Polanco *et al.*, 2016). By contrast, a recent study concluded that tau is released from cells through a pathway that is distinct from exosome-mediated secretion (Fontaine *et al.*, 2016). This pathway requires heat shock cognate 70 (Hsc70), its co-chaperone DnaJ and the SNARE protein SNAP23. These findings have suggested that chaperones may be central in the removal of misfolded proteins from the cytoplasm. However, it remains to be shown that aggregated tau is released from cells through these mechanisms.

It will be important to know which percentage of released tau aggregates is membrane-enclosed in different cells, because antibodies used for immunotherapy need to have access to tau aggregates. Anti-tau antibodies have been shown to reduce hyperphosphorylated and aggregated tau in mice transgenic for human P301S tau (Yanamandra *et al.*, 2014; Sankaranarayanan *et al.*, 2015). Although it is known that tau aggregates can spread between connected neurons, it is unclear if their release is entirely through synaptic mechanisms. A study using non-neuronal tau inclusion donor cells and acceptor hippocampal neurons showed that synapses enhanced the propagation of tau inclusions (Calafate *et al.*, 2015). However, non-synaptic mechanisms were also at work.

Most studies used transfected non-neuronal cells, some of which were of human origin, or primary nerve cells from rodents. However, human neurons derived from induced pluripotent stem cells also took up tau seeds and exhibited cytoplasmic seed-induced tau aggregation (Usenovic *et al.*, 2015; Verheyen *et al.*, 2015).

Aggregated α-synuclein

α-Synuclein aggregates are characteristic of Parkinson's disease (the second most common neurodegenerative disease), dementia with Lewy bodies, Parkinson's disease dementia, rapid eye movement sleep behaviour disorder, primary autonomic failure and multiple system atrophy (MSA) (Goedert, 2015). In these diseases, α-synuclein, a normally soluble protein of 140 amino acids, assembles into a filamentous, β-sheet-rich conformation that is able to seed aggregation of soluble α-synuclein (Rodriguez *et al.*, 2015; Tuttle *et al.*, 2016). Lewy pathology in nerve cells characterizes these diseases, with the exception of MSA, where filamentous α-synuclein inclusions are present in both nerve cells and glial cells, in particular oligodendrocytes and Schwann cells (Goedert *et al.*, 2013; Cykowski *et al.*, 2015; Nakamura *et al.*, 2015). Pathologically, the brains of patients with some *SNCA* mutations (G51D and A53E) have characteristics of both Parkinson's disease and MSA (Kiely *et al.*, 2013; Pasanen *et al.*, 2014)

Lewy pathology appears to spread along neural pathways in the brain, beginning in dorsal motor nucleus of the glossopharyngeal and vagal nerves, olfactory bulb and anterior olfactory nucleus (Fig. 1) (Braak *et al.*, 2003). Lewy inclusions are also found in spinal cord, autonomic ganglia, adrenal medulla, submandibular gland, heart and enteric nervous system (Goedert *et al.*, 2013). α-Synuclein deposits may form early in the enteric nervous system, which is connected to the brain through the vagal nerve, and in the peripheral nervous system. Disease mechanisms could originate in the gut and move retrogradely to the brain via the vagal nerve, or they could start in the vagal dorsal motor nucleus and move from there to the spinal cord and the gut in an anterograde fashion. Gut-to-brain spreading would be analogous to variant Creutzfeldt-Jakob disease (vCJD). In support, vagotomy has been reported to be associated with a reduced risk of Parkinson's disease (Svensson *et al.*, 2015) and Lewy pathology was detected in the gastrointestinal tract up to 20 years before a clinical diagnosis of Parkinson's disease (Stokholm *et al.*, 2016).

In overexpressing rodents, human α-synuclein can transit to nerve cells grafted into the striatum, reminiscent of what

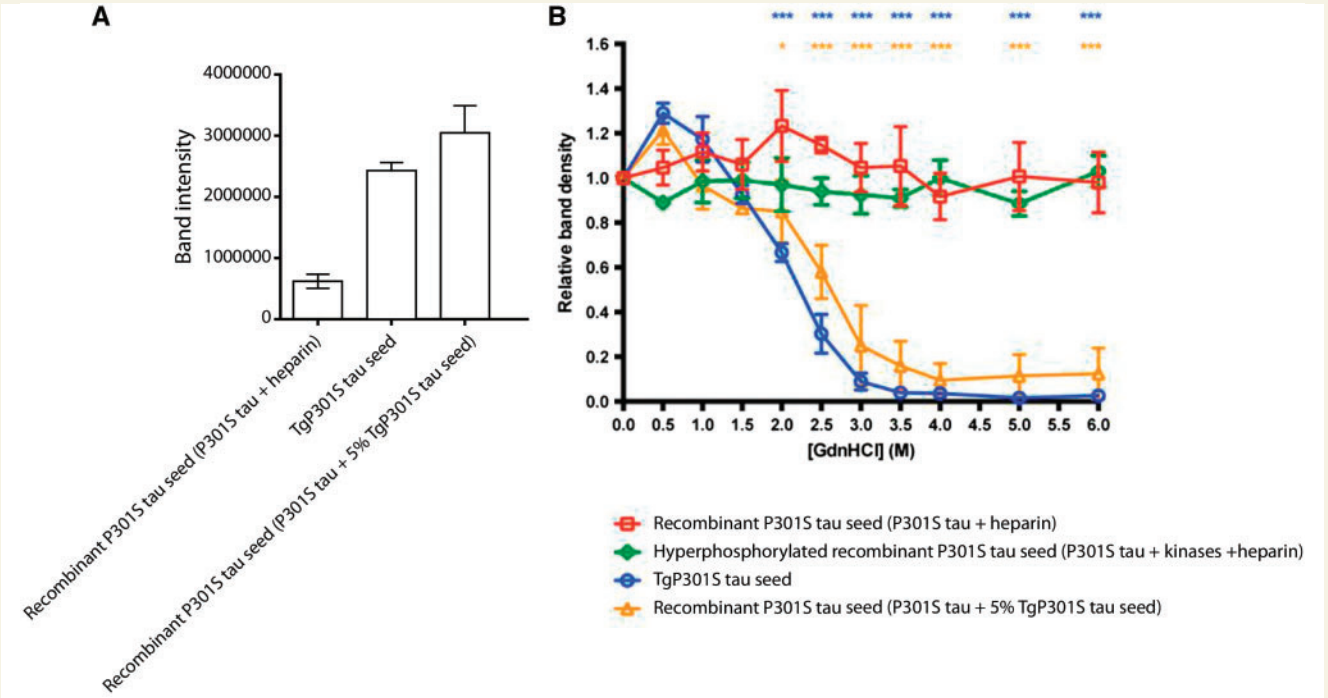

Figure 5 Conformation determines the seeding potencies and resistance to disaggregation of tau aggregates. (**A**) Quantitation by western blotting of insoluble fraction from tau-expressing HEK cells seeded with equivalent amounts of aggregated recombinant P301S tau (P301S tau + heparin), TgP301S tau aggregates and aggregated P301S tau (P301S tau + 5% TgP301S tau aggregates). (**B**) Guanidine hydrochloride (GdnHCl) treatment of tau seeds.

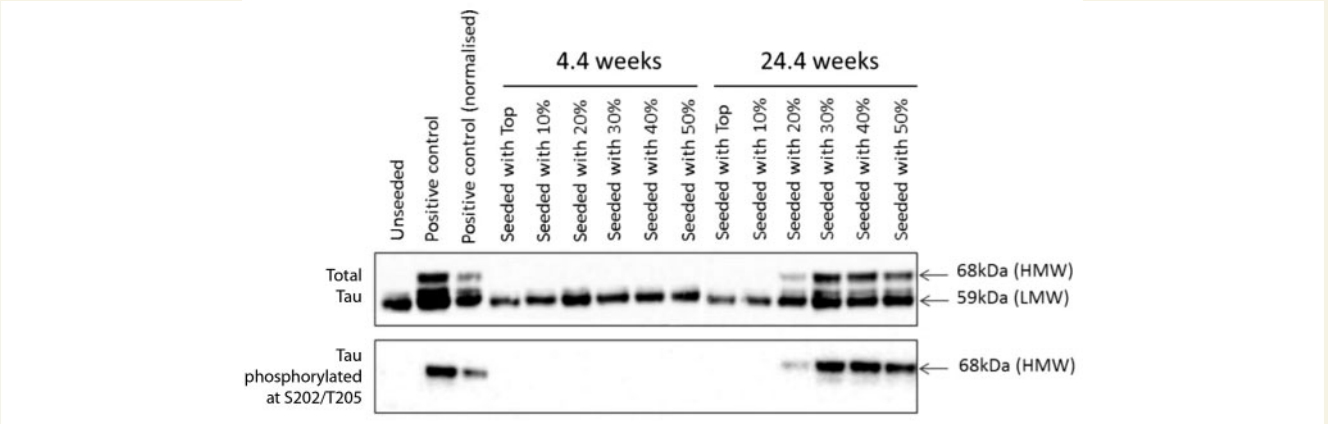

Figure 6 Seeding of tau aggregation with sucrose gradient fractions from the brains of mice transgenic for human mutant P301S tau in a cell-based assay. The mice were aged 4.4 weeks (no symptoms, no tau filaments) or 24.4 weeks (symptoms, abundant tau filaments). Sucrose gradient fractions were used to seed aggregation of tau in HEK cells overexpressing 1N4R tau with the P301S mutation. The pellet from a 100 000 *g* spin of the seeded cells was analysed by western blotting for total tau and tau phosphorylated at S202/T205 (anti-tau antibodies DA9 and AT8). Filamentous tau runs at ~68 kDa (high molecular weight, HMW); non-filamentous tau runs at ~59 kDa (low molecular weight, LMW). Positive control was seeding with sarkosyl-extracted tau from unfractionated brains of symptomatic transgenic P301S tau mice and the normalized positive control was seeding with sarkosyl-extracted tau from symptomatic mice, normalized for total tau levels relative to those of the sucrose gradient fractions. Seeding ability correlated with the presence of the 64 kDa band in 24.4-week-old mice (20–50% sucrose gradient fractions). No seeding was observed upon addition of sucrose gradient fractions from 4.4-week-old mice.

may happen in grafted Parkinson's disease cases (Hansen *et al.*, 2011; Kordower *et al.*, 2011; Angot *et al.*, 2012). Moreover, acceleration of the formation of aggregated α-synuclein phosphorylated at S129 and a reduced survival time have been described in presymptomatic transgenic mice (line M83) following intracerebral inoculation of brain homogenates from symptomatic mice (Luk *et al.*, 2012*a*; Mougenot *et al.*, 2012). The formation of α-

synuclein inclusions was accompanied by neurodegeneration. Accelerated pathology and neurodegeneration were absent in α-synuclein knockout mice that had been injected with the same brain homogenates.

Using long-term *in vivo* imaging, it has been shown that aggregated recombinant α-synuclein can seed the ordered assembly of expressed α-synuclein (Osterberg *et al.*, 2015). Inclusion-bearing neurons degenerated, demonstrating that inclusion formation was linked to cellular toxicity. However, the toxic α-synuclein species are not known and could be oligomers. Two types of oligomers formed *in vitro*, one of which exhibited proteinase K resistance and neurotoxicity to rat primary neurons (Cremades *et al.*, 2012).

Further evidence for assembled α-synuclein behaving like a prion has come from the injection of MSA brain extracts into heterozygous mice transgenic for A53T α-synuclein, which express the human mutant protein predominantly in nerve cells (Prusiner *et al.*, 2015; Woerman *et al.*, 2015). Intracerebral injection led to the formation of abundant α-synuclein inclusions and their spreading, accompanied by motor impairment. By contrast, the injection of Parkinson's disease brain extracts did not cause pathology or symptoms. Unlike in MSA, where they are also found in oligodendrocytes, inclusions were only present in nerve cells, the major site of production of the transgene. It is unclear why oligodendrocytes develop α-synuclein inclusions in MSA. One possibility is that α-synuclein is normally produced at low levels by oligodendrocytes, which overexpress or fail to clear it in MSA. α-Synuclein has been detected in oligodendrocyte lineage precursors, but its levels decreased during oligodendrocyte maturation (Djelloul *et al.*, 2015). Alternatively, α-synuclein aggregates may propagate from neurons to oligodendrocytes. Experimentally, α-synuclein aggregates have been shown to transfer from neurons to oligodendrocytes (Reyes *et al.*, 2014). After injection into hind limb muscles of heterozygous A53T transgenic mice, recombinant α-synuclein assemblies induced cerebral α-synuclein aggregation and disease symptoms (Sacino *et al.*, 2014). Transection of the sciatic nerve mitigated these effects.

The intracerebral injection of sarkosyl-insoluble fractions from the brains of patients with MSA and incidental Lewy body disease into mice expressing wild-type human α-synuclein in nerve cells on a mouse α-synuclein knockout background, led to the formation of abundant neuronal α-synuclein inclusions and their spreading, in the absence of clinical symptoms (Bernis *et al.*, 2015; Prusiner *et al.*, 2015).

Transport of α-synuclein aggregates from periphery to brain has been demonstrated in wild-type rats (Peelaerts *et al.*, 2015). However, it remains to be shown that these aggregates can seed aggregation of endogenous α-synuclein. To date, the peripheral injection of aggregated α-synuclein has not resulted in the formation of brain or spinal cord inclusions in wild-type animals.

This contrasts with the central injection of α-synuclein aggregates. Intrastriatal or intranigral injection of recombinant α-synuclein assemblies into wild-type rodents gave rise to α-synuclein inclusions and some brain dysfunction (Luk *et al.*, 2012*b*; Masuda-Suzukake *et al.*, 2013, 2014; Paumier *et al.*, 2015). Depending on the injection sites of α-synuclein aggregates, spreading to different brain regions was observed (Masuda-Suzukake *et al.*, 2014). Injection of α-synuclein assemblies from the brains of patients with dementia with Lewy bodies and Parkinson's disease into wild-type mice also resulted in the formation of inclusions (Masuda-Suzukake *et al.*, 2013; Recasens *et al.*, 2014). Moreover, the intracerebral injection of Lewy body extracts from Parkinson's disease patients into macaque monkeys gave rise to α-synuclein inclusions and signs of nerve cell dysfunction (Recasens *et al.*, 2014). Similar experiments remain to be carried out using brain extracts from human tauopathies.

Although these findings support the view that aggregated α-synuclein interacts directly with monomeric protein and induces its aggregation, it is also possible that this happens indirectly. It has been shown that the *in vitro* assembly of monomeric α-synuclein requires large numbers of seeds (Iljina *et al.*, 2016). In cells, reactive oxygen species were generated in response to a much lower concentration of α-synuclein aggregates. These findings suggest that effective seeding of monomeric α-synuclein may require both seeds and cellular stress.

Morphological differences between disease-associated α-synuclein filaments have been described (Goedert, 2015). Polymorphs of recombinant aggregated α-synuclein in the form of ribbons or fibrils have also been reported (Bousset *et al.*, 2013). When injected into the rat substantia nigra, ribbons gave rise to Lewy pathology, whereas fibrils, which did not seed Lewy pathology, led to the loss of dopaminergic neurons (Peelaerts *et al.*, 2015). It remains to be determined if ribbons and fibrils have their counterparts in human synucleinopathies.

In separate work, some α-synuclein filaments seeded both tau and α-synuclein aggregation, whereas others seeded only α-synuclein aggregation (Guo *et al.*, 2013). These conformers of aggregated α-synuclein exhibited different properties after proteinase K digestion. They were like prion strains in that they showed structural variations, differences in seeding properties and heritability of phenotypic traits.

Studies in cultured cells have shown that α-synuclein fibrils are internalized, seed aggregation of expressed protein, are axonally transported, released and taken up by adjoining neurons (Lee *et al.*, 2008; Guo and Lee, 2014). Like aggregated tau, uptake of aggregated α-synuclein was through macropinocytosis and required heparan sulphate proteoglycans (Hansen *et al.*, 2011; Holmes *et al.*, 2013). Unlike tau aggregates, induced α-synuclein aggregates were resistant to degradation and inhibited macroautophagy (Tanik *et al.*, 2013). Like tau aggregates, they were p62-positive. Fibril-induced inclusions can be formed from endogenously expressed α-synuclein (Volpicelli-Daley *et al.*, 2011). Like

tau, α-synuclein has to be aggregation-competent (Danzer *et al.*, 2009; Luk *et al.*, 2009), suggesting that aggregation inhibitors can reduce α-synuclein-induced seeding and spreading. Stabilization of α-synuclein fibrils resulted in a reduction of seeding, confirming that fibril stability was inversely related to seeding activity (Lam *et al.*, 2016). Addition of A30P α-synuclein seeds to cells expressing wild-type protein led to the generation of fibrils with the same characteristics as the seeds, consistent with the prion concept (Yonetani *et al.*, 2009).

In cell culture, α-synuclein is released through unconventional exocytosis (Lee *et al.*, 2005) and it has been reported that its release is enhanced when lysosomal function is reduced (Alvarez-Erviti *et al.*, 2011). Dopaminergic neurons derived from induced pluripotent stem cells harbouring a triplication of *SNCA* secrete α-synuclein that is taken up by neighbouring neurons (Reyes *et al.*, 2015). A fraction of extracellular α-synuclein is associated with exosomes and may promote aggregation (Emmanouilidou *et al.*, 2010; Stuendl *et al.*, 2016).

Misfolding-associated protein secretion (MAPS), which targets misfolded cytoplasmic proteins for secretion when proteasome activity is impaired, may also play a role (Lee *et al.*, 2016). It uses the endoplasmic reticulum-associated chaperone/deubiquitinase USP19, which has been shown to promote α-synuclein secretion. Seeded α-synuclein aggregates are ubiquitinated. It will be interesting to see if released α-synuclein is not ubiquitinated. In this pathway, de-ubiquitinated cargoes are packaged into endoplasmic reticulum-associated late endosomes and secreted. It is not known how MAPS (Lee *et al.*, 2016) relates to the secretion pathway involving Hsc70, DnaJ and SNAP23 (Fontaine *et al.*, 2016). Given that most secreted α-synuclein is free, immunotherapy may be beneficial in human synucleinopathies (Bae *et al.*, 2012; Tran *et al.*, 2014). It remains to be seen if secreted α-synuclein can also be aggregated.

Acquired protein aggregates

Prion diseases are sporadic, inherited or acquired (Prusiner, 2013). Only 1% of cases of CJD are acquired, with 99% being sporadic or inherited. Acquired cases include iatrogenic CJD, vCJD and Kuru. Iatrogenic CJD occurred under unusual circumstances, such as injections of cadaveric growth hormone and gonadotropin, cadaveric dura mater grafts, blood transfusions, corneal transplants and implantation of improperly sterilized depth electrodes. Injections of growth hormone and dura mater grafts gave rise to most cases of iatrogenic CJD. Recent work on the propagation of aggregated amyloid-β, tau and α-synuclein has raised the question if these protein deposits can also be acquired. As Alzheimer's disease and Parkinson's disease are much more common than prion diseases, even if 1% of cases were acquired, this would represent hundreds of thousands of cases.

Between 1958 and 1985, ~15 000 individuals with short stature received intramuscular injections of human growth hormone extracted from the pituitary glands of pooled cadavers (Brown *et al.*, 2012). It has been estimated that more than 400 000 pituitaries were used and some had probably been collected from patients with CJD. In the mid-1980s some recipients of growth hormone died of CJD many years after cessation of their growth hormone treatment. Fortunately, recombinantly produced human growth hormone became available in 1985, at the time when some of the cadaver-derived hormone was known to be contaminated with prions. From then on, recombinant growth hormone was mostly used. However, human pituitary-derived growth hormone continues to be available on the black market and is being abused, because of its anabolic and lipolytic properties (Holt and Sönsken, 2008). Unlike recombinant human growth hormone, cadaveric growth hormone is indistinguishable from endogenously produced growth hormone.

To date, ~220 individuals injected with cadaver-derived pituitary human growth hormone developed iatrogenic CJD. Most cases occurred in France, the UK and the USA. In a recent study, of eight patients dying of CJD between the ages of 36 and 51 following the injection of cadaver-derived human growth hormone, four had abundant amyloid-β plaques (Jaunmuktane *et al.*, 2015). Three patients had smaller amounts of deposits and only one patient lacked amyloid-β pathology. Three of the four patients with abundant amyloid-β plaques also had cerebral amyloid angiopathy. Pituitary extracts may have been contaminated with amyloid-β seeds, possibly originating from the pituitaries of Alzheimer's disease patients. Tau-positive inclusions were not present. These findings are reminiscent of work showing that the intracerebral injection of Alzheimer's disease brain extracts into wild-type marmosets caused the formation of amyloid-β deposits, but not tau inclusions (Ridley *et al.*, 2006).

Another route of transmission of iatrogenic CJD has been through cadaveric human dura mater grafts. About 230 individuals, mostly in Japan, developed CJD (Brown *et al.*, 2012). Some years ago, amyloid-β deposits were detected in a 28-year-old patient who died from CJD 23 years after receiving a dura mater graft (Preusser *et al.*, 2006). More recently, of six additional patients with dura grafts dying from CJD between the ages of 33 and 63, four had abundant amyloid-β plaques and cerebral amyloid angiopathy (Frontzek *et al.*, 2016). They all died more than 20 years after surgery. Patients lacking amyloid-β pathology died 11 and 12 years after surgery.

Although these studies suggest that amyloid-β seeds can transfer between individuals, the patients died from iatrogenic CJD. They did not have Alzheimer's disease, either clinically or neuropathologically, possibly because they lacked abundant tau inclusions. Most patients treated with cadaver-derived human growth hormone before

1985 did not develop a prion disease. It will be important to determine if they have an increased risk of developing Alzheimer's disease.

Conclusion

Overexpression of tau and α-synuclein in cells does not result in inclusions. However, the addition of seeds causes inclusion formation, which makes it possible to investigate the mechanisms underlying their formation and those causing nerve cell dysfunction. For example, it has been shown that mitochondrial stress is downstream of α-synuclein aggregation (Dryanovski *et al.*, 2013), where α-synuclein oligomers appear to bind to the mitochondrial receptor TOM20 (Di Maio *et al.*, 2016).

These models do not speak to nucleation-dependent aggregation. The evidence so far indicates that small fibrils are required for the seeding and spreading of tau and α-synuclein inclusions, with sonication of seed preparations increasing the amounts of seeded aggregation, probably through an increase in free ends. The ends of existing fibrils are believed to recruit soluble monomers into aggregates. The propensity of fibrils to fragment is probably an important determinant of self-propagation (Knowles *et al.*, 2009). Besides their intrinsic tendency to fragment, fibrils may also break in cells as a result of cleavage by disaggregases (Gao *et al.*, 2015; Nillegoda *et al.*, 2015). A fibril that does not break might be relatively harmless in the context of propagation of pathology. Seeded aggregation of tau and α-synuclein must be accompanied by amplification of seeds for sustained propagation.

In these assays, soluble monomeric protein is the substrate and seeding is concentration-dependent. Not surprisingly, therefore, most studies required overexpression of monomeric proteins to work well. However, upon intracerebral injection of wild-type mice with aggregates, some tau or α-synuclein inclusions formed (Clavaguera *et al.*, 2009, 2013*b*; Luk *et al.*, 2012*a*; Masuda-Suzukake *et al.*, 2013). Interestingly, an age-related increase in the staining of nigral cell bodies for α-synuclein has been described in humans and in rhesus monkeys (Chu and Kordower, 2007). Might this have something to do with the age-dependent development of Parkinson's disease, or does it reflect a defect in axonal transport resulting from the aggregation of α-synuclein in nerve terminals? It remains to be seen if the propagation of protein aggregates changes with age. This appears likely, because an increase in age leads to a reduction in proteostasis (Vilchez *et al.*, 2014).

Protein aggregation may take place all the time, but in most individuals, brain cells may be able to degrade small aggregates, preventing the formation of large aggregates and their propagation. With age, the ability of cells to degrade aggregates of a particular protein may decrease and this may differ between cells. Intrinsic differences in the efficiency of lysosomal clearance may also exist. They may explain, for instance, the differential vulnerabilities of dopaminergic neurons in substantia nigra and ventral tegmental area to toxic concentrations of α-synuclein (Decressac *et al.*, 2013).

Prion replication and toxicity may be caused by different species of aggregated PrP^{Sc} (Sandberg *et al.*, 2011), akin to secondary nucleation (Knowles *et al.*, 2009). Subclinical states and large amounts of PrP^{Sc} have been described, as has neurodegeneration in the presence of small amounts of PrP^{Sc}. It remains to be seen if the species of aggregated tau and α-synuclein that are responsible for propagation and neurodegeneration are also different. In transmission experiments, signs of nerve cell dysfunction and neurodegeneration have been observed more commonly following the injection of α-synuclein aggregates than that of aggregated tau.

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