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Pathogenic Protein Seeding in Alzheimer's Disease and Other Neurodegenerative Disorders

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Abstract

The misfolding and aggregation of specific proteins is a seminal occurrence in a remarkable variety of neurodegenerative disorders. In Alzheimer's disease (the most prevalent cerebral proteopathy), the two principal aggregating proteins are β-*amyloid* (Aβ) and *tau*. The abnormal assemblies formed by conformational variants of these proteins range in size from small oligomers to the characteristic lesions that are visible by optical microscopy, such as senile plaques and neurofibrillary tangles. Pathologic similarities with prion disease suggest that the formation and spread of these proteinaceous lesions might involve a common molecular mechanism – corruptive protein templating. Experimentally, cerebral β-amyloidosis can be exogenously induced by exposure to dilute brain extracts containing aggregated Aβ seeds. The amyloid-inducing agent probably is Aβ itself, in a conformation generated most effectively in the living brain. Once initiated, Aβ lesions proliferate within and among brain regions. The induction process is governed by the structural and biochemical nature of the Aβ seed, as well as the attributes of the host, reminiscent of pathogenically variant prion strains. The concept of prion-like induction and spreading of pathogenic proteins recently has been expanded to include aggregates of tau, α-synuclein, huntingtin, superoxide dismutase-1, and TDP-43, which characterize such human neurodegenerative disorders as frontotemporal lobar degeneration, Parkinson's/Lewy body disease, Huntington's disease, and amyotrophic lateral sclerosis. Our recent finding that the most effective Aβ seeds are small and soluble intensifies the search in bodily fluids for misfolded protein seeds that are upstream in the proteopathic cascade, and thus could serve as predictive diagnostics and the targets of early, mechanism-based interventions. Establishing the clinical implications of corruptive protein templating will require further mechanistic and epidemiologic investigations. However, the theory that many chronic neurodegenerative diseases can originate and progress via the seeded corruption of misfolded proteins has the potential to unify experimental and translational approaches to these increasingly prevalent disorders.

Keywords

Alzheimer's disease; amyloid; amyotrophic lateral sclerosis; frontotemporal lobar degeneration; Huntington's disease; inclusions; neurofibrillary tangles; Parkinson's disease; prion; proteopathy; senile plaques; tauopathy

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It is a concept that is compelling in its power and simplicity: the misfolding and aggregation of specific proteins underlies many of the chronic neurodegenerative diseases that afflict aging humans. But what initiates this injurious cascade, and how does the pathology ramify throughout the nervous system? A wave of recent research, initially driven by tantalizing pathologic similarities between prion diseases and Alzheimer's disease (AD)[1-3], has begun to address these questions, and the findings increasingly implicate *corruptive protein templating*, or *seeding*, as a prime mover of the neurodegenerative process. The prion-like corruption of proteins may also be involved in the pathogenesis of such clinically and etiologically diverse neurological disorders as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal lobar degeneration, and chronic traumatic encephalopathy. Currently there is no evidence that these maladies are infectious in the same sense as are prion diseases. However, understanding protein misfolding and aggregation could reveal general principles, and thus similar therapeutic targets, of a pathogenic mechanism that impels some of the most devastating diseases of the elderly, AD foremost among them.

The proteopathic basis of Alzheimer's disease

Alois Alzheimer linked senile plaques and neurofibrillary tangles to the dementia of AD over a century ago, but fundamental insights into the pathogenesis of the disorder emerged only with the identification of the principal proteins that comprise these lesions: **Aβ** in senile plaques and cerebral amyloid angiopathy (CAA), and **tau** in neurofibrillary tangles[4] (for historical references see[5]). Whereas the degree of tauopathy correlates strongly with cognitive decline in AD[6-8], genetic, pathologic and biochemical evidence implicates the aggregation of Aβ as a critical, early trigger in the chain of events that leads to tauopathy, neuronal dysfunction and dementia[9].

Advancing age is the most prevalent risk factor for the accumulation of Aβ in the brain[10, 11], possibly because of the decline of cellular protein quality control processes[12]. All firmly established genetic risk factors for AD promote the buildup of Aβ, either by increasing its production, promoting its aggregation, or impeding its elimination[9, 13]. Recent longitudinal imaging studies indicate that cerebral Aβ deposition precedes the clinical symptoms of AD by a decade or more[4]. How Aβ aggregates impair neuronal function remains uncertain, but evidence is growing that oligomeric forms of the protein, which can range in size from dimers to dodecamers or larger[14-18], are more deleterious to brain function than are histologically obvious Aβ lesions such as senile plaques and CAA. Moreover, at least some of the Aβ toxicity appears to be tau-dependent[19, 20].

Cross-sectional analyses of postmortem human brains reveal a characteristic progression of β-amyloid plaques and a highly stereotypical appearance of neurofibrillary tangles (Fig 1). β-amyloid plaques develop first in the neocortex, followed by the allocortex and then the subcortex, and the progression of their appearance often corresponds to functionally and anatomically coupled brain regions[21-23]. Neurofibrillary tangles first arise in the locus coeruleus and entorhinal/limbic brain areas, and then spread to interconnected neocortical regions[8, 24]. The pattern that emerges from these studies implicates neuronal transport and synaptic exchange mechanisms in the spread of AD lesions within the brain[24-26]. Overall, the incidence of plaques and tangles correlates positively in AD, but a consistent anatomical relationship between the lesions is not apparent. Imaging ligands that bind selectively to pathogenic protein deposits *in vivo*, such as the β-amyloid binding agent Pittsburgh Compound B[27], will increasingly enable the longitudinal analysis of lesion spread in AD patients[28].

Misfolded Proteins as Infectious Seeds: Prion Diseases

In humans, the prion diseases (spongiform encephalopathies) are relatively uncommon but uniformly fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD), variant CJD, fatal familial insomnia, Gerstmann-Sträussler-Scheinker Syndrome and Kuru. In other mammals, spongiform encephalopathies can be more prevalent; the nonhuman prionoses include scrapie, chronic wasting disease, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy, and others[29-31]. Prion diseases are unusual in that they can be genetic, idiopathic, or infectious (transmissible) in origin[29]. Experimentally, prion disease has been transmitted to a broad range of species and models[29, 32, 33]. Fortunately, the transmission of prion disease to humans is unusual, having occurred in fewer than 700 known cases, most under extraordinary circumstances such as treatment with human growth hormone derived from cadaveric pituitary glands, or in conjunction with the BSE outbreak that peaked in the late 20th century[34].

Prions 'infect' via an unconventional mechanism whereby misconformed, β-sheet-rich prion protein induces the templated misfolding of other prion protein molecules[29, 30, 35-37]. The biological functions of the normal ('cellular') prion protein (PrP^{C}) remain indeterminate, but it is not essential for survival[38]. In the disease state, the replication of infectious particles is sustained because cells continually produce PrP^{C} [29, 30], which serves as the raw material for templated conversion to the pathogenic form ('PrP Scrapie', or PrP^{Sc}). The conformationally corrupted molecules are predisposed to self-aggregation, in which form they can become injurious to neurons[29, 30]. Though the mechanism of spread remains uncertain, there is evidence that prions can be conveyed between neurons by trans-synaptic transport[39]. Clinically, pathologically, and molecularly, the prion diseases exhibit variability suggestive of polymorphic and polyfunctional *strains* of the agent[35-37, 40]. While prion disease is the only demonstrably infectious cerebral proteopathy, mounting evidence implicates prion-like molecular mechanisms in the initiation and spread of a variety of neurological and systemic diseases[39, 41-45]. This emerging principle of pathogenesis has the potential to unify experimental and therapeutic approaches to these seemingly disparate disorders.

Induction and spread of Aβ aggregates in experimental animals

The pathologic similarities between prion disease and AD have long engendered speculation that AD might be inducible in a prion-like manner[1-3]. Early, long-term studies in nonhuman primates reported evidence both against[46] and for[47, 48] the exogenous inducibility of senile plaques. The introduction of transgenic mouse models of β-amyloidosis has provided a more efficient and definitive means of testing the hypothesis that AD-like lesions can be seeded *in vivo*. Studies in our laboratories and others have shown that the deposition of Aβ can be instigated in the brains of Aβ precursor protein (APP)-transgenic mice by the intracerebral infusion of brain extracts containing minute amounts of aggregated (multimeric) Aβ[49-53] (Fig 2).

Several lines of evidence[49-52] collectively argue that aggregated Aβ in the brain extract is critical for *in vivo* seeding: (1) The extract is able to seed only if it contains aggregated, human-sequence Aβ; (2) Immunoneutralization/depletion of Aβ in the extract by anti-Aβ antibodies impedes seeding; (3) Seeding of Aβ deposition is ineffective in non-transgenic mice, which (because of three amino acid differences from human-sequence Aβ) do not develop cerebral β-amyloidosis; (4) The phenotype of the induced Aβ deposits mirrors that of the deposits in the extract, suggesting an Aβ-templating mechanism; (5) Aβ-rich brain extracts from transgenic mice seed as effectively as do AD brain extracts, ruling out such factors as a cross-species immune reaction or human-specific microbes; and (6) Seeding can

be abrogated by denaturation of the extract with formic acid. Recent findings reveal that the β-amyloid-inducing seeds do not consist of a single type of Aβ aggregate, but rather can occur as proteinase K (PK)-resistant species in the pellet fraction and as a soluble, PK-sensitive species in the 100,000×g soluble fraction[54]. Sonication of the extract, and thus presumed fragmentation of the "insoluble" Aβ seeds into smaller "soluble" Aβ multimers, enhances seeding, and thus hints at a continuum of Aβ aggregates of various sizes that can act as amyloid-inducing agents[54].

The induction of Aβ deposits following injection of Aβ-rich brain extracts initially is most evident within the injected brain area. However there is also spreading between non-contiguous but axonally interconnected regions[50, 52] (Fig 2), suggesting that seeds can migrate along defined neuronal pathways. Interestingly, seed placement in one region also can foster CAA in separate locations[51], implicating perivascular fluid drainage channels[55] and/or vascular transport mechanisms in the dissemination of the seeds[51]. Moreover, the intraperitoneal injection of Aβ-rich brain extracts into APP-transgenic mice induces β-amyloidosis in the brain after prolonged incubation[56]. How the seeds travel from the periphery to the brain in this model is an important open question.

Aggregated synthetic Aβ thus far has not induced significant cerebral Aβ-deposition in APP-transgenic mice[51]. The failure of seeding by synthetic Aβ was not unanticipated, in that the induction of prion disease also has been difficult to achieve with PrP generated *in vitro*[57]. It is possible that the aggregation of synthetic Aβ under suitable (but as yet undefined) conditions will yield a more effective *in vivo* seed, as was recently demonstrated for PrP[58]. The differential seeding ability of synthetic vs. natural Aβ aggregates (which may contain additional factors) suggests the possibility that Aβ, like prions, can misfold into polymorphic and polyfunctional strains[51, 59-62].

Induction and spread of tau filaments in experimental animals

Using a paradigm similar to *in vivo* Aβ-seeding, neurofibrillary (tau) tangles can be exogenously induced by the intracerebral infusion of brain extract containing abnormal tau filaments into mice bearing a human tau transgene[63]. This finding is remarkable in that the host (Alz17 mouse) expresses non-mutant human tau and does not normally develop tau filaments; moreover, the seeded tangles, unlike Aβ plaques and CAA, are *intra*cellular. The induction of tauopathy is time- and brain region-dependent, and immunodepletion of tau from the donor brain extract prevents seeding[63]. Initially, the induced tau filaments are confined to the injected brain region, but over time, they extend to neighboring and/or axonally connected areas, suggesting directed spreading of seeds by neuronal transport processes. Although final evidence is still lacking that the *in vivo* induction of tau occurs via prion-like corruptive protein templating (versus, for example, activation of a signaling cascade that promotes tau aggregation), *in vitro* studies favor a prion-like mechanism[64-66]. Specifically, (1) aggregated tau is taken up via endocytosis from the cell medium and can induce the aggregation of soluble, endogenous tau in cells; (2) tau aggregates can transfer among co-cultured cells; and (3) distinct conformational properties of recombinant tau fibrils can be propagated. The latter finding, albeit achieved in a cell-free system[65], is intriguing because tau inclusions characterize a variety of sporadic and genetic neurodegenerative diseases in which the aggregates display polymorphic conformations[67, 68]. Thus, as with other proteopathies, distinct tau strains may explain the pathogenic and phenotypic variations among the tauopathies[45].

The coexistence of Aβ- and tau-lesions in AD still lacks a mechanistic explanation. Experiments in transgenic mice have shown that tauopathy can be augmented by aggregated, synthetic Aβ[69] or by Aβ-rich brain extracts[70]. While this phenomenon may result from the

direct cross-seeding of tau by aggregated Aβ[71, 72], indirect pathways such as Aβ-induced tau phosphorylation, inflammation, and/or disruption of proteostasis[20, 73-75] have not been ruled out.

Induction and spread of protein aggregates linked to other neurodegenerative diseases

Increasing evidence implicates the templated corruption of disease-specific proteins in other neurodegenerative diseases. In the brain, the α-synuclein-rich lesions that typify Lewy body disease/Parkinson's disease first arise in the lower brainstem (notably the dorsal motor nucleus of the vagus nerve), and in the anterior olfactory nucleus and the olfactory bulb; they subsequently appear in a predictable sequence in mesencephalic and neocortical regions[76, 77]. The concept that α-synuclein lesions ramify within the CNS by a seeding-like process is bolstered by the observation that fetal dopaminergic neural transplants in the striatum of Parkinsonian patients can eventually exhibit α-synuclein-positive Lewy bodies in some cells, implying that synuclein seeds propagate from the host to the graft[78, 79]. In support of this observation, neural grafts placed into transgenic mice expressing human α-synuclein take up the human protein and form synuclein-positive aggregates[80-82]. The *in vivo* approaches in these studies could not discriminate between a prion-like corruptive templating mechanism, i.e. host-derived, misfolded α-synuclein inducing the misfolding of α-synuclein generated in the graft, versus the simple translocation of aggregated synuclein from the host to the graft. In cell culture, however, the prion-like propagation of α-synuclein lesions has been demonstrated[80, 81], as has the induction of proteinaceous lesions associated with other neurodegenerative diseases, such as aggregates of superoxide dismutase 1 (SOD1)[83, 84], which are characteristic of SOD1-mutant and some idiopathic cases of amyotrophic lateral sclerosis (ALS), cytosolic aggregates of TDP-43[85], which are present in ALS and frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP), and aggregates of polyglutamine[86], which typify Huntington's disease and spinocerebellar ataxias.

These studies, along with those of Aβ and tau (above), imply that disease agents can be disseminated by cells, but the means whereby protein aggregates travel between cells, and the cellular domain(s) in which the templated conversion occurs, remain poorly understood[39, 43, 44, 87]. *In vitro*, aggregates of tau, α-synuclein, polyglutamine, and SOD1 all can be taken up by endocytosis and induce the misfolding of the corresponding intracellular proteins; moreover, cytoplasmic protein aggregates can translocate from one cultured cell to another[64, 66, 80, 81, 83, 84, 86, 88]. The mode of cell-to-cell transfer is unclear[39], but at least α-synuclein and SOD1 are secreted into the cell medium[89, 90]. Furthermore, tau and α-synuclein have been measured at robust levels in the cerebrospinal fluid, suggesting secretion of these proteins *in vivo*[91]. The intercellular transfer of cytosolic protein aggregates may also occur through nanotubes, exosomes or microvesicles[39]. Like other pathogenic proteins, Aβ can be taken up, modified and secreted by cells in vitro[92, 93], and it also is present in the CSF[94].

Clinical, Practical and Theoretical Implications

The induction and proliferation of proteinaceous aggregates by corruptive protein templating appears to be a common feature of multiple, clinically diverse disorders, although many questions remain to be addressed (Table 1). The unexpected prevalence of this pathogenic mechanism raises a number of clinical and practical issues, and highlights the corruptive seeds as both potential biomarkers and therapeutic targets[44] (Table 1).

In AD, cerebral β-amyloid deposition begins in humans at least a decade prior to the onset of cognitive decline, and hence is an early and predictive indicator of the disease[4]. It is likely, therefore, that an effective disease-modifying therapy must be initiated prophylactically, before the disease has inflicted irreversible damage on the brain. Early intervention will require an early and informative biomarker[4, 95]. *In vitro*, the formation of corruptive seeds is a relatively slow, stochastic process[96], though once a seed is present, aggregation proceeds quite rapidly. *In vivo*, then, the appearance of soluble Aβ seeds may precede appreciable Aβ-deposition in plaques and blood vessels. Because the most potent Aβ seeds, like prions[97], appear to be relatively small[54], soluble Aβ seeds could serve as informative biomarkers in bodily fluids.

The ability of Aβ-rich brain extracts injected into the peritoneal cavity to induce Aβ deposition in the brain[56] indicates that Aβ seeds resemble prions in their ability to reach the central nervous system from the periphery. In the absence of direct evidence linking non-prion neurodegenerative diseases to seeds arising outside the central nervous system or taken up from the environment (e.g. in food or air), the practical implications of this finding are uncertain. It is probable, however, that a fuller understanding of the trafficking of pathogenic seeds will yield insights into the endogenous progression of disease, and hence denote novel points of intervention. For example, the early appearance of α-synuclein-containing Lewy bodies in the peripheral nervous system, and their relatively systematic spread within the brain[98, 99], suggest that seeds-in-transit (i.e., those traveling between cells or from one region to another) might be profitable objectives for therapeutic interference[44].

In some cases, protein misfolding and aggregation can be initiated by heterologous, β-sheet-rich proteins[41, 100-102]. This ‘cross-seeding’ is generally less potent than is homologous seeding, but the potential corruption of proteins by exogenous nanoscale materials, some of which may feature amyloid-like structural properties[103, 104], should be factored into safety evaluations of such materials[41]. Furthermore, the cell-to-cell transfer of seeds and the induction of pathogenic protein aggregates in cellular grafts[44, 79, 105] underscores the need for measures to protect grafted cells from host-induced corruptive protein templating, e.g., by selectively engineering the cells so that vulnerable proteins are either absent or resistant to seeding.

Beyond its importance as a therapeutic objective, there is a clear need for studies on the clinical and epidemiological implications of corruptive protein templating as a disease mechanism (Table 1). Given our current state of knowledge, we feel that it is unlikely that non-prion proteopathies are communicable under everyday circumstances. However, it is worth considering the possibility that non-prion proteopathies can be promoted under certain extraordinary circumstances. The most efficient induction of prion disease and Aβ deposition is achieved by direct introduction of the seeding agent into the brain[30, 56]. In rare instances, prion disease has been transmitted to humans by contaminated neurosurgical instruments[34]. Experimentally, β-amyloid induction can be triggered in transgenic mice by intracerebrally implanted stainless steel wires coated with minute amounts of brain extract rich in aggregated Aβ[52] (Fig 2). While the transmission of non-prion proteopathies by tainted instruments thus is a theoretical possibility, proof of such a phenomenon (which could be obscured by a protracted incubation period) has not been demonstrated in humans. Nevertheless, when considered alongside the (slight) risk of prion transmission by instruments used on patients with undiagnosed prion disease[106], the hypothetical risk that non-prion proteopathies might be similarly induced suggests a need for more research into the epidemiology of such disorders in long-term, post-neurosurgical patients. In addition, risk analysis of the incidence of proteopathic diseases in the recipients of donated organs, tissues, extracts or fluids is needed to establish with confidence the inclusion criteria for donors. For example, inasmuch as advancing age is a salient risk factor for most human

neurodegenerative diseases, should there be an age-limit for organ and tissue donation? It is still premature to provide answers to these questions, but the growing prevalence of cerebral proteopathies necessitates a comprehensive quest to illuminate the causes and consequences of corruptive protein templating in human disease.

Conclusion

The seeded proliferation of misfolded proteins, a concept that arose and evolved in the prion field, holds considerable explanatory power for the pathogenesis of many of the neurodegenerative diseases that afflict our burgeoning elderly population. The theoretical framework underlying this paradigm recently has expanded to include an extraordinary array of neurological and systemic disorders. Moreover, the templated modification of protein structure also subserves the transfer and storage of useful biological information in systems ranging from microbes to mammals[107-110]. Unfortunately, much of the research on this far-reaching phenomenon is fragmentary, and the clinical implications remain uncertain. A concerted inquiry into the biophysics, biochemistry, and cell biology of protein aggregation is needed to decipher the molecular underpinnings of a large and growing constellation of age-related disorders of the nervous system, and thereby accelerate the discovery of efficacious therapies.

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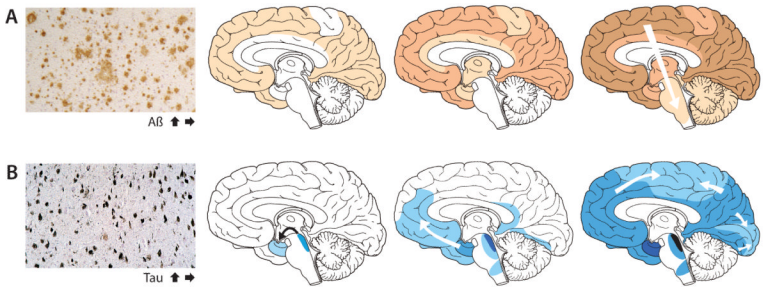

Figure 1. The accumulation of misfolded proteins in AD follows characteristic and predictable patterns

Cross-sectional autopsy studies indicate that β-amyloid plaques (**A**) first appear in the neocortex, followed by the allocortex and finally subcortical regions[21]. In the brain, neurofibrillary tangles (**B**) occur first in the locus coeruleus and transentorhinal area and then spread to the amygdala and interconnected neocortical brain regions[8, 24]. The relatively stereotyped patterns of expansion suggest the involvement of neuronal transport mechanisms in the spread of proteopathic seeds. Increasing density of shading indicates increasing pathology. The schemata with the progression of the Aβ and tau lesions have been modified from previous publications[21].

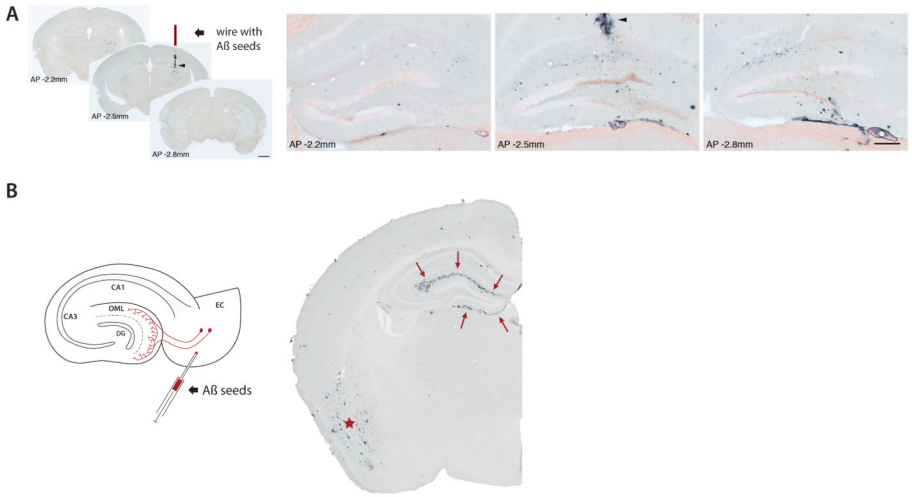

Figure 2. Induction and spread of Aβ lesions in a transgenic mouse model

(**A**) Stainless-steel wire segments were coated with Aβ-rich brain extract, dried, and implanted unilaterally into the hippocampus of APP23 transgenic mice. Four months later, immunohistochemical analysis with an anti-Aβ-antibody revealed strong local induction of Aβ-deposits in the vicinity of the wire (arrowhead in the middle section of three coronal sections along the anterior-posterior (AP) axis through the hippocampus). Higher magnification of the dentate gyrus double-stained with anti-Aβ-antibody and Congo red revealed spreading of Aβ-deposition thoughout the dentate gyrus (distance between the sections shown: 600μm). Reproduced from[52] with permission. (**B**) The injection of Aβ-rich brain extracts induces Aβ aggregation within the injected brain region, as shown here for the entorhinal cortex (EC) in APP23 transgenic mice (asterisk). However EC injections also induce β-amyloid deposition (arrows) in the outer molecular layer (OML) of the hippocampal dentate gyrus (DG), a region that is non-contiguous but is axonally interconnected with the injection site. For details of the methods, see[52].

Table 1

Pathogenic protein seeding: Open questions

Mechanistic and theoretical issues:	
1	What is the molecular structure of pathogenic proteins?
2	Why are synthetic and recombinant protein aggregates often poor seeds?
3	What co-factors influence the formation and activity of seeds?
4	Are there polystructural and polyfunctional strains of misfolded proteins?
5	What accounts for the selective vulnerability of neurons in the proteopathies?
6	How do protein assemblies move from cell to cell, and from region to region?
7	What are the mechanisms of intracellular vs. extracellular seeding?
8	Are some seeded aggregates protective, e.g. by binding toxic oligomers?
9	Is there cross-seeding between proteins, or between non-protein seeds and proteins?
10	Can exogenous corruptive seeds trigger non-prion neurodegenerative diseases?
Practical and clinical issues:	
1	Can soluble protein seeds be targeted therapeutically or as disease biomarkers?
2	Is it necessary to impede the seeding cascade for cell transplantation or genetic therapies to be fully effective against neurodegenerative diseases?
3	Until the nature of non-prion seeds is better understood:
	a. Should more rigorous decontamination of neurosurgical instruments be considered?
	b. Should there by age limits for donating organs, tissues, extracts or fluids for medical purposes?
	c. Should potential donors with known non-prion proteopathies be excluded from donating biological materials?
	d. Are special precautions needed for professionals who handle biological materials from affected patients?
	e. How can the hypothetical risk of exogenous induction of non-prion proteopathies best be assessed epidemiologically?