

Prions and protein aggregates as pathogens, self-propagating structures, biomarkers, and therapeutic targets

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SUMMARY Many mammalian diseases appear to be caused primarily by the abnormal accumulation of self-propagating assemblies of specific host proteins such as A β and tau in Alzheimer's disease, α -synuclein (aSyn) in Parkinson's disease, and prion protein (PrP) in classical prion diseases. Most proteinopathies involve a prion-like spreading of the aggregates from localized sites of initiation within the host and, sometimes, between individuals. Often, the pathological assemblies take the form of amyloid fibrils, the cores of many of which have been solved by cryo-electron microscopy, revealing disease-specific, strain-like conformers of the given protein. Amyloids grow via seeded polymerization, a mechanism that is being widely exploited to develop ultrasensitive and specific amplification assays for pathological seeds as biomarkers. Such assays can aid fundamental research, diagnostics, prognostics, and clinical trials for multiple proteinopathies that

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B.C., C.D.O., and A.G.H. have patents on RT-QuIC seed amplification technologies.

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have been challenging to diagnose and treat. Here, we review the structural biology, transmissibilities, spreading mechanisms, and detection of proteopathic aggregates as well as therapeutic approaches to limiting their accumulation.

KEYWORDS prions, proteinopathies, structural biology, clinical therapeutics, Alzheimer's disease, Parkinson's disease, tauopathies, synucleinopathies, diagnostics, transmissibilities

INTRODUCTION

To function properly, most proteins must fold into discrete three-dimensional structures. However, during this process, or after the native fold is achieved, misfolding can expose sticky surfaces that may promote abnormal aggregation (Fig. 1). Initially, small oligomers may form that progress to either non-amyloid or amyloid fibrillar deposits inside or outside of cells, but such events are poorly understood *in vivo*. Along the path of aggregation, major changes in the conformation of the protein constituents may occur. The assembly of amyloid fibrils (or filaments), which have highly ordered β -sheet cores, often involves the formation of intermolecular β -sheets from regions of proteins that are intrinsically disordered in their native state (1, 2). However, sometimes even predominantly α -helical domains of proteins (e.g., the normal cellular prion protein [PrP^C] (3) and serum amyloid A (4)) refold into β -sheets (5–12). Amyloid formation by ~70 distinct proteins characterizes the pathologies of ≥ 50 disorders in humans including Alzheimer's disease (AD) and Parkinson's disease (PD) (1, 2, 12, 13). Usually, protein quality control (proteostatic) mechanisms limit the accumulation of such aggregates, but occasionally such controls can be progressively overwhelmed—sometimes with catastrophic consequences.

Prion protein (PrP)-based prion diseases represent the first, and particularly well documented, examples of how even femtograms of an infectious misfolded protein assembly, such as certain PrP amyloid fibrils, can propagate in the host to cause fatal neurodegenerative disease (Fig. 1). Early in the last century, it became apparent that a disease called scrapie was transmissible among sheep (20, 21). Transmissions of scrapie into rodents in the 1960s (22, 23) emphasized the existence of distinct strains and a host gene controlling scrapie incubation time (24) (later shown to be the *Prnp* gene for PrP by Manson and colleagues [25]). Also in the 1960s, the human prion diseases Creutzfeldt-Jakob disease (CJD) and kuru were initially transmitted to non-human primates by Gibbs, Gajdusek, and colleagues (26, 27). Due to these agents' unusual resistance to inactivation by treatments harmful to nucleic acids, they were presciently proposed in 1967 to be a toxic, self-propagating form of a host protein that lacked a nucleic acid genome by J.S. Griffith (28). Consistent with this possibility were many earlier and subsequent accounts of abnormal amyloid fibril/plaque deposits in brain tissue from humans with kuru, CJD, and Gerstmann-Straussler-Scheinker (GSS) and animals with scrapie (reviewed in reference 29), including a report of scrapie strain-dependent differences in fibril morphology (30). In 1982, Stanley Prusiner coined the term prion to denote "a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids" (31) and isolated the main protein in infectious preparations (including fibrils) (32–34). This protein has since generically been called PrP^{Sc}, for PrP scrapie, in transmissible prion diseases. The Weissmann, Prusiner, Chesebro, and Robakis labs soon cloned the first *Prnp* genes from rodents (34–37), which Weissmann later proved to be essential for prion infections (38). Decades of ensuing research eventually proved that PrP^{Sc} could cause PrP^C to convert to PrP^{Sc} (39, 40) in a strain-dependent manner (41) and is primarily an infectious abnormal conformer and aggregation state of host-encoded PrP molecules (5–8, 35–37, 42–44). These studies confirmed and elaborated on nucleated polymerization models proposed by Griffith (28), Gajdusek (45), and Lansbury (46, 47) for infectious self-propagating states of host proteins as well as Bessen and Marsh's PrP^{Sc} conformation-based explanation of prion strains (48). However, PrP^{Sc} is usually also associated with poorly defined (*in vivo*) peripheral ligands/cofactors/

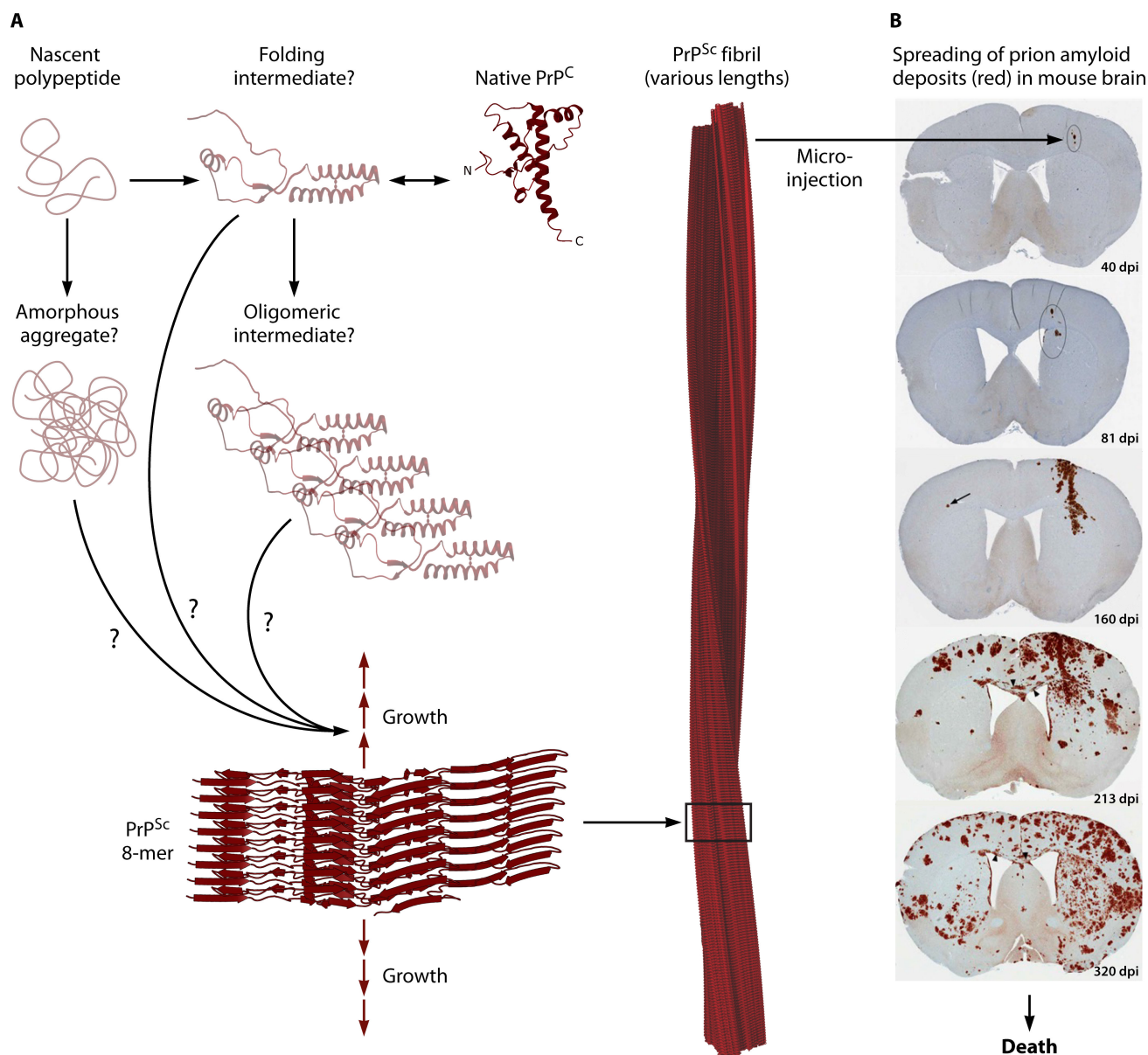


FIG 1 Folding, misfolding, and aggregation of proteins into amyloid fibrils using mouse prion protein (PrP) as an example of an infectious amyloid. (A) Folding pathway of a nascent PrP polypeptide *en route* to the final native PrP^C structure determined by nuclear magnetic resonance (NMR) spectroscopy (3). Faded structures are hypothetical and have not been empirically determined. The cartoons depict only the PrP residues known to form the ordered fibril core of the mouse aRML PrP^{Sc} prion as determined by cryo-EM (shown, for example, as an octameric unit on the bottom of A) (8, 14–16). Further growth of the PrP^{Sc} oligomer generates elongated fibrils. The immediate precursors that add onto the PrP^{Sc} templating ends remain unclear (question marks) but might be amorphous or partly folded oligomeric intermediates or monomers that must ultimately undergo complete refolding relative to PrP^C to assume the amyloid conformation (8). Growth rates (red arrows) may be different at the opposite ends (17). (B) Stereotactic microinjection of RML prions into the brains of transgenic mice expressing glycosylphosphatidylinositol (GPI)-free PrP^C has shown the spreading of PrP^{Sc} over the ensuing months until the terminal stage at 320 days post-inoculation (dpi) (coronal brain sections). Adapted from reference 18, published under a Creative Commons Attribution (CC BY) license. Analogous spreading has been documented in prion-infected wild-type mice (19).

components that can influence prion infectivity and strain characteristics (reviewed in reference 49).

From a biochemical perspective, at least, other protein aggregation diseases, that is, proteinopathies, also have the potential to be transmissible between humans as prions, but definitive epidemiological evidence of such events remains scarce (50, 51). Nonetheless, multiple reports have provided evidence consistent with iatrogenic transmission

of AD (52) and cerebral amyloid angiopathy (CAA), which involves the deposition of the amyloid β (A β) peptide derived from amyloid precursor protein (APP) (e.g. [53–56]). Furthermore, experimental injections of tissue from patients with other types of proteinopathies into susceptible animal models have suggested risks of transmission of those disorders between humans, if only by invasive medical procedures such as surgeries, transplants, or transfusions (51, 57).

Even without transmission between individuals, many human proteinopathies feature prominent, often neuroanatomical, prion-like spreading of aggregates and the associated pathological lesions over the course of disease. For example, the Braak stages of AD describe the sequential spreading of microtubule-associated protein tau-containing (58) neurofibrillary tangle pathology within the brain (59). In PD, Braak staging is based on the distribution of deposits of α -synuclein (aSyn) in Lewy bodies (60). A current debate in the synucleinopathy field is the extent to which aSyn aggregation might begin in the brain and migrate to the periphery to cause symptoms such as gut and cardiac dysfunction, or vice versa (i.e., the “brain first” versus “body first” hypotheses, respectively) (e.g. [60–62]). Amyotrophic lateral sclerosis (ALS) is also a progressive disease with spreading motor, and sometimes sensory, neuron degeneration involving abnormal deposition of proteins such as superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), and fused in sarcoma (FUS) (63–65). With these and multiple other proteinopathies, important current topics include (i) the origins of aggregation cascades in individuals; (ii) prion and prion-like aggregate structures and their correlations with transmission and strain properties; (iii) propagation and fragmentation mechanisms; (iv) clearance mechanisms; (v) risks of transmission; (vi) the detection of pathological seeds as biomarkers; and (vii) the inhibition of aggregation for therapeutic purposes. Here, we provide updated overviews and perspectives on these topics. We have used PrP prions as more detailed points of reference given their well-documented infectivity and the fundamental precedents in protein aggregation-based pathogenesis established by the long-term availability of fully disease-recapitulating animal models (66) and revealing *in vitro* models (39, 67, 68).

ORIGINS OF PATHOLOGICAL PROTEIN AGGREGATION AND PROPAGATION CASCADES

Many proteinopathies involving the aggregation of a specific protein or proteins have both sporadic (i.e., irregular, of unknown cause) and genetic types, such as the familial forms of Alzheimer’s or Parkinson’s diseases. With PrP prion diseases, cases have also been shown convincingly to be acquired due to infections with exogenous aggregates (69, 70). In humans, acquired prion diseases include: kuru from ritual cannibalism (71, 72); iatrogenic CJD from the use of CJD-contaminated growth hormone, dura mater, corneas, surgical instruments, electrodes, or transfusions (73); and variant CJD from exposures to bovine spongiform encephalopathy (BSE) contaminated beef products (74) or human blood (75). Acquired cases account for <1% of human prion disease cases (73, 76) but can be much more prominent in cervids (the ongoing chronic wasting disease [CWD] epidemic), cattle (the former bovine spongiform encephalopathy [BSE] epidemic), and sheep (recurring scrapie). Both sheep scrapie (77) and CWD are known to spread via the environment (78), with CWD being particularly difficult to control. As such, CWD is spreading widely among deer, elk, and moose in many parts of the United States and Canada and is also affecting cervids in Korea and Northern Europe. The North American and Korean CWD strains appear to be indistinguishable from each other, but distinct from European strains (79). The zoonotic potential of BSE has been demonstrated by the surge of >200 human variant CJD cases after the onset of the BSE epidemic in the 1990s. However, to date, no cases of human prion disease are known to have been caused by CWD or scrapie prions (80) (https://www.cidrap.umn.edu/sites/default/files/CWD%20Report%202025_0.pdf). Animal and *in vitro* models of human susceptibility have also usually indicated high barriers to CWD transmission (81–86). However, adaptation of CWD to pathogenicity in human PrP^C-overexpressing transgenic mice

can occur with serial passages (87) or prior *in vitro* amplification, and possible strain alteration, by protein misfolding cyclic amplification (PMCA) (88). Nonetheless, we have difficulty conceiving of how any of these experimental CWD strain adaptation methods might represent naturally occurring processes.

Inherited proteinopathies can often be linked to mutations in specific genes that often, but not always, encode the aggregating protein itself (1). In this situation, the mutations likely affect the biosynthesis, conformational stability, ligand interactions, and/or clearance of the offending protein in a way that potentiates aggregation.

The vast majority of human proteinopathy cases are classified as sporadic. In these cases, it appears that the aggregation process begins spontaneously and stochastically but is likely enhanced by physiological or inflammatory stresses (e.g., reference 89), deficiencies in the patient's protein quality control (proteostatic) systems, and/or transcription errors in amyloidogenic proteins (13). The age dependence of most proteinopathies, together with the accumulation of a spectrum of detergent-insoluble proteins in tissues later in life (90, 91), suggests that proteostatic mechanisms can eventually wane and/or become overwhelmed by the accumulation of aggregated and abnormally cross-linked proteins. Chronic inflammation and overexpression of amyloid precursors in the blood, such as immunoglobulin light chain, transthyretin, or serum amyloid A, are known to cause multiple systemic amyloidoses (12). Inflammation may also promote the development or spreading of proteinopathies by enhancing vascular leakage, molecular crowding, and trafficking.

Evidence also suggests that some of the proteins that are centrally involved in various diseases, such as aSyn, tau, TDP-43 (92), FUS (93), p53 family tumor suppressors (94), and PrP (95–102) can form condensates or liquid-liquid phase separations, and perhaps other agglomerations as part of physiological responses to traumatic brain injury (103–105), infection (106), and stimuli or stresses within cells and tissues (95, 98, 106–111). Even if such agglomerations do not usually involve amyloid fibril formation, the protein concentration effects may enhance the likelihood of spontaneous fibril nucleation (94, 112), which is usually exponentially dependent on monomer concentration (47). On the other hand, recent evidence suggests that condensate formation can also suppress amyloid fibril formation (113). Perhaps most nascent aggregates are cleared by proteostatic mechanisms (see "Clearance mechanisms," below) as the damage or stresses are resolved. Such a phenomenon has been seen with PrP aggregates or condensates formed along needle tracks in brain tissue of mice inoculated with normal (uninfected) brain homogenate (97, 99) which are largely resolved by 2 weeks after formation (99). However, age-dependent deficiencies or other perturbations of protein homeostasis in vulnerable anatomical sites might allow some nascent aggregates to propagate faster than they are cleared. Such perturbations are thought to include environmental factors, infections, inflammatory events, traumas, and intercurrent co-pathologies such as diabetes, due in part to their being implicated as risk factors for the development of dementia, PD, and other neurodegenerative disorders (104, 114–121).

Interestingly, tau can form fibrils, or at least seeding-competent assemblies, in the context of normal physiology or stress responses (122). Again, such seeds may normally be cleared but occasionally escape routine proteostatic mechanisms. If such controls are overwhelmed in an individual cell or site to allow accumulation of self-propagating aggregates, then transfer to adjacent cells might initiate an overwhelming prion-like spreading of the aggregates and any associated pathology within and between tissues. Alternatively, or additionally, more generalized deficiencies in clearance mechanisms in a patient may allow a multifocal initiation of insufficiently controlled cascades of aggregate propagation. However, whether the initial seeds are formed normally or due to stochastic insufficiencies of protein homeostasis, the neuroanatomical spreading features of AD, Lewy body disorders, ALS, prion diseases, and others suggest that certain tissue sites are more vulnerable than others to the initial prion-like propagation of specific proteins. We speculate that relative vulnerabilities might be influenced by the availability of polyionic cofactors such as sulfated glycans and nucleic acids that are

known to associate with many types of protein aggregates, notably amyloid fibrils, and either positively or negatively influence their propagation depending upon circumstances (49, 123–126).

STRUCTURAL BIOLOGY OF PRION AND PRION-LIKE FIBRILS

The most fundamental issue in proteinopathy structural biology is how the structures of pathological amyloid fibrils (or perhaps other types of aggregates) propagate and interact with their *in vivo* environments in conformation-dependent ways to cause characteristic disease phenotypes. Recently determined high-resolution 3D structures of amyloid fibrils using cryo-EM have shown that distinct proteinopathies involve the accumulation of particular fibrillar conformers of specific proteins (127, 128). Solid-state (ss) NMR analyses have also revealed structural features and dynamics of amyloid fibrils (129, 130). However, such ssNMR studies are often complicated by the need to amplify brain-derived seeds *in vitro* to introduce the necessary isotopic labels, a process that frequently allows conformational deviations from the original pathological seeds (examples below). In the following subsections, we will emphasize the structures of tissue-derived (*ex vivo*) fibrils.

Amyloid- β (A β)

AD is defined in part by the accumulation of amyloid plaques composed mainly of a 42-residue form of A β (A β 42) (131). In the terminal phase, many AD patients also have CAA amyloid deposits composed primarily of a 40-residue peptide of A β (A β 40) in the leptomeninges and blood vessel walls (132). CAA can also occur independently of AD (133), and, while most cases are sporadic, some can be familial (134, 135). A recent cryo-EM study reported two distinct polymorphs of A β 42 fibrils from AD brains, with Type I found mainly in sporadic AD and Type II in familial AD and other conditions (136). The Arctic APP mutation, encoding an E693G substitution, leads to the accumulation of A β 42 fibrils with substructures like those of AD Types I and II fibrils, but with subtle differences (137). Some missense mutations in the presenilin gene can lead to inherited AD with the accumulation of “cotton wool” plaques of A β comprised predominantly of Type I A β fibrils, but with some novel interprotofilament arrangements (138). Both cryo-EM analyses of brain-derived A β fibrils and ssNMR studies of *in vitro*-amplified fibrils have further highlighted the diversity of possible A β fibril structures as well as a tendency of A β fibril preparations propagated *in vitro* to diverge conformationally from the *ex vivo* fibrils that were used to seed them (139, 140). Such divergence has been suggested to be due to the lack of cofactors required to maintain amplification of the *in vivo* fold and/or to spontaneous, untemplated formation of seeds that are able to propagate faster *in vitro* than the brain-derived fold (140).

Tau

Tauopathies are typically categorized by the inclusion of specific tau isoforms in their fibrils/filaments (Fig. 2A). These isoforms are differentially spliced from the *MAPT* gene and contain either three or four microtubule binding (MTB) repeats. Pick's disease (PiD) has preferential incorporation of the three tau isoforms with only three MTB repeats (3R), whereas diseases such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and argyrophilic grain disease (AGD) incorporate the three tau isoforms with four MTB repeats (4R). Filaments in diseases such as AD and chronic traumatic encephalopathy (CTE) contain all six tau isoforms and have a mixture of both 3R and 4R isoforms. The solution of many *ex vivo* tau fibril structures has supported a structure-based classification of the aforementioned tauopathies and subtypes thereof (2, 128, 141). Interestingly, biochemical evidence suggests that rapidly progressing AD cases appear to have tau aggregates that differ structurally from those of more slowly progressing sporadic AD cases (142). AD-like tau fibrils also form in the brains of patients with certain genetic prion diseases: PrP cerebral amyloid angiopathy (PrP-CAA) due to mutations leading to early termination of PrP translation at Q160; and GSS due to an

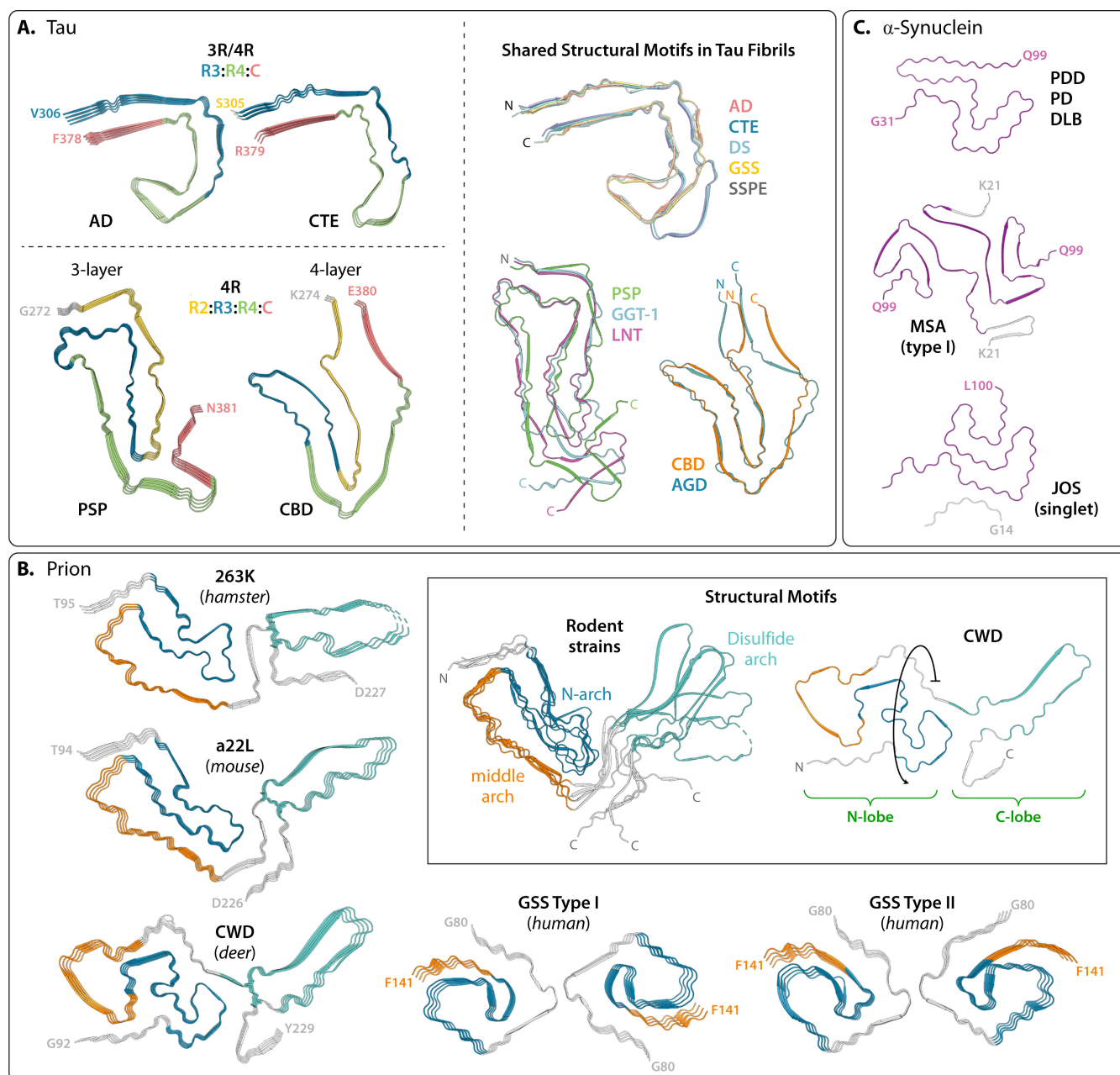


FIG 2 Representative cryo-EM-based models of brain-derived tau, prion, and aSyn fibrils (cross-sectional views of the ordered fibril or protofilament cores). (A) Structures of AD (148) and CTE (149) fibrils in structure class R3:R4:C with color-coded MTB repeats: repeat 2 (R2, yellow); repeat 3 (R3, blue); repeat 4 (R4, green); C-terminal (C, coral). Structures of PSP (141) and CBD (150) fibrils in structure class R2:R3:R4:C displaying a three-layer or four-layer core, respectively. 3R/4R structural core similarities are displayed in an overlay of tau fibrils from AD (148), CTE (149), DS (Down syndrome) (151), GSS (143), and SSPE (subacute sclerosing panencephalitis) (152). Three-layer 4R structural core similarities displayed in an overlay of PSP (progressive supranuclear palsy), GGT-1 (globular glial tauopathy), and LNT (limbic-predominant neuronal inclusion body 4R tauopathy) (141). Four-layer 4R structural core similarities are displayed in an overlay of CBD (150) and AGD (argyrophilic grain disease) (141). (B) Representative prion structures of strains in hamster (263K) (8), mouse (a22L) (15), deer (CWD) (153), and human (GSS types 1 and 2) (154). Color coding represents major structural motifs N-arch (teal), middle-arch (C-terminal flank, orange; its N-terminal flank is shared with the N-arch), and disulfide arch (cyan). The inset includes an overlay of all rodent strains solved to date (ME7 [155], aRML [14], RML [16], a22L [15], 263K [8]), as well as a CWD cross-sectional monomer for comparison, depicting the twist of the N-lobe relative to the rodent-adapted scrapie prions. (C) single chains of aSyn fibrils from PDD (Parkinson's disease dementia), PD (Parkinson's disease), and DLB (dementia with Lewy bodies) (156), MSA type-1 (multiple system atrophy) (145), and JOS (juvenile-onset synucleinopathy) (146). Corresponding sequences are shown in purple. The images in this figure were prepared using PyMol v. 2.6.0 and Protein Data Bank structure ID numbers for *ex vivo* fibrils of tau (AD, 5O3L; CTE, 6NWP; DS, 9BXI; GSS (tau), 7MKH; SSPE, 8CAQ; PSP, 7P65; CBD, 6VHA; GGT-1, 7P66; LNT, 7P6A; AGD, 7P6D); aSyn (PDD, PD, DLB, 8A9L; MSA-I, 6XYO; JOS, 8BQV); and prions (263K, 7LNA; aRML, 7TD6; RML, 7QIG; ME7, 8A00; a22L, 8EFU; CWD, 9DMY; GSS-I, 7UMQ; GSS-II, 7UN5).

F198S missense mutation (143). In addition, cryo-EM analyses showed the formation of >70 conformational intermediates over time *in vitro* as synthetic tau monomers assemble spontaneously into AD-like or CTE-like fibrils under different salt conditions (144). However, as with A β and multiple other amyloidogenic proteins, it can be difficult to accurately amplify brain-derived tau seed conformations *in vitro*.

α -Synuclein (aSyn)

High-resolution structures of brain-derived aSyn fibrils from humans with Lewy body pathology (e.g., Parkinson's disease [PD], Parkinson's disease dementia [PDD], or dementia with Lewy bodies [DLB]) have a protofilament core conformation called the Lewy fold (153) (Fig. 2C). The Lewy fold is distinct from that of fibrils from brains of multiple system atrophy (MSA), frontotemporal lobar degeneration-synuclein (FTLD-Synuclein), and juvenile onset synucleinopathy patients (145–147). As with other types of *in vitro*-amplified fibrils, many distinct conformers of synthetic aSyn fibrils have been shown to form under various conditions, but full recapitulation of the Lewy or MSA folds *in vitro* has been difficult (e.g. [129, 147]).

TDP-43 and annexin A11

Cryo-EM has also established the structures of homomeric TDP-43 fibrils that accumulate in the brains of patients with ALS with FTLD type B and FTLD-TDP types A (64, 157). In contrast, patients with FTLD-TDP type C have heteromeric pathological fibrils composed of a co-assembly of two different proteins, that is, TDP-43 and annexin A11 (ANXA11) (158). Unlike the other types of FTLD-TDP, type C is not associated with any known pathogenic genetic variants. The deposition of this fibril suggests a key role for the aggregation of wild-type ANXA11, as well as TDP-43, in FTLD-TDP type C pathogenesis. Attempts to form TDP-43 fibrils from recombinant monomers have yielded structures distinct from *ex vivo* fibrils (159).

Amyloid A (AA)

The structure of serum AA-derived *ex vivo* amyloids that accumulate in systemic AA amyloidosis of humans, mice, and cats has also been solved (9–12). Striking differences between the fibrils from these different species were observed, as well as major deviations from fibrils synthesized *in vitro*. The native serum AA tetramer is largely α -helical (4), so the conversion to cross- β amyloid fibrils involves dramatic refolding, at least in the N-terminal domain that forms the fibril core (12).

Superoxide dismutase 1 (SOD1)

To our knowledge, no detailed structures of tissue-derived SOD1 fibrils have yet been solved. However, recent reports of cryo-EM structures of *in vitro*-generated wild type and ALS-causing mutants of human SOD1 have revealed tendencies of these sequences to form distinct fibrillar conformations (160, 161).

PrP prions (fully infectious)

The overt and readily testable infectivity of PrP prions makes them particularly revealing models for detailed studies of structure-activity relationships of self-propagating, pathological aggregates. Building on work with synthetic recombinant PrP amyloid fibrils with no known infectivity (e.g., references 162–168), we and Manka et al. have reported the structures of several conformers (strains) of highly infectious, brain-derived prions (8, 14–16, 153, 155) (Fig. 2B). These isolates have been estimated to contain $\sim 10^9$ lethal doses of prion infectivity per mg of protein if injected into the brains of susceptible hosts. Like most other pathological protein aggregates mentioned above, these infectious prions take the form of amyloid fibrils with parallel in-register intermolecular β -sheet (PIRIBS) cores. However, unlike most of the others, the *ex vivo* prion fibrils have a single filament rather than two or more closely aligned protofilaments. This difference may

be dictated by other key differences between infectious PrP prion fibrils and other *ex vivo* amyloid fibrils, namely, the laterally projecting N-linked glycans and, usually, glycoposphatidylinositol (GPI) membrane anchors. These bulky and structurally variable post-translational modifications, as well as other laterally associated electron densities of unknown composition (perhaps cofactors), are likely to block the formation of consistent, well-ordered lateral interfaces between protofilaments. Another striking feature of the infectious prion fibrils is an unusually long polypeptide chain of ~136 residues comprising the ordered fibril cross-section.

Analogous to the disease-dependent fibrillar polymorphisms that characterize other pathological protein fibrils, each prion strain appears to have a distinct core conformation that provides a unique template for fibril growth (Fig. 2B). This templating presumably ensures the consistent propagation of phenotypically distinct prion strains, which can be maintained passage after passage in laboratory animals. The first prion strains to be solved were experimentally adapted from scrapie-infected sheep, in some cases via goats, in the UK into hamsters (263K [8]) or mice (RML [169], aRML [14], a22L [15], or ME7 [155]). Each of these rodent-adapted strains has two major N- and C-terminal lobes comprising the fibril cross-section, and a similar combination of arch/hairpin, loop, and steric zipper motifs. However, the conformational details within those shared motifs vary substantially. Comparison of the aRML and a22L strains propagated in the same genotype of mouse revealed the purely conformational determinants of the respective phenotypes of these two strains (15). In more microbiological terms, these structures have finally demonstrated in near-atomic detail the protein conformational, rather than genetic, basis for prion strain differentiation, as had been proposed decades earlier (41, 48, 170–172).

The first natural prion structure to be solved came from a CWD-infected deer expressing the most common wild-type *PRNP* genotype (153). CWD fibrils resembled those of the rodent-adapted prion strains in terms of the core residues forming major N- and C-terminal cross-sectional lobes. However, strikingly, these lobes are swiveled 180° relative to one another when compared to the rodent prions. Also, the N-lobe residues of the CWD structure lack the main N-lobe arches and steric zipper of the rodent structures and take a more circuitous path through the fibril cross-section. The origin(s) of North American CWD are not known, but are suspected by some to have been sheep scrapie. However, the fundamental difference in the relative orientations of the N- and C-lobes makes it unlikely that this strain of North American CWD could have been templated initially by the UK isolates of sheep scrapie that were used to derive the rodent-adapted strains described above.

GSS F198S PrP amyloid

Although, due to biosafety concerns, we know of no one being allowed to study fully infectious human-transmissible prions on a cryo-electron microscope (yet), Hallinan et al. have solved the structure of fibrils from patients with a much less infectious genetic prion disease, namely GSS linked to the F198S mutation in the *PRNP* gene (154). These fibrils differ strikingly from the CWD and rodent-adapted scrapie fibrils (Fig. 2B), most notably, by having a protofilament core cross-section with a much shorter PrP polypeptide fragment of 62 residues (no. 80–141) that partially overlaps only a portion of the N-lobe of the fully infectious prion cores. Moreover, the GSS F198S fibrils have 2–4 protofilaments in contrast to the single filament of the more infectious fibrils. These ordered protofilament interactions may be permitted by the lack of glycans or GPI attached directly to the core residues.

Additional structural correlates with transmissibility

Another GSS-like genetic prion disease in humans is caused by the Y145Stop mutation in the *PRNP* gene (173, 174). The expressed C-terminally truncated PrP leads to the assembly of amyloid fibrils and cerebrovascular amyloidosis. Although the structure of such fibrils from the brain is not known, the cryo-EM structure of synthetic fibrils of

the truncated human PrP 23–144 sequence has been solved (175). This structure has four identical protofilaments, each containing a relatively small, ordered core of residues ~108–141 that is quite distinct from conformations of overlapping sequences within the *ex vivo* fibrils of GSS F198S patients and animals with CWD or rodent-adapted scrapie. The transmissibility of these human PrP 23–144 fibrils was not reported, but their murine counterparts are capable of causing serially transmissible prion disease in mice that overexpress murine PrP, at least when 3 µg, that is, >10⁶-fold more than the fg-pg lethal doses of typical highly infectious natural prions, are injected into the brain (176). Finally, many synthetic recombinant PrP fibrils have been made with ordered cores limited to C-terminal residues of roughly 160–231 that have no demonstrated infectivity (e.g., references 57, 177). Therefore, so far, the most infectious of PrP prion fibrils have much larger ordered cores comprising residues ~92 to near the C-terminus (~231). Importantly, however, even small differences (e.g., ~1 kDa) in the size of monomers comprising protease-resistant PrP fibril cores have been linked to multi-log differences in infectivity (178).

In more general terms, amyloid fibrils of a given protein, such as PrP, can range from being extraordinarily infectious and pathogenic to seemingly innocuous *in vivo*, depending upon their respective conformations.

INTER-SPECIES TRANSMISSION BARRIER MECHANISMS

The transmission of BSE into humans demonstrated that at least one non-human PrP prion strain has zoonotic potential. A particular current concern is human and livestock exposures to CWD from cervids (80) (Fig. 3). Multiple lines of *in vitro* and *in vivo* evidence indicate that with other prion diseases, differences of as little as a single key residue between an inoculated PrP^{Sc} and the recipient's PrP^C sequences can protect against new PrP^{Sc} formation (81, 179–182) and disease (e.g., references 183–188). The cryo-EM structures of deer CWD and hamster 263K prions have prompted consideration of the molecular mechanisms of their barriers to transmission to humans and mice, respectively (8, 153, 189–191). In each case, as shown in transgenic animals (184, 188) and cell cultures (185), particular highly localized residue differences are primarily responsible for the lack of transmission. Subsequent molecular modeling suggested that conformational conversion of those heterologous PrP^C molecules onto the infecting prion template would cause major steric clashes or the burying of uncompensated side chain charges into a tightly packed structure to likely slow or stop conversion (8, 153). Increasing insights into prion structures and conversion reactions should help to reveal mechanisms of transmission barriers and why protections against prion disease can be afforded by heterozygosity (such as M/V at residue 129 in humans) in the host's *PRNP* gene (192).

Concerns have also been raised about the zoonotic potential of amyloid A (AA) amyloid, for example, in duck or goose foie gras, to initiate AA amyloidosis in humans (12). AA amyloidosis has been accelerated in transgenic mouse models by inoculation of AA amyloids from a variety of host species, including humans. The sequences of the amyloid precursor (serum amyloid A) in humans, ducks, and other species have regions of both similarity and divergence, and it remains unclear which, if any, of these sequence differences may be protective against inter-species transmissions.

GROWTH, FRAGMENTATION, AND SPREADING MECHANISMS

Pathological amyloids grow primarily by seeded polymerization (46). The fibril ends provide conformational templates that bind monomers, or perhaps small oligomeric intermediates, and induce their refolding into the structure of the template as they are incorporated into the growing fibril. The detailed mechanisms by which this process occurs for various types of fibrils are usually poorly understood. However, a generic feature of PIRIBS amyloid fibrils or protofilaments is that the polypeptide backbone of the terminal monomer on each end exposes interdigitated hydrogen bond donors (R-NH groups) and acceptors (carbonyls) that are poised to attach in a zipper-like fashion with the complementary groups on the backbone of an incoming monomer (193, 194). Initial

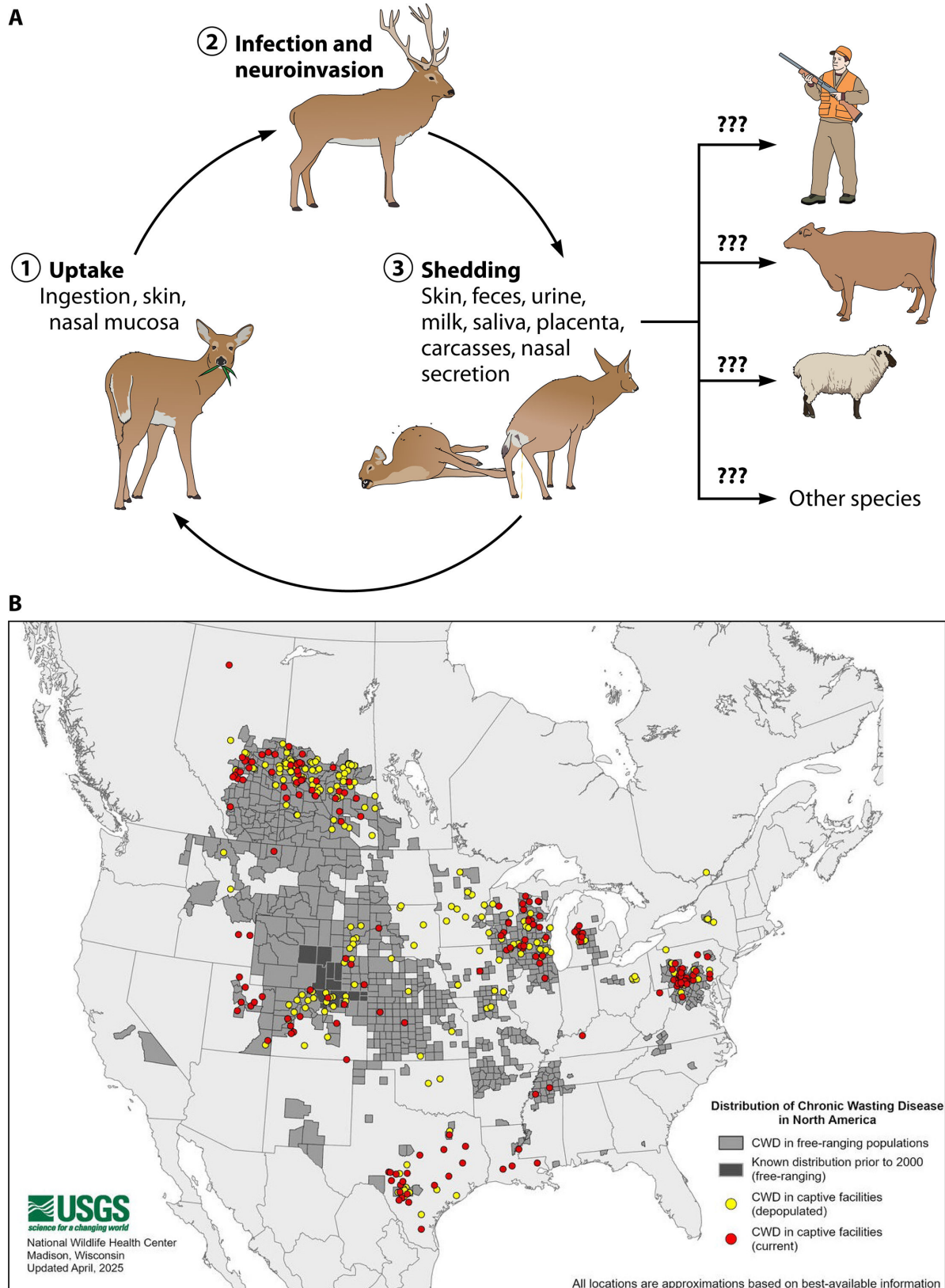


FIG 3 CWD epidemic in North American cervids (deer, elk, moose). (A) Transmission cycle of CWD with poorly understood potential for natural transmission to humans and livestock. CWD is difficult to control and appears to be the most causally contagious of the proteinopathies via direct contact and the environment, where CWD prions can remain infectious for years. (B) Distribution of CWD in North America. Reproduced from <https://www.usgs.gov/media/images/distribution-chronic-wasting-disease-north-america-0> (public domain).

contacts between the template and monomer would also likely be aided by interactions between exposed hydrophobic residue side chains that, as with all side chains, splay out to either side of the backbone perpendicular to the fibril axis on the template. Alternatively, or additionally, polyionic cofactors that can promote fibrilization and align with stacks of charged side chains along the fibril axis might help to bring monomers into proper alignment with a template to promote parallel-in-register backbone-backbone interactions.

Not only does prion-like propagation of aggregates require elongation, but also fragmentation (195, 196), to increase aggregate number and decrease particle size to enhance mobility within and between cells and tissues (Fig. 4). The dimensions of many pathological protein deposits, at least in their mature forms, often rival, or exceed, the dimensions of neuronal or glial cell somas and their projections, likely impeding movement through tissues, especially through narrow axons, dendrites, small (30–150 nm) extracellular vesicles, and intercellular spaces. Also, the spreading of particles bigger than ≥ 500 kDa (e.g., a PrP^{Sc} ~ 16 mer) through interstitial fluid flow is severely limited (197, 198), apparently by the narrow spacing (30–70 nm) and matrices between cells. Indeed, scaling studies of prion replication *in vivo* have provided evidence that fragmentation is a key kinetic determinant (196).

The need for fragmentation raises the issue of the extent to which various types of fibrillar aggregates might break spontaneously as opposed to being fragmented under the influence of other physiological processes. Computational analyses of A β and other peptide fibril structures have begun to describe the tensile/nanomechanical properties of cross- β fibrils as a function of morphology, conformation, sequence, and length (194, 202, 203). For example, longer A β (1–40) fibrils (>100 nm) appeared to be more stable than shorter fibrils (202). Our recent all-atom molecular dynamics (MD) analyses of the cryo-EM-based fibril core structures of three strains of infectious, brain-derived prion fibrils indicated a high degree of conformational stability (to a lesser extent for the terminal monomers) in oligomeric fragments as small as tetramers (only 2 nm long). No fragmentation was seen within 0.5–2 μ s, even at elevated temperatures of up to 400K (127°C) in tests of 8- and 25-mers (204). Although longer MD simulation times might be needed to allow fragmentation, our data to date are consistent with a key role of physiological stressors in prion fragmentation. Importantly, these simulations have suggested so far that short oligomeric aggregates (as small as 3–4 mers) with the same PIRIBS architectures of elongated amyloid fibrils can be stable. However, although some reports have suggested that prion strains spontaneously disassemble into mostly dimeric-tetrameric units in non-disinfecting detergents based on size exclusion chromatography and light scattering analyses (31, 205), we have recently found that this is not the case, and that the previous suggestions were likely based on confounding experimental artifacts (206).

Tissue activities that might assist aggregate fragmentation include proteostasis systems, innate immune factors, acidification of autophagic vacuoles, interactions with cytoskeletal elements and motors, and the shearing forces of membrane dynamics. The latter might be especially relevant in the case of wild-type prions, which are typically anchored to membranes via an array of GPI anchors along the fibril axis (8, 16, 153, 155). Consistent with this possibility are observations of disease-associated PrP with distorted membranes, some of which appear to be abortive attempts at internalizing PrP aggregates from the cell surface (207). We have speculated (8) that fragmentation of GPI-anchored PrP^{Sc} fibrils might be facilitated by curvature-induced stresses imposed by endocytosis, membrane recycling, autophagy, multivesicular body, and exosome formation, as well as the pulsing of blood vessels. Even a small proportion of accumulated fibrils in or around affected cells that are broken into small, more mobile units might account for the spread of seeds and their associated pathology through cells and tissues.

Although molecular chaperones facilitate some clearance of PrP^{Sc} (see “Clearance mechanisms,” below, and references 208–210), they also seem capable of promoting the propagation of PrP^{Sc} by fragmenting it and releasing more mobile units (211).

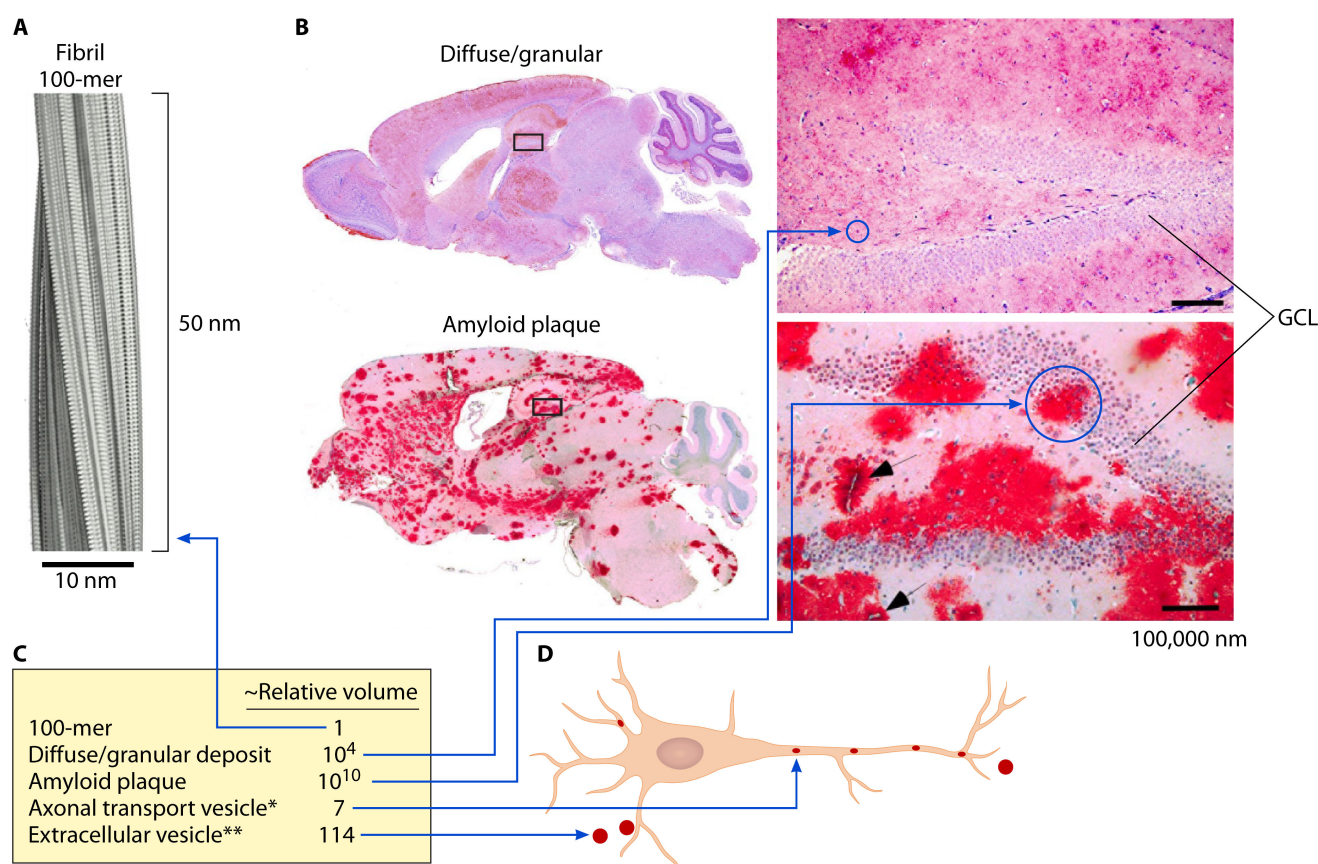


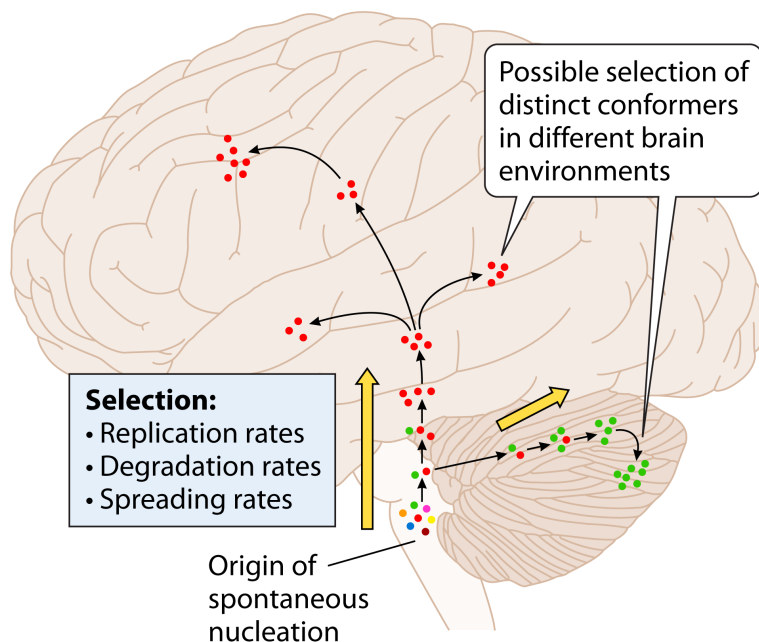
FIG 4 Relative sizes of prion amyloid fibrils, granular deposits, amyloid plaques, axonal transport vesicles, and extracellular vesicles. (A) A cryo-EM-based model of an aRML prion amyloid fibril core (14) containing 100 PrP monomers. (B) Sagittal brain sections (left) and expanded views of the dentate gyrus (right; corresponding to the inset boxes in the whole brain sections) stained for PrP^{Sc} (red) from either wild-type (top) or GPI-anchorless PrP transgenic (bottom) mice, showing predominant diffuse or granular staining (wild type) versus amyloid plaque (GPI-anchorless), respectively. For comparison, the closely apposed nuclei of the granular cell layer (GCL) are counterstained in blue and are much smaller than many of the diffuse/granular and amyloid PrP^{Sc} deposits. Adapted from Chesebro et al. (199), published under a Creative Commons attribution (CC BY) license. (C) Estimated relative volumes of a 100-mer fibril, the indicated PrP^{Sc} aggregates, a mid-sized (*) 60 nm axonal transport vesicle, and a mid-sized (**) extracellular vesicle (150 nm; i.e., at the border between the “small” and “large” extracellular vesicle ranges) normalized to the 100-mer. (D) Cartoon of a neuron illustrating vesicles through which prions and other pathological aggregates are thought to often spread (200, 201). Both small and large extracellular vesicles have been shown to carry prions, at least in cell culture (201).

Chaperone-promoted propagation has also been observed with fibrils of tau (212), Sup35 (213), and α -synuclein (214). The yeast prion Sup35 has exposed chaperone binding sites along its core that allow chaperones to unfold internal subunits, driving fragmentation (213). An analogous mechanism of exposed inter-core binding sites might also assist the fragmentation of mammalian prions and other disease-associated amyloids. Chaperone-promoted propagation of PrP^{Sc} is also facilitated by other cellular processes like acidification (215), which can destabilize PrP^{Sc} and help chaperones to bind and break it (210).

EMERGENCE OF DOMINANT DISEASE-ASSOCIATED CONFORMERS

The structural diversity and significance of protein aggregates may differ markedly between the early and late stages of pathogenesis. In sporadic or genetic types of proteinopathies, the initial nucleation of aggregates may occur in a stochastic and conformationally haphazard manner akin to the early formation of a multitude of fibrillar polymorphs *in vitro* when tau is allowed to fibrillize spontaneously (144). However, in the latter experiment, the most efficiently propagated conformers soon become dominant, with the population converging on a single majority conformer under a given set of

A Sporadic or genetic (spontaneous misfolding)



B Acquired infection (with predominant, preselected conformer)

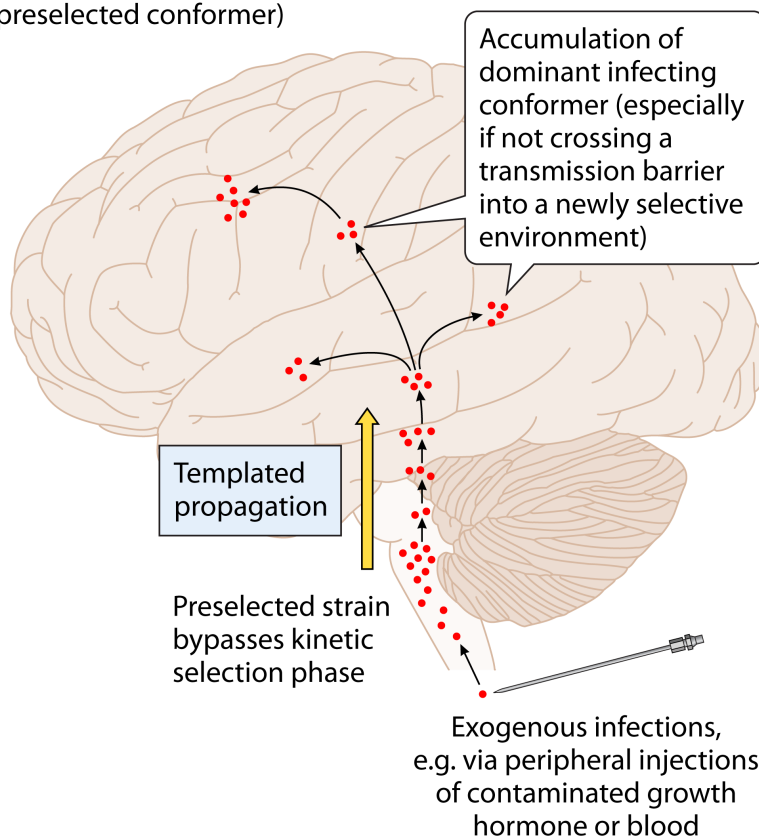


FIG 5 Hypothetical conformation selection scenarios in sporadic or genetic (A) versus acquired (B) proteinopathies. (A) Model of kinetic selection of the most “fit” conformers from many possible initial, spontaneously arising nuclei/seeds in a given locus, especially in the contexts of sporadic and (Continued on next page)

Fig 5 (Continued)

genetic proteinopathies. (B) In acquired proteinopathies, in which the host is exposed to a predominant conformer that was preselected in another individual of similar genotype, much (but perhaps not all) of the selection processes in the recipient would be bypassed. This scenario is amply exemplified by the consistent maintenance of distinct predominant strain-specific PrP^{Sc} conformers (8, 14–16, 155) and other strain phenotypes upon serial passaging of a given prion strain in a given type of host (reviewed in references 66, 223). In humans, such infections can be initiated by peripheral injections of contaminated growth hormone or blood (represented by the needle) or other iatrogenic modes (not represented) such as the use of contaminated transplant tissue (corneas, dura mater), electrodes, etc. (73).

conditions (Fig. 5A). Analogously, it seems likely that in the brain, the most fit of various initial, spontaneously formed aggregates would overtake others kinetically (216) to yield a predominant disease-associated conformer(s) through conformational templating and spreading between cells. The overall relative fitness of a conformer would likely be determined by the net effects of replication (growth + non-inactivating fragmentation), degradation, and spread within and between cells. Much of this selection might occur within a cell or localized group of cells, after which conformation-dependent templating would drive the accumulation of the selected conformer (Fig. 5A, red dots). This scenario is consistent with the identification of single predominant tau, A β , and aSyn fibril conformers in the terminal stages of distinct disorders as described above. However, much like the effects of different salts on the *in vitro* selection of AD vs CTE tau fibril conformers *in vitro* (144), fitness might also be modulated locally or regionally by variables such as cofactors, proteostatic responses, cell subtypes (e.g., neurons and glia in their different activation states), physiologic stresses, and copathologies, allowing for the emergence of another conformer/polymorph (green). Indeed, this scenario is consistent with reports that there can be more than one PrP^{Sc} subtype in 30%–100% of individual brains from sCJD patients (217, 218) and that these can vary with brain region (219, 220). However, a subsequent report highlighted the technical challenges associated with sCJD subtyping and concluded that “co-occurrence of PrP^{Sc} types in Creutzfeldt-Jakob disease is not the rule” (221). Nonetheless, a more recent report describes the transmission to animals of different sCJD subtypes from distinct regions of a single sCJD brain (222).

With acquired prion diseases, in which the predominant conformer is inoculated to template aggregation, the initial kinetic conformer/strain selection process appears to be largely bypassed, at least in hosts of the same genotype (Fig. 5B). The ability of distinct fibril polymorphs to provide growth templates provides a plausible mechanism for conformationally faithful propagation within the context of a given disease (8, 14–16, 41, 128, 155, 224–226). However, when biologically cloned prion strains have been transmitted into different host species or genotypes, conformational adaptations to the new host have been observed, presumably due to selective pressures applied by new host environments (223, 227–230). Prion strain adaptation or “evolution” under the selective pressure of a prion inhibitor has also been observed in cell culture (231).

If other conformers accumulate to meaningful levels as well, they might represent either similarly fit (in terms of accumulation) polymorphs or secondary byproducts of the accumulation of the predominant conformer(s) without direct conformational templating. For example, the latter might occur via secondary nucleation or scaffolding of monomers of the same protein along the lateral surfaces, rather than the ends, of fibrils. We suspect, however, that such lateral scaffolding would sometimes be limited *in vivo* by lack of access of monomers to the ordered cores of amyloid fibrils due to the less ordered “fuzzy coats,” which, in the case of PrP prions, include glycans and GPI anchors as noted above (8, 16, 153, 155). Moreover, direct access would likely also be blocked by the ligands mentioned above, as well as binding to membranes. Even if such putative secondary byproducts of fibrilization were more toxic than the fibrils themselves, it should follow that blocking the propagation of the primary conformer should limit new production of such byproducts if the latter were not themselves independently self-propagating.

CLEARANCE MECHANISMS

As noted above, the proteostatic degradation and/or clearance of pathological aggregates from tissues such as the brain should help to limit their accumulation. Extensive studies on PrP^{Sc} prions have shed early light on the cellular capabilities and mechanisms involved in clearing such aggregates. Surprisingly, given its extreme resistance to proteases, PrP^{Sc} can be degraded by multiple cell types, not just professional phagocytes (232). While the rate of degradation can be impacted by prion strain (233, 234), aggregate size (234), and the presence of cofactors (235, 236), the ability of a cell to degrade PrP^{Sc} does not appear to depend upon either the initial stability or the relative protease resistance of the PrP^{Sc} aggregate (237). This may be because cellular interactions with PrP^{Sc} via the proteasomal pathway (238, 239), acidic microenvironments such as those in endocytic vesicles and lysosomes (234, 236, 240–242), chaperones (210, 211), or cellular proteases (240, 242, 243) are needed to modify PrP^{Sc} prior to degradation. In general, the lysosome appears to be the primary site for degradation of PrP^{Sc} (242, 244, 245), with contributions from the ubiquitin proteasome system (244, 246) and an indirect role for autophagy-mediated degradation (247). It remains unclear how cytosolic proteasomes come into contact with PrP^{Sc}, which is thought to be on the cell surface or in the lumen of vesicles. Interestingly, however, distorted abnormal PrP-containing plasma membrane invaginations seen in prion-infected brain can also be stained on the cytosolic side with anti-ubiquitin antibodies (207, 248). In any case, multiple cellular pathways may contribute to the degradation of prions (249).

Recent studies examining how PrP^{Sc} is degraded support the hypothesis that PrP^{Sc} is modified during a degradation process that occurs in two distinct phases (234). After PrP^{Sc} is taken up into cells, it first undergoes a pH-dependent conformational change (234, 236). This conformational change impacts all PrP^{Sc} aggregates regardless of size (234) and likely occurs in early endocytic compartments (234) and/or the lysosome (242, 245, 249). The change in structure makes PrP^{Sc} more susceptible to cellular protease activity, leading to the loss of some proportion of PrP^{Sc} (234, 236). The change in PrP^{Sc} conformation is followed by aggregate disassembly (233, 234), some of which may be driven by chaperones (210, 211). Smaller aggregates are disassembled more rapidly than larger aggregates, but the overall result is a decrease in the amount of larger PrP^{Sc} aggregates (234). As this cycle of cell-induced conformational change and disassembly repeats over time, there is a downward shift in PrP^{Sc} aggregate size and an increase in protease resistance (234). One outcome of PrP^{Sc} degradation is therefore the accumulation of smaller aggregates that the cell cannot break down (234). Small PrP^{Sc} aggregates have been identified as the most infectious prion particles per unit protein (250), perhaps because of a greater mobility and proportion of their surface area being PrP^C conversion templates/catalysts. Indeed, chaperone-mediated disaggregation of PrP^{Sc} can generate smaller PrP^{Sc} aggregates capable of seeding new PrP^{Sc} formation (211). Thus, in trying to protect themselves by degrading PrP^{Sc}, cells may inadvertently facilitate prion propagation.

Overexpression of molecular chaperones can reduce the concentration of PrP^{Sc} in chronically infected cells (208) and animals (209); however, chaperone overexpression has never resulted in complete clearance. PrP^{Sc} fibrils may elude complete destruction due to structural features and post-translational modifications such as glycation- and dityrosine-mediated cross-linking (251, 252). This may explain why at least small percentages of PrP^{Sc} can survive even harsh conditions (e.g. [253]). As noted above, the binding of ligands (235, 254–256) could also help stabilize PrP^{Sc} and block chaperone binding.

The tenuous balance between propagation and clearance of PrP^{Sc} by proteostasis has also been observed with other amyloids. In yeast, the [URE3] and [PSI⁺] prions are formed by fibrils of Ure2p and Sup35, respectively (257), and chaperone variations can modulate their propagation. For example, a moderate level of the disaggregase Hsp104 is needed to fragment [PSI⁺] fibrils to allow them to distribute effectively to daughter cells upon cell division or cytoplasmic exchange during mating to propagate

the [PSI⁺] phenotype in culture. However, both overexpression and underexpression can cure yeast of [PSI⁺] prions, although some [PSI⁺] variants can be cured by normal levels of Hsp104, representing an example of an “anti-prion system” (213, 257, 258). Chaperones can also help propagate disease-associated fibrils of tau (212) and α -synuclein (214) through fragmentation, but overexpression of chaperones can diminish, but not eliminate, both types of fibril (259, 260). As with PrP^{Sc}, total clearance of other amyloids may be compromised by the cross-linking, specifically mediated by glycation in α -synuclein (251) and dityrosines in tau (261).

Tau and α -synuclein fibrils can be digested by cytoplasmic proteases (262, 263), but are predominantly degraded in lysosomes (263–265), similarly to PrP^{Sc} (266). However, cells' attempts to deal with such fibrils in lysosomes appear to have severe impacts on their fitness. Notably, PrP^{Sc} (267), tau (268), and/or aSyn (269) fibrils can induce lysosomal dysfunction, for example, leakage (270, 271), and their degradation products can affect mitochondria (272, 273). Lysosomal leakage may allow both tau and aSyn fibrils that have been internalized by endocytosis to escape into, and propagate, within the cytoplasm. Collectively, these observations suggest that the overall effects of manipulations of proteostasis for therapeutic purposes may be difficult to predict.

DETECTING MISFOLDED PROTEINS AS BIOMARKERS AND BIOHAZARDS

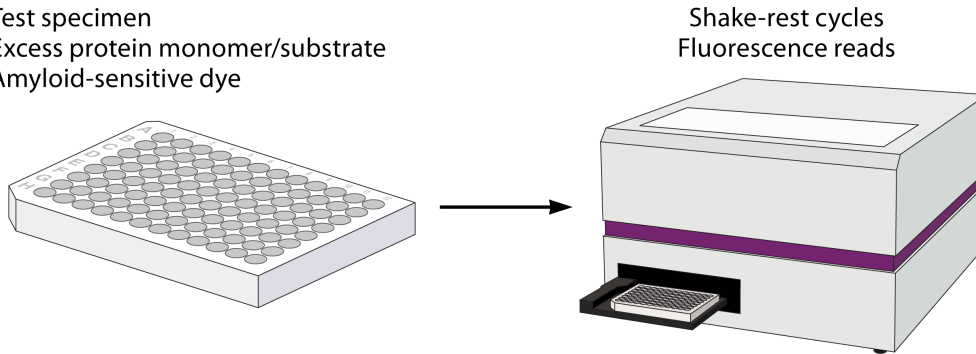
Proteinopathies can be difficult to clearly diagnose, especially early, when clinical indices tend to be variable and shared among multiple disorders. The self-propagating (seeding) activities of pathological aggregates have been exploited to develop highly sensitive and specific seed amplification assays (SAAs) for proteopathic seeds as etiological biomarkers (274–279). SAAs such as protein misfolding cyclic amplification (PMCA) (67) and real-time quaking-induced conversion (RT-QuIC) (280, 281) were first developed for PrP prion diseases, but are now being rapidly extended to other types of proteinopathies. As originally defined, PMCA assays amplified prions in sonicated reactions with brain extracts as a source of the normal PrP^C substrate (67), which were then detected by immunoblotting. RT-QuIC assays arose from efforts to develop more practical tests in multi-well plates using recombinant PrP^C substrates, shaking as the source of agitation, and amyloid-dependent thioflavin-T fluorescence as a “real-time” readout (Fig. 6) (280–284). Recent SAA innovations that should help to broaden and/or simplify applications to proteinopathies include the following: improved quantitation strategies (285–287); seed detection on solid surfaces and/or in environmental samples (288–295); colorimetric- (Micro-QuIC) (296–298) or capillary-based (Cap-QuIC) (299) readouts; higher throughput 384-well plate assay formats (300); artificial intelligence-assisted analysis of data (AI-QuIC) (301); and, more automated, standardized, and streamlined RT-QuIC/SAA data analysis software (QuICSeedR) (302). A troubling observation that we have made using surface detection methods is that prions and pathologic seeds of tau and aSyn that have been dried onto surgical instruments can retain seeding activity even after treatment with a standard clinical cleaning and disinfection protocol (291). Others have recently reported that four successive attempts to use hypochlorite bleach to decontaminate a barn used to house scrapie-infected sheep failed to prevent subsequent infections of naïve sheep (303). These findings raise the possibility that broader testing, more effective disinfection strategies, and/or increased use of disposable devices and materials should be developed and implemented in situations in which proteinopathy transmissions might be a concern.

PrP prion RT-QuIC

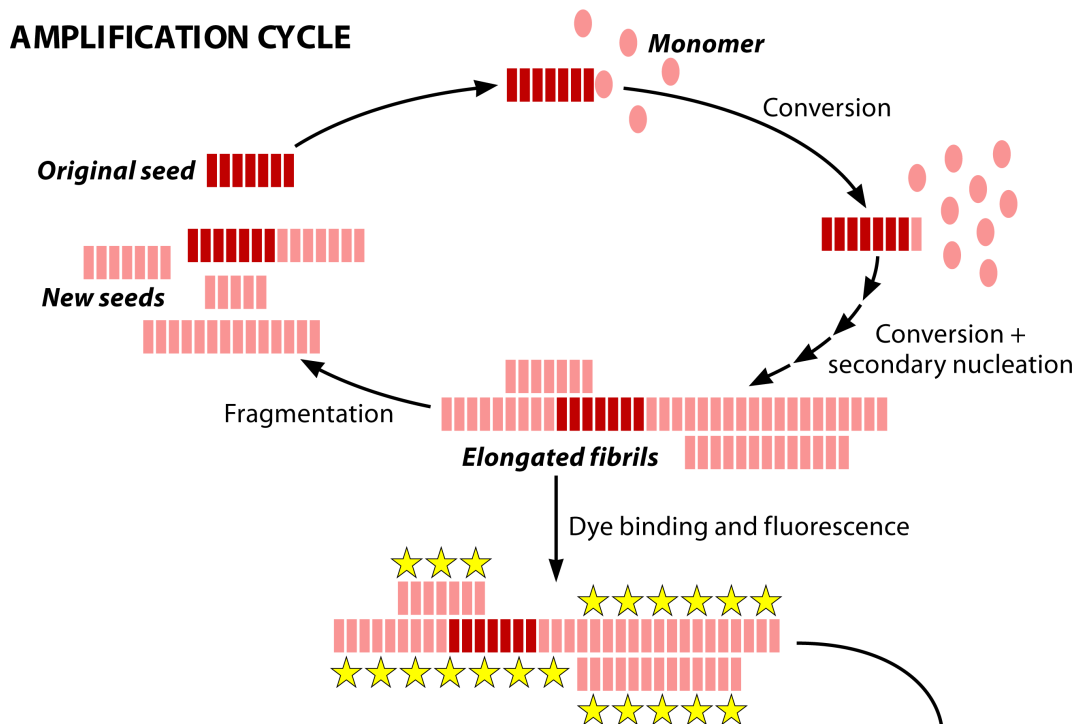
RT-QuIC assays have markedly improved prion disease diagnosis, with RT-QuIC testing of cerebrospinal fluid now being included in the official diagnostic criteria for sCJD in the United States and Europe (<https://www.cdc.gov/creutzfeldt-jakob/hcp/clinical-overview/diagnosis.html>; <https://www.ecdc.europa.eu/en/infectious-disease-topics/variant-creutzfeldt-jakob-disease/disease-information/eu-case-definition>). Round robin studies

SETUP

- Test specimen
- Excess protein monomer/substrate
- Amyloid-sensitive dye



AMPLIFICATION CYCLE



READOUT

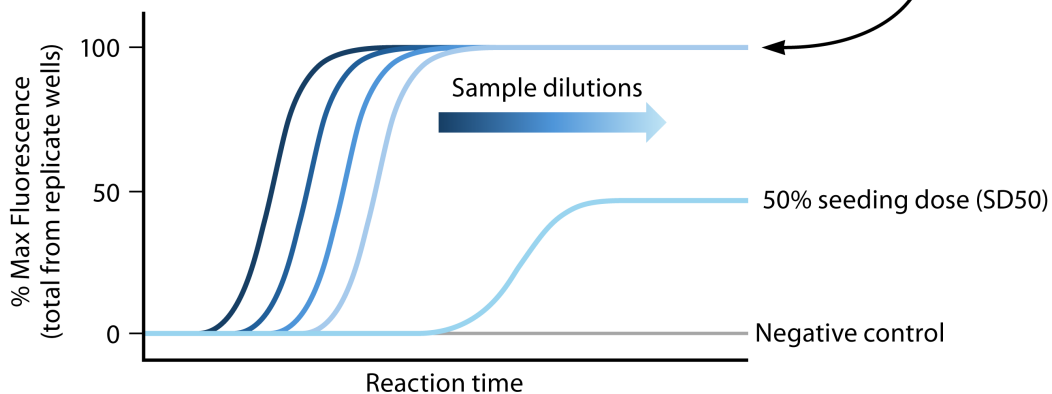


FIG 6 Proteopathic seed amplification assay: RT-QulC format (280, 281). **SETUP:** The designated key ingredients are mixed with a variety of other analyte-dependent reaction constituents in multi-well plates and subjected to cycles of shaking and rest in a temperature-controlled fluorescence plate reader, with periodic fluorescence reading. **AMPLIFICATION:** Proteopathic seeds in the specimen bind, refold, and incorporate monomers (or perhaps small oligomeric precursors) onto their growing tips (elongation) and perhaps their sides (secondary nucleation). Agitation fragments the elongated fibrils to generate many more

Fig 6 (Continued)

seeds than were in the original sample. Amplifications of prion seeding activity of up to a trillion-fold have been observed (e.g., reference 304). READOUT: An amyloid-sensitive dye such as thioflavin T emits increasing fluorescence as new fibril forms until the supply of free substrate is exhausted, as the fluorescence plateaus. With serial dilutions of the test specimen, the lag phase, or time to a given fluorescence threshold of positivity, tends to increase. Eventually, an endpoint dilution is reached at which some of the replicate reactions fail to become positive. An SD50 is defined as the amount of sample that gives positive reactions in 50% of the replicates.

have reported high interlaboratory reproducibility and concordance in RT-QuIC of testing cerebrospinal fluid (CSF), olfactory mucosa (nasal swabbings), and retropharyngeal lymph node specimens, often using different reagents (305–308). RT-QuIC testing has proven to be more practical than animal bioassays and PMCA and, importantly, in terms of biohazards to assayists, the highly amplified sCJD-seeded RT-QuIC products are not themselves infectious (309).

The adaptation of RT-QuIC assays to peripheral tissues of diagnostic interest (279), such as CSF (278, 281, 305, 306, 310–316), olfactory mucosa (317–319), skin (320–322), tears (323), urine (324–326), and feces (327, 328) has improved accurate and sensitive early detection of most known mammalian prion strains. Some prion strains, such as human sCJD and vCJD, C-, H-, and L-BSE, and classical and atypical Nor98 scrapie in sheep, can be readily discriminated (329–331). Pre-symptomatic detection of prions in CSF or peripheral tissues has been shown for hamster-adapted scrapie (332, 333), CWD (326, 333), and genetic human diseases (315). Although significant progress has been made toward the use of RT-QuIC in more routinely collected peripheral biofluids such as blood (304, 333, 334), improvements in the practicality and quantitative capabilities of the assay would be helpful.

α -synuclein RT-QuIC/SAA

aSyn SAAs are widely used for research purposes and are being increasingly validated for clinical purposes (335–337). The first aSyn RT-QuIC SAA was first reported by Green et al. (338), followed by a similar assay called aSyn PMCA by the Soto lab (339). These initial assays took 5–13 days, but we have since reported aSyn RT-QuIC assays that take only 48 h (340, 341), or even 12 h (316). So far, aSyn SAAs have detected pathological aSyn aggregates in a variety of biospecimens including brain tissue, cerebrospinal fluid (CSF) (e.g., 287, 335, 337–347), olfactory mucosa (348, 349), blood components (350, 351), submandibular gland (352), skin (346, 353, 354), and intestinal mucosa (IM) (355). Current assays can detect seeds even in prodromal phases of disease (e.g., 62, 343, 345, 356–361). PD and DLB seed detection with CSF-based aSyn RT-QuIC often yields roughly 90% sensitivity and 90%–100% specificity (e.g., 287, 335, 338–340, 342–345, 347). Sensitivities of olfactory mucosal analyses have ranged from 45% to 90%, while maintaining ~90% specificity (348, 357). SAA kinetic parameters can help discriminate PD from PSP with aSyn co-pathology (337). Faster seeding was seen with *GBA1*-Parkinson's disease and predicts cognitive decline in Parkinson's disease. Although significant progress has been achieved in α -synuclein RT-QuIC assays, further improvements, such as greater consistency, more effective applications to easily accessible biospecimen samples like blood, automation, and standardization of data analysis (362), and advanced quantitative capabilities (285, 286, 336, 362) would be valuable.

Tau RT-QuIC

Also promising, but less developed for diagnostic purposes, are tau SAAs. Using previously described tau constructs (363), the Colby (364) and Margittai (365) groups initially showed that both truncated (K18 [4R] and K19 [3R]) and full-length (htau40) recombinant tau constructs could be seeded by AD brain extracts. Building upon these principles, we established the first ultrasensitive and selective tau RT-QuIC assay using a modified 3R K19 substrate (366). This assay detects Pick's disease seeds in *postmortem*

(Continued on next page)

CSF and brain tissue dilutions as extreme as 10^{-9} . We then developed tau RT-QulC assays for 3R/4R tauopathies (AD, CTE, and primary age-related tauopathy), called AD RT-QulC (367) and K12 RT-QulC (368). AD RT-QulC uses a primary tau construct encompassing the core residues of brain-derived AD tau fibrils (306 [148]). Carlomagno et al. used a related approach to detect seeds in *antemortem* AD CSF (369). A modified K12 (3R) construct, which is C-terminally extended to residue 400 (368), allows detection and differentiation of both 3R/4R (e.g., AD) and 3R (Pick's) tau seeds in the brain based on ThT amplitudes and FTIR spectra. A modified K18 construct alone, as well as a combination of all six full-length human tau isoforms, can also detect seeds in AD brain homogenate (370). Discrimination of amplified PSP- and CBD-seeded conformers has been shown with the original 4R tau RT-QulC assay (371). This assay, which uses both 3R (K19CFh) and 4R (K18 CFh) constructs, also detects seeds in *postmortem* and, to a lesser extent, *antemortem* PSP and CBD CSF specimens. Later improvements led to a 4R-sensitive tau RT-QulC assay using a single construct (K11CFh), which also differentiates between CBD and PSP and supported the identification of a novel case of mixed pathology (372). Safar et al. have shown that reactions containing a K18 (4R) substrate can kinetically discriminate rapidly progressing AD cases from more slowly progressing sporadic AD cases, suggesting that the tau seeds differ structurally between these AD subtypes (142). A 4R tau RT-QulC assay has also been adapted for the detection of PSP and corticobasal syndrome in *postmortem* and *antemortem* skin samples (373). Encouragingly, the *antemortem* skin panel yielded 87.5% sensitivity and 95% specificity for PSP diagnosis. Tau seeding activity has also been detected in *postmortem* skin samples collected from AD, PSP, CBD, and Pick's disease patients with 75%–80% sensitivity and 95%–100% specificity for tauopathy (374). Although the above reports are promising, much remains to be done with tau RT-QulC/SAAAs to fully establish high diagnostic accuracy in living patients using accessible biospecimens.

SOD1 seeding activity as a biomarker for ALS

As noted above, ALS often involves the accumulation of SOD1 aggregates in ALS linked to *SOD1* (375) and *C9ORF72* mutations (376, 377). Aggregates have also been reported in sporadic ALS cases (378), but the latter finding is controversial (379). Recently, Leavens and colleagues developed an SOD1 RT-QulC assay that detects SOD1 seeds in *postmortem* spinal cord (sporadic and familial cases) and motor cortex (sporadic cases) tissue from ALS patients (380). These results are consistent with SOD1 seeding activity being a useful ALS biomarker, particularly if SOD1 seed detection in accessible patient biospecimens is more fully documented. Such a capability may be especially helpful in confirming sporadic ALS cases when genetic testing would not be indicative. On a more fundamental level, the presence of SOD1 seeding activity in diseased ALS neural tissues provides evidence of a SOD1 self-propagating activity that could be a molecular basis for the neuroanatomical spreading feature of ALS neuropathology.

TDP-43 seeds in ALS and FTLT

TDP-43 aggregates have been linked to diseases such as ALS, frontotemporal lobar degeneration (FTLD), and AD. TDP-43 normally regulates RNA metabolism and splicing in neurons and shuttles between the nucleus and cytoplasm (381). TDP-43 is usually highly α -helical; however, with pathological mutations and/or conditions, TDP-43 can also form β -sheets, and its nuclear import can be disrupted (382). Aberrant TDP-43 can recruit native TDP-43 in a prion-like fashion to form fibrils that can move from cell to cell, causing a TDP-43 loss of function and cellular dysfunction (383). Spreading pathology can also be induced by inoculation of extracts from diseased human brain tissue into animal models (384). TDP-43 RT-QulC SAAAs have been developed and applied to CSF from ALS and FTLT patients, yielding 94% diagnostic sensitivity and 85% specificity overall (385). Subsequent testing of olfactory mucosa samples from patients with frontotemporal dementia gave 82.4% sensitivity (349), while ALS patients yielded

46.9% sensitivity and 88.9% specificity (386). However, in our experience at least, TDP-43 RT-QuIC assays can be challenging to reproduce consistently. Thus, as with many types of SAA, the development of more robust and simpler TDP-43 RT-QuIC assays should be helpful for routine clinical applications.

INHIBITING AGGREGATE ACCUMULATION FOR THERAPEUTIC PURPOSES

Whether the accumulation of protein aggregates is the primary cause or a toxic consequence of a given proteinopathy, inhibiting further aggregation and spreading would likely be beneficial for the patient (Fig. 7). Prominent therapeutic strategies include the following: eliminating (38, 387) or reducing the concentration of the native monomers required for aggregate growth (388–396); stabilizing the native protein monomer against incorporation into amyloid (397, 398); and blocking the templating surface(s) on the aggregates (399).

PrP prion diseases

The early availability of prion disease-recapitulating animal models supported the discovery of the first prophylactic or therapeutic proteinopathy inhibitors in the 1970s (400–402). These inhibitors were initially chosen for their anti-viral activities, but subsequent studies in infected cell cultures (403, 404) and cell-free PrP conversion assays (405, 406) indicated anti-PrP^{Sc} mechanisms and enabled much broader searches for inhibitors of prion propagation (405, 407–411). The best of those compounds could then be tested *in vivo* (401, 402, 412, 413). Inhibitors have also been further tested, and in some cases newly identified, using mouse organotypic slice cultures (414, 415), human prion-infected brain organoids (416), and/or toxicity-based assays in mouse hippocampal neuronal cultures (417, 418).

These approaches have identified numerous inhibitors that markedly delay the onset of prion disease, especially if administered early before peripheral prion infections have reached the brain. However, most of these compounds have poor bioavailability to the CNS and are much less able to enhance survival once prion infections have reached the brain (401, 402, 405). Crossing the blood-brain barrier is a common obstacle in CNS pharmacology. Direct injections of the compounds into the brain improved the efficacy of some compounds later in the disease course in rodents (419–422) but would not be a preferred dosing modality in practice. One anti-prion compound with favorable brain penetration is the antibiotic doxycycline (423). Conflicting results were obtained from doxycycline treatments of patients with preexisting prion disease, but trials of long-term preventative treatments of subjects with genetic risk of fatal familial insomnia (a prion disease) are ongoing (423). Further studies have explored anti-prion vaccines (424–427), antibodies (428, 429), and aptamers (430–434) (Fig. 7). However, so far, no non-antibody drugs have proven to be effective enough in human trials to be approved as treatments for prion disease in humans. One promising inhibitory antibody, PRN100, was approved for more extensive human clinical trials in the UK, but to our knowledge, sufficient funding for such trials has failed to materialize.

Additional, and potentially complementary, approaches to prion disease therapeutics have been applications of short hairpin RNAs (396) and antisense oligonucleotides (ASOs) to reduce the CNS levels of PrP mRNA, and hence PrP^C protein, as a substrate for further conversion to PrP^{Sc} (Fig. 7). The ASO approach showed considerable promise in rodent models infected with multiple prion strains, even with a single ASO dose given near the onset of clinical prion disease (390, 435, 436). Subsequent “humanization” of the ASOs has enabled an ongoing phase 1/2a clinical trial in humans (PrProfile) of intrathecally administered ASO ION717 by Ionis Pharmaceuticals and multiple international clinical centers.

Another approach to reducing human PrP^C expression in a “humanized” mouse model by *in vivo* adeno-associated virus-mediated PRNP gene base editing has been described recently (388) (Fig. 7). Encouragingly, this strategy proved to be protective against challenges with a variety of sporadic and genetic human prion subtypes. Also

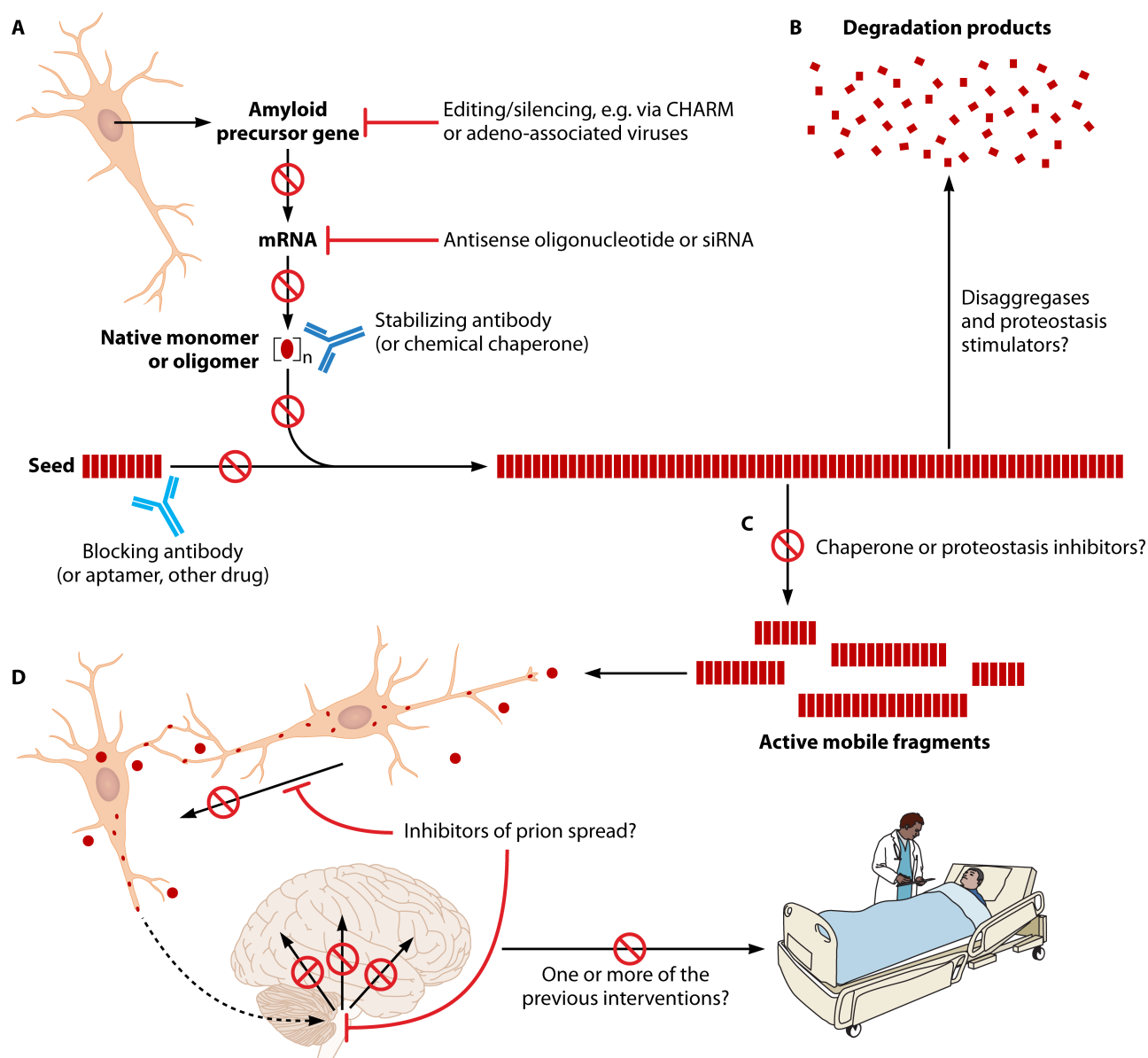


FIG 7 Therapeutic targets to reduce aggregate accumulation and spread. The indicated approaches have, or might be (?), pursued to (A) reduce aggregate/seed growth; (B) increase aggregate degradation; (C) prevent fragmentation into smaller and potentially more active, mobile, infectious, and/or toxic seeds; or (D) limit the spread of seeds within and/or between cells, tissues, and individuals.

promising is an approach to silencing of genes in the brain called Coupled Histone Tail for Autoinhibition Release of Methyltransferase (CHARM) (389). Although CHARM has been demonstrated using the prion protein as an example, it represents an approach that could be broadly applied to the silencing of other genes for amyloidogenic proteins or modulators thereof.

The delivery of mesenchymal stem cells and activation of the glymphatic system have also been demonstrated as a promising approach to prion disease therapy (437). However, so far, this approach has only been tested in mice against a single prion strain. Unfortunately, many previously tested therapies have shown encouraging anti-prion activities in rodents but have proven to be ineffective or difficult to translate into human therapeutics (401, 402, 438).

The availability of the high-resolution cryo-EM structure for the PrP fibrils of GSS patients with the F198S *Prnp* mutation (154) should support more rational

structure-based approaches to the screening and design of therapeutics for at least this one familial form of human prion disease. It remains important to follow a similar approach with sporadic CJD, but, as noted above, the much greater infectivity of sCJD prions has prevented cryo-EM analyses so far.

Other proteinopathies

Despite considerable effort over decades, there has been little success in establishing therapies that markedly affect the underlying disease process for most proteinopathies. However, a notable exception is the FDA approval of the small molecule tafamidis for the treatment of transthyretin type familial amyloid polyneuropathy (TTR-FAP) (398, 439). Tafamidis stabilizes the native transthyretin tetramer, preventing its incorporation into amyloid fibrils. Other prominent therapeutic approaches to limiting aggregate accumulation in proteinopathies are immunological (392, 440, 441) or RNA-based (e.g., antisense oligonucleotides) (391, 393, 394, 442) (<https://ionis.com/science-and-innovation/pipeline>). Multiple drugs derived from such efforts are in clinical trials, and some are already approved as drugs. As examples, we will highlight three antibodies that have been applied to AD in humans.

Both active and passive immunotherapies against A β accumulation have been explored (reviewed in reference 441). Vaccination approaches have been plagued by undesirable autoimmune responses in some recipients. Passively administered antibodies against A β fibrils (Aducanumab), protofilaments/oligomers (Lecanemab), and specific pyroglutamated plaque subspecies (Donanemab) have significantly reduced A β plaque burdens, other AD biomarkers, and some clinical indices of disease progression in clinical trials. However, these trials have also identified occasional side effects such as microhemorrhaging and edema that seem likely to be consequences of antibody binding to perivascular CAA deposits that are frequently found in AD patients. Nonetheless, each of these antibodies has been approved by the FDA for the treatment of mild cognitive impairment or mild dementia stages of AD. Even so, aducanumab was removed from further development by the manufacturer in 2024.

Improved biomarkers are guiding earlier therapeutic interventions and lessening dependence on clinical syndromes alone (441), but arguments are also being raised against the approval of amyloid-targeting therapies based primarily on biomarker data (443). A limitation of attacking only A β -based pathology in AD is that this may do little to impede the progression of concurrent tau-based pathology that is also a key element of AD. Fortunately, trials of therapeutics that simultaneously target the aggregation of both A β and tau are underway to address this issue (reviewed in references 441, 444).

Conclusions

A multitude of related approaches to treating other proteinopathies in animal models, and, to a lesser extent, human patients, have shown encouraging results that may be built upon to more effectively treat these diseases. Overall, the optimization of much-needed therapeutic approaches for proteinopathies will likely be greatly aided by the strides made towards earlier and more accurate diagnoses and improved understanding of diverse protein aggregate structures and the various pathologies that they cause. Indeed, structure and machine learning based approaches are beginning to allow high-throughput screening and identification of potent new small molecule inhibitors of fibril growth (399). Efforts to ameliorate proteinopathies are of great importance given the huge burdens that these diseases collectively impose on society, especially with an aging population and exposures to environmental risk factors (reviewed in references 117, 445).

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Byron Caughey completed his Ph.D. in biochemistry from the University of Wisconsin-Madison in 1985 and postdoctoral studies at Duke University in 1986. He has since been at the NIAID/NIH Rocky Mountain Laboratories, becoming a tenured senior investigator in 1994. His work has focused mostly on prion diseases, including structure, cell biology, transmissibility, disinfection, diagnostics, and therapeutics. Recently, he and his collaborators have determined the first high-resolution structures of several infectious mammalian prion strains. His lab has also developed ultrasensitive RT-QuIC prion seed amplification assays are widely used in prion detection and prion disease diagnosis, and adapted the assay platform to Parkinson's, Alzheimer's, and related human diseases. His collaborative findings with antisense oligonucleotides in animal models have underpinned ongoing human clinical trials of ASOs for prion disease. Dr. Caughey is a Fellow of the American Academy of Microbiology.

Efrosini Artikis is a postdoctoral researcher at the NIH/NIAID Rocky Mountain Laboratories in Hamilton, MT. She earned her B.Sc. in Chemistry from Florida State University and completed her Ph.D. in Biophysics from the University of Michigan in 2020. During her academic journey, she has cultivated a strong interest in structural biology and its application to neurodegenerative disease. Her current work focuses on understanding the role of protein structure and dynamics in mechanistic aspects of prion diseases.

Daniel Shoup received his Ph.D. in Biochemistry and Biophysics from Texas A&M University in 2016 for characterizing the intricacies of protein aggregate disassembly by molecular chaperones using a combination of novel single particle and ensemble fluorescence-based techniques. He is currently a Research Fellow at NIAID/NIH Rocky Mountain Laboratories. Using a combination of immunoassays and fluorescence-based techniques, his current research focuses on uncovering how interactions between cellular machinery and infectious prions alters both cellular physiology and the properties of infectious prions. His work not only provides a deeper insight into prion behavior in cellular environments but also provides a better understanding of the cellular mechanisms involved in other neurodegenerative diseases.

Christina D. Orrú is a Staff Scientist in the Prion Biochemistry section at Rocky Mountain Laboratories, National Institutes of Health (NIH). Over the past 15 years, Dr. Orru has played a key role in the development of the Real Time Quaking Conversion assay (RT-QuIC) for sensitive and specific amplification of disease associated prion protein. She has continued to contribute to the improvement and adaptation of this technology, which has given rise to more modern-day diagnostic seed amplification assays. More recently Dr. Orru aided in adapting this technology to pathological alpha-synuclein detection in diagnostically relevant samples as well as detection from surfaces contaminated with prion, alpha-synuclein, and Tau. Dr Orru has been principal & co-principal investigator on several international grants/collaborations leading, for example, an international ring trial validation for the CSF and OM RT-QuIC for sCJD intra vitam diagnosis.

Parvez Alam received his master's and Ph.D. in Biotechnology from Aligarh Muslim University, Aligarh, India. His Ph.D. research focused on investigating the inhibitory effects of small molecules on protein amyloid formation. He pursued postdoctoral training at Aarhus University, Denmark, where he worked on alpha-synuclein and in vitro blood-brain barrier model. Currently, he is a Visiting Fellow at the National Institutes of Health, United States. His research focuses on developing ultrasensitive diagnostic assays for Parkinson's and other neurodegenerative disorders. Additionally, he is exploring the structural details of brain-derived prion strains. Parvez has been working in the field of amyloids and neurodegenerative disorders for over a decade.

Sabiha Parveen completed her Ph.D. in Chemistry from Aligarh Muslim University, India, focusing on the synthesis of anti-cancer therapeutic molecules. She later joined the Department of Chemistry at Aligarh Muslim University as an Assistant Professor, where she taught various undergraduate level chemistry courses. Currently, she is working as a Visiting Fellow at the National Institutes of Health. Her research primarily focuses on developing RT-QuIC seed amplification assays for Alzheimer's and Parkinson's diseases.

Samantha King received her B.S. in chemistry at the University of Montana. After spending two years working in industry as an analytical chemist, she decided to pursue a research opportunity to establish and fortify her interest in biochemistry. Samantha worked in the Caughey lab as a post-baccalaureate fellow for one year and is now pursuing her PhD at Colorado State University through the Biochemistry and Molecular Biology department. She plans to continue researching neurodegenerative diseases in the future.

After receiving his M.Sc. in Virology, **Jakub Soukup** began his Ph.D. in prion research in 2016 at Charles University, Prague, and has continued in the field through his current postdoctoral work. Trained in both light and electron microscopy since his M.Sc., he has developed expertise in applying advanced imaging approaches to biological research. His work focuses on high-resolution visualization of prions in cells and extracellular vesicles, exploring how their spatial organization and interactions contribute to disease processes, alongside structural studies using cryo-EM.

Andrew G. Hughson received his MS in Genetics and Cell Biology at Washington State University studying bacterial transmembrane receptor signal transduction. He has worked for the past 25 years in Dr. Byron Caughey's research group at Rocky Mountain Labs as a technician to support research on prion and prion like neurodegenerative diseases.

Suzette Priola received her Ph.D. in microbiology and immunology in 1990 from the University of California, Los Angeles. In 1991, she joined the Rocky Mountain Laboratories where she is now a Senior Investigator. She is a former Chair of the FDA TSE Advisory Committee and is currently Deputy Chief of the Laboratory of Neurological Infections and Immunity and Chief of the TSE/Prion Molecular Biology Section. Her laboratory focuses on the molecular basis of disease in transmissible spongiform encephalopathy (TSE) or prion diseases. Using both in vitro and in vivo model systems, Dr. Priola's laboratory studies the role of PrPC and PrPSc in several aspects of prion pathogenesis, including: 1) the molecular pathogenesis of prion species barriers and strains; 2) the establishment of acute versus chronic prion infection; 2) the contribution of mitochondria to prion pathogenesis; and 4) the development of prion vaccines and therapeutics.