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Expanding Spectrum of Prion Diseases

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Running title: Expanding spectrum of prion diseases

SUMMARY POINTS

- Prions are host-encoded proteins that adopt alternative conformations, which are self-propagating and found in organisms ranging from yeast to humans.
- Some prions acquire alternative biological activities that correlate with alternative protein structures.
- Prions were initially discovered in studies of scrapie, a transmissible neurodegenerative disease of sheep and goats.
- Prions cause common neurodegenerative disorders including Alzheimer’s and Parkinson’s disease, as well as rare illnesses including Creutzfeldt-Jakob disease.
- Some prions have normal functions that alter metabolism while others modify immunological functions and still others modify long-term memory.

ABSTRACT

Prions were initially discovered in studies of scrapie, a transmissible neurodegenerative disease (ND) of sheep and goats thought to be caused by slow viruses. Once scrapie was transmitted to rodents, it was discovered that the scrapie pathogen resisted inactivation by procedures that modify nucleic acids. Eventually, this novel pathogen proved to be a protein of 209 amino acids, which is encoded by a chromosomal gene. Once the absence of a nucleic acid within the scrapie agent was established, the mechanism of infectivity posed a conundrum and eliminated a hypothetical virus. Subsequently, the infectious scrapie prion protein (PrP^{Sc}) enriched for β-sheet was found to be generated from the cellular prion protein (PrP^{C}) that is predominantly α-helical. The post-translational process that features in nascent prion formation involves a templated conformational change in PrP^{C} that results in an infectious copy of PrP^{Sc}. Thus, prions are proteins that adopt alternative conformations, which are self-propagating and found in organisms ranging from yeast to humans. Prions have been found in both Alzheimer's (AD) and Parkinson's (PD) diseases. Mutations in APP and α-synuclein genes have been shown to cause familial AD (fAD) and PD. Recently, AD was found to be a double prion disorder: both Aβ and tau prions feature in this ND. Increasing evidence argues for α-synuclein prions as the cause of PD, multiple system atrophy, and Lewy body dementia.

INTRODUCTION

The identification and description of prions has revolutionized the way scientists think about neurodegenerative diseases (NDs). The idea of an infectious agent lacking a nucleic acid was initially the subject of intense debate. Such a notion challenged the “central dogma of molecular biology” that had been adopted by the scientific community following the discovery of the double helical structure of DNA. It seemed inconceivable that an infectious agent could replicate or exist as distinct “strains” without a nucleic acid genome. The extreme resistance of scrapie to methods normally used to inactivate viruses, its failure to induce an immune response following infection, and its observed long incubation periods indicated a novel infectious agent was at play and led to the idea that an infectious agent could be composed solely of protein (1-3). The protein-only hypothesis is now widely accepted and is believed to feature in a wide range of other NDs.

Over the last two decades, as NDs have become better characterized, a growing body of evidence has shown that prions are the cause of many of these illnesses (4). The proteins implicated in these disorders (such as tau in Alzheimer’s disease [AD] and α-synuclein in Parkinson’s disease [PD]) share remarkable similarity to the first prion (PrP) described in scrapie, kuru, and Creutzfeldt-Jakob disease (CJD). The studies summarized in this review provide a historical perspective on the identification of various prions and

shed light on the characteristics that have led to the classification of the common NDs such as AD and PD as prion disorders.

HISTORICAL PERSPECTIVE ON PRIONS AND PRION BIOLOGY

For more than two centuries, a disease of sheep and goats had been described that caused animals to itch incessantly, stop eating, become emaciated, and die. This disease continues to be called “scrapie.” If shepherds identified one of their animals to have the condition, they would isolate it from the healthy animals, as the disease was known to be infectious and cause serious harm to the flock. During the 1930s and 1940s, veterinarians in England, France, and Germany began experiments to better understand and characterize this illness. Through transmission studies, researchers discovered that the incubation period for the disease was extraordinarily long—much longer than for other infectious agents known at the time. For this reason, the term “slow virus” was coined by Björn Sigurdsson in 1954 to describe the clinicopathologic features of the disease. Given its unusual properties, the infectious agent was bestowed with many other names including “unconventional virus,” “latent virus,” and “atypical virus.”

In the middle of the twentieth century, the double-helical structure of DNA was established, and genes were identified as linear strands of DNA (5-9). Subsequent experiments established the colinearilty between a gene and its corresponding protein by elucidating the transfer of biological information

from gene to messenger RNA (mRNA) to protein (10, 11). This sequence of steps became known as the “central dogma of molecular biology,” and the information encoded by nucleic acid was shown to be used by all biological entities, including infectious pathogens such as bacteria and viruses.

With the central dogma in place and knowledge of viral biology becoming better characterized, multiple studies showed that viruses contain DNA or RNA that enciphers the information needed to reproduce new virions. These findings led researchers to explore the biochemical features of the scrapie agent and search for a nucleic acid core. Early studies by Tikvah Alper and her colleagues demonstrated that the scrapie agent was extremely resistant to inactivation by ultraviolet light and ionizing radiation (12, 13). Additionally, using ionizing radiation, Alper and colleagues determined the size of the infectious pathogen to be roughly 90% smaller than the smallest known virus particles (14). Based on these data, Alper and colleagues surmised that the scrapie agent was devoid of nucleic acid (12). Due to the central dogma and the role of nucleic acid in the replication of all organisms, the hypothesis that the scrapie agent is devoid of DNA or RNA was generally rejected. The proposal of a pathogen free of nucleic acid initiated what would become a contentious and widely debated topic: ultimately resulting in the recognition of an unprecedented infectious pathogen.

CJD is a rapidly progressive human ND that was first described in the early twentieth century. Around 85% of CJD cases are sporadic with no

known origin, whereas the remaining ~15% of cases are primarily inherited with an autosomal-dominant pattern of expression and variable penetrance. The familial forms of CJD are now known to be due to mutations in the *PRNP* gene that encodes the prion protein, as are both Gerstmann-Sträussler-Scheinker (GSS) disease and fatal familial insomnia (FFI). As these illnesses progress, a range of neurological signs are observed, including a rapidly progressive dementia, myoclonus, visual or cerebellar signs, weakness of extremities, and, in some cases, coma. Prior to understanding the pathogenic role of the prion protein (PrP), several neuropathological characteristics were essential in the diagnosis of CJD, including neuronal loss, spongiform degeneration, and reactive gliosis. Interestingly, these same neuropathological observations had been described to occur in the brains of scrapie-infected animals, though the hypothesis of similar etiologies did not arise until the description and study of another similar neurological disorder.

While scientists were trying to better understand the infectious agent responsible for scrapie, a neurological disease among the Fore tribe in Papua New Guinea called kuru was described. This condition primarily affected women and presented with features similar to those observed in animals with scrapie and individuals with CJD. The transmission of kuru was later attributed to ritualistic cannibalism of deceased family members, including the brain, practiced within the tribe; a ritual that only the women and children performed. Although the symptoms associated with CJD vary, the uniform clinical presentation of kuru remains fascinating (15, 16). The

affected individuals presented with headache and joint pain, which rapidly progressed to difficulty walking in 6 to 12 weeks and death in 12 months. Like scrapie, the brains of kuru patients revealed neuronal loss, spongiosis, and reactive gliosis, which led William Hadlow to question whether a similar etiology might be responsible for these two spongiform encephalopathies (17). Another similarity between scrapie and kuru was their extremely long asymptomatic incubation periods. Transmission studies of scrapie had revealed incubation periods as long as 30 months, while epidemiological studies of kuru had suggested incubation periods of decades in the Fore people. Remarkably, approximately 2,700 cases of kuru were documented over the course of the epidemic, which was initially described in the late 1950s. After the cause of the outbreak was identified and ritualistic cannibalism stopped, there have been no known Fore people born in the South Fore region of Papua New Guinea after 1959 who developed kuru.

As noted above, Hadlow hypothesized that an infectious agent similar to that causing scrapie might be similar to the one responsible for kuru (17). To address this question, Carleton Gajdusek, Michael Alpers, and Clarence Gibbs inoculated homogenates of kuru brains into the brains of chimpanzees and monkeys (18). In less than three years, the animals developed progressive neurological dysfunction with neuropathological features resembling those observed in kuru patients. Noting the pathological similarities with CJD, Gajdusek and colleagues intracerebrally injected chimpanzees with brain homogenates from CJD patients. These animals

developed neurological signs 13 months after inoculation and were euthanized 3 months later due to progressive neurological dysfunction. Autopsies revealed spongiform encephalopathy in the brains of these non-human primates (19). Together, their studies revealed that both kuru and CJD were human transmissible disorders resulting in brain dysfunction, progressive neurological dysfunction, and severe spongiform degeneration.

The unprecedented pathogens causing scrapie, kuru, and CJD became increasingly mysterious as the studies of these transmissible NDs became more intensely investigated. These mysterious pathogens were initially anointed as “slow viruses” and later “unconventional viruses” (20, 21). Such transmissible diseases had much longer incubation periods than generally observed for viruses and exhibited extreme resistance to inactivation by formalin and heat (22, 23). Also perplexing was the lack of a detectable immune response in scrapie-infected animals, a common feature for foreign pathogens (24). Alper’s assertion that the infectious agent causing scrapie might lack a nucleic acid genome generated several hypotheses offering novel mechanisms for the production of unprecedented proteins (1, 2).

The identification of the protein responsible for scrapie infectivity led to the discovery of prions. To accomplish this, the infectious agent first had to be purified. Although mice had commonly been the experimental model used in scrapie transmission studies, transmission into Syrian golden hamsters significantly shortened incubation periods and led to higher titers of the

infectious agent in their brains when compared to mice (25). This switch in experimental model allowed the production of a large number of scrapie-infected animals in a relatively short time frame and was essential in the subsequent steps used to purify the infectious scrapie agent (3). By treating these partially purified hamster brain fractions with chemicals that modify or hydrolyze proteins, yet have no effect on nucleic acid, scrapie infectivity was found to be diminished (26). Based on these observations, the term “prion” was introduced to distinguish this novel infectious agent from viruses or other infectious pathogens known to depend on nucleic acids for replication. A prion was originally defined as “a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids” (3). As more has been learned about prions, a refined definition has been proposed: “Prions are composed of host-encoded proteins that adopt alternative conformations, which are self-propagating” (27).

Subsequent studies revealed that highly purified fractions prepared from the brains of scrapie-infected hamsters retained significant infectivity in animals, and the protein was later identified and named PrP for “prion protein” (3, 28). Interestingly, the mRNA that encodes PrP was also identified in uninfected hamsters; however, the PrP produced in uninfected animals was found to be susceptible to limited proteolysis, denoted PrP^{C}, whereas the disease-specific form was protease-resistant, denoted PrP^{Sc} (29). It became clear when the amino acid sequences of PrP^{C} and PrP^{Sc} were found to be identical that the prion protein was encoded by a cellular gene (30). The

identification of PrP led to the production of mouse monoclonal antibodies specific to PrP epitopes and allowed the development of immunohistochemistry, Western blots, and other immunological tests, which greatly enhanced the study and diagnosis of prion diseases caused by PrP^{Sc} (31-36).

The identification of a novel infectious agent lacking nucleic acid led predictably to the question of how a protein-only agent was capable of replicating itself. To address this question, studies began to explore the differences between PrP^{C} and PrP^{Sc}. Using spectroscopy, PrP^{C} was revealed to be enriched for α-helical structure, whereas the PrP^{Sc} secondary structure was found to be rich in β-sheet (37-41). These findings led to the proposal that a conformational change of the PrP^{C} protein resulted in formation of PrP^{Sc}. In animal studies, replication of PrP^{Sc} was found to depend on the interactions between PrP^{C} and PrP^{Sc}. A transgenic (Tg) line of mice was produced that expressed both mouse and Syrian hamster PrP^{C}, whose amino acid sequences differ by ~10%. When these mice were inoculated with mouse PrP^{Sc} prions, only mouse PrP^{C} was capable of being converted to PrP^{Sc}, whereas when inoculated with hamster PrP^{Sc} prions, only hamster PrP^{C} was converted into prions (42). This study revealed the importance of homotypic interactions between PrP^{C} and PrP^{Sc} for the replication of nascent prions. Based on these and other findings, prion replication appeared to occur via the binding of PrP^{Sc} to PrP^{C} that was accompanied by conversion of PrP^{C} to PrP^{Sc}, thereby propagating the infection.

Though the majority of transmission studies of PrP induce disease by intracerebral inoculation of PrP^{Sc}, natural transmission of prion diseases is believed to occur via peripheral routes. Perhaps most significant and common is the oral route (43, 44), though other possible portals exist including the nasal route in PrP prion diseases in animals, such as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) in deer and elk (45-47). Initial studies investigating the transport of prions examined the spatial and temporal accumulation of PrP^{Sc} following intraocular injection (48). Over time, spongiosis appeared in only those neuronal structures, like the superior colliculus, whose axons project to the inoculated eye and was absent in structures associated with the uninoculated eye. Subsequent studies of inoculation into the sciatic nerve of hamsters also demonstrated the accumulation of PrP^{Sc} in only those structures whose axons project down the inoculated nerve, implicating retrograde transport as a means of propagation. These findings revealed the ability of PrP^{Sc} to be transported along defined neuroanatomical pathways in both the central and peripheral nervous systems and provided a mechanism to explain the accumulation of prions throughout the nervous system (49).

AMYLOID PLAQUES

As mentioned above, amyloid plaques are a neuropathological feature observed in a subset of prion diseases caused by PrP. Over a century ago, Rudolf Virchow introduced the term “amyloid” (50). Subsequent studies with vital dyes demonstrated amyloids in neuropathological structures that have

characteristics such as unbranched fibrils, a crossed β-sheet structure, a birefringence in polarized light after staining with Congo red, and a shift in fluorescence emission spectra after staining with thioflavin T (51-55). Amyloid plaques are known to accumulate in many NDs—for example, it has been known for decades that both senile plaques in AD and plaques observed in kuru exhibit a green birefringence upon staining with Congo red (56, 57). Interestingly, when the purified fractions of the scrapie agent were stained with Congo red, they also exhibited green birefringence demonstrating their amyloid structures (37). Subsequent immunological studies demonstrated that the amyloid fibrils that accumulate in kuru, CJD, and scrapie are composed of PrP^{Sc}, whereas those that accumulate in AD are composed of the amyloid-β (Aβ) protein (37, 58). These studies and others opened a new field of neuropathological investigation; these findings raised the question whether other amyloidogenic proteins are also transmissible.

Aβ PRIONS

AD is the most common ND and is characterized clinically by a gradual decline in cognitive function. Pathologically, AD is defined by the accumulation of Aβ-amyloid plaques and neurofibrillary tangles (NFTs), the presence of neuropil threads, as well as neuronal and synaptic loss (59). Aβ-amyloid fibrils are also found in cerebral amyloid angiopathy (CAA) where it accumulates in and around small blood vessels in the brain producing micro-hemorrhages in the absence of substantial tau accumulation (60, 61). NFTs composed of the tau protein are an additional hallmark of AD; the tau protein

is responsible for a host of other diseases, known as tauopathies, and are discussed below.

The Aβ peptide is derived through the sequential endoproteolytic cleavage of the amyloid precursor protein (APP) (62, 63). Though primarily sporadic, a small number of familial AD cases have provided insight into the underlying causes of the disease. Aside from mutations in the *APP* gene itself, many mutations are in genes whose proteins are responsible for the processing of APP, yielding a range of proteolytic Aβ fragments, with the Aβ40 and Aβ42 peptide isoforms being the predominant species in AD (64-66). Studies have demonstrated that Aβ42 is the most aggregation-prone of these peptides and the major species that accumulates in Aβ plaques.

Similar to the studies demonstrating the transmissibility of kuru and CJD, Carleton Gajdusek and Joseph Gibbs also explored the transmissibility of AD (67, 68). Of the 52 AD brain homogenates they injected into nonhuman primates, animals injected with two cases of fAD developed a disease that was indistinguishable from CJD (67). However, these results could not be replicated, and some investigators believe the symptomatic animals may have been contaminated with PrP prions. Similar experiments were later performed in marmosets. After almost 10 years of incubation, the majority of the infected animals were found to have accumulated amyloid plaques that stained positive with anti-Aβ antibodies (69, 70). Though these investigations provided convincing evidence for the transmissibility of Aβ, they also

highlighted the difficulty in performing such studies given the long incubation periods and high cost of housing nonhuman primates.

In search of a rodent model for studying the transmissibility of AD, Mathias Jucker, Matthias Staufenbiel, and Lary Walker created transgenic mice that expressed a mutant form of APP (71). Both intracerebral and intraperitoneal injections of AD homogenates into these mice produced an accelerated deposition of amyloid plaques (72, 73). Additional transmission studies used bigenic mice co-expressing mutant APP under control of the *Thy1.2* promoter and luciferase under control of the glial fibrillary acidic protein (GFAP) promoter. Intracerebral injections of synthetic mutant Aβ fibrils induced Aβ plaque deposition in these bigenic mice, and depending on the particular APP mutation, the site of proteolytic cleavage was modified and resulted in amyloid fibrils with different characteristics (74, 75).

Though rare, iatrogenic cases of PrP prion diseases (iCJD) have been reported that originated through medical procedures using cadaveric human growth hormone, dura mater grafts, and corneal transplants from patients who had accumulated PrP^{Sc} in these tissues (76, 77). Recent studies by John Collinge and colleagues re-examined these cases to determine whether, in addition to the transmission of PrP^{Sc}, there may have been transmission of Aβ prions. The iCJD cases originating from human growth hormone contaminated with PrP^{Sc} resulted in the death of patients at a relatively young age—well before the onset of sporadic AD or the point at which one

would expect to find the accumulation of Aβ plaques in the brain. In four of the eight brains Collinge examined, there was severe gray matter loss and vascular Aβ plaque deposition (78). The Collinge group later characterized the growth hormone that caused the supposed iCJD cases and detected Aβ prions (79). Furthermore, injections of these fractions into transgenic mice expressing mutant human APP induced the accelerated accumulation of Aβ plaques and CAA. This set of experiments strongly supports the transmissibility of Aβ prions and, as Collinge concludes, sheds light on the risk of iatrogenic AD and CAA (78).

TAU PRIONS

As described above, although the accumulation of tau in the form of NFTs, neuropil threads, and dystrophic neurites is a hallmark of AD, accumulation of the protein is also observed in a spectrum of other neurodegenerative diseases, which are collectively referred to as tauopathies. Tau is a microtubule-associated protein expressed from the *MAPT* gene that undergoes alternative RNA splicing leading to the production of six different major tau isoforms (80-83). Similar to prion diseases caused by PrP and Aβ, clinically relevant mutations in tau have provided researchers the tools to better understand the mechanisms involved in tau pathogenesis (84, 85). Many of these mutations are found in an area of the tau protein containing microtubule-binding repeat domains (RD), a region of the protein critical for its binding to microtubules. Depending on splicing of its mRNA, the tau protein can contain either three or four of these domains. Mutations within

the RD lead to an impairment in the protein's ability to bind to microtubules, and for some mutations, can potentiate tau aggregation (86, 87).

Similar to the transport of PrP prions, the deposition of tau prions over the course of AD has been hypothesized to spread in a predictable pattern throughout the brain along known neuroanatomical pathways (88). Recent findings from longitudinal studies using tau-PET tracers support this theory by revealing the accumulation of pathological tau in brain regions that are neuroanatomically connected (89-91). The localization of tau prions in the brain and the clinical features of the disease can vary dramatically among different tauopathies. This finding led researchers to speculate that tau was capable of adopting multiple distinct strains, or misfolded conformations, that could lead to these separate diseases; a trait known to account for distinct PrP prion diseases (92, 93). In studies using cultured cells expressing a truncated version of tau fused to a fluorescent protein, Marc Diamond revealed that when added to the cells, the tau prions isolated from different tauopathies induced morphologically distinct tau aggregates (94, 95). Subsequent studies in similar cell lines demonstrated that tau prion propagation required matching isoforms between the prion and its substrate (96). Cryo-electron microscopy (cryo-EM) studies have now enabled investigators including Michel Goedert, Sjors Scheres, Bernardino Ghetti, and Anthony Fitzpatrick to isolate tau prions from the brains of postmortem patients and determine their structures at the atomic level. Such cryo-EM studies have shown that distinct structures of tau are responsible for

diseases such as AD (97, 98), chronic traumatic encephalopathy (CTE) (99), corticobasal degeneration (CBD) (100), and Pick's disease (98). These findings have had a profound impact on the understanding of strains not only for tau prions but also for prions implicated in all other NDs.

Michel Goedert, Markus Tolnay, and colleagues were the first to investigate the transmission of tau prions in experimental mouse models. They found that tau prions extracted from the brains of a mouse line expressing P301S mutant tau were able to induce the aggregation and spread of wild-type human tau following injection into a separate line of mice overexpressing tau (101). In addition, their group and others injected synthetic tau fibrils, polymerized in the presence of heparin, into the brains of the P301S mutant mouse line to reveal a similar induction and spread of tau pathology (102, 103). Tau prions isolated from the brains of humans with several tauopathies were also capable of inducing pathology in mice overexpressing wild-type human tau; furthermore, the morphology of the induced tau aggregates appeared indistinguishable from that of the aggregates of the injected tau prions (102).

TWO PRIONS FEATURE IN ALZHEIMER'S DISEASE

The previous sections describe the contributions of Aβ and tau prions in several NDs. AD, however, is unique among these diseases in that the majority of patients accumulate both Aβ and tau inclusions. Many hypotheses exist to explain the pathogenesis of AD in regard to the importance, or lack

thereof, of these two proteins and how they may cooperate to cause disease; the most well-known being the amyloid cascade hypothesis, which posits that changes in Aβ initiate a cascade of events, including pathological changes to tau (104). To address this, multiple *in vivo* studies in transgenic mice have reported that Aβ is capable of inducing and enhancing the accumulation of tau aggregation (105-108). In a recent study from Atsushi Aoyagi, Carlo Condello, and colleagues, the prion activities of Aβ and tau isolated from the brains of sporadic and inherited AD patients were investigated using a highly sensitive cellular assay (109). This assay allowed for the distinction between biologically active Aβ and tau prions and inactive and inert forms of the proteins. The authors found that the prion activity in Aβ and tau decreased with age despite the increase in pathological hallmarks like NFTs and phosphorylated tau. In addition to elucidating a distinction between biologically active and inactive forms of Aβ and tau prions, this study revealed that AD is a double prion disorder.

α-SYNUCLEIN PRIONS

Genetic linkage studies into the cause of inherited forms of PD identified the A53T mutation in the gene encoding α-synuclein, *SNCA* (110). Antibodies to the protein were produced, and it was soon realized that the hallmark pathology observed in both sporadic and inherited forms of PD, called Lewy bodies, contained α-synuclein (111). Shortly thereafter, α-synuclein was identified in the glial cytoplasmic inclusions (GCIs) found in the brains of patients with multiple system atrophy (MSA) (112, 113). Studies using

recombinant α-synuclein, which is a natively unfolded protein and is easily assembled into fibrils when incubated with shaking at high concentrations, showed that the ultrastructural and biochemical properties of the fibrils derived from PD and MSA patients shared many features (114).

Benoit Giasson and colleagues created TgM83 mice overexpressing the A53T point mutation in α-synuclein that causes inherited PD. Tg(*SNCA**A53T)M83$^{+/-}$ mice, which are hemizygous for the α-synuclein transgene, do not exhibit signs of neurological dysfunction before 18 months of age, while mice homozygous for the transgene (TgM83$^{+/+}$) begin to develop a paralytic phenotype at ~12 months of age (115). Using the TgM83 mice, Mougenot and colleagues injected brain homogenates from ill TgM83$^{+/+}$ mice into healthy TgM83$^{+/-}$ mice. About 180 days after inoculation, the TgM83$^{+/-}$ mice began to display signs of neurological dysfunction and widespread deposits of α-synuclein (116). Subsequent studies by others reported similar results (117). The next year, we reported that two human cases of MSA could be transmitted to the TgM83$^{+/-}$ mouse (118). An additional set of 14 MSA cases were soon thereafter injected into more TgM83$^{+/-}$ mice to ensure the reproducibility of this remarkable finding. All 14 of the Tg mouse brains were found to induce disease in a similar time frame as in the initial experiments, whereas none of the control or PD patient inocula caused neurological dysfunction (119). Injections of MSA homogenates also led to the widespread accumulation of phosphorylated α-synuclein in the brains of symptomatic TgM83$^{+/-}$ mice. In parallel studies, α-

synuclein prions from MSA showed the ability to induce α-synuclein aggregation in a stable cell line expressing a full-length version of human synuclein containing the A53T mutation and fused to yellow fluorescent protein (α-syn140*A53T–YFP) (119, 120). Notably, injections of α-synuclein fibrils via peripheral routes were capable of inducing α-synuclein neuropathology and disease in this mouse line (121, 122).

As discussed earlier, the unusual resistance of PrP prions to treatments that normally inactivate viruses is a defining feature of PrP prions. To better understand α-synuclein prions, several studies were undertaken to explore how similar inactivation methods would affect MSA transmissibility (123). MSA brains that had been stored in formalin, commonly used to inactivate viruses for vaccine production, were inoculated into the brains of TgM83$^{+/-}$ mice. The data revealed that treatment with formalin, for even as long as 244 months in one case, had no effect on the transmissibility of MSA α-synuclein prions. The stability of α-synuclein prions was also investigated by extraction in increasing concentrations of the detergent sarkosyl (123). In sarkosyl extractions of 10% or higher, α-synuclein was detected more abundantly as a monomer when analyzed by Western blot analysis, indicating the solubilization of the protein. This finding correlated well with the infectivity of these fractions, as extractions in concentrations of 10% or higher were also found to eliminate the infectivity in our α-syn140*A53T–YFP cultured cell model. From these studies, we concluded that MSA is a transmissible human ND caused by α-synuclein prions.

IN QUEST OF EFFECTIVE THERAPUETICS FOR PRION DISEASES

As described here, an expanding body of evidence argues that prions cause an array of NDs. Each of these NDs is caused by a different protein that can fold into a prion and is capable of propagating and causing a particular ND. As consensus in the field grows, more investigators are leveraging the principles of prion biology in discovering effective therapeutics. In this context, a brief reflection on the state of therapeutic discovery for PrP diseases may prove instructive. Building on a modest survival benefit of the polyanion, antiviral HPA-23 in rodent models of scrapie transmission, polyanions have been investigated in scrapie models for over 30 years (124-126). Among these, pentosan polysulfate (PPS) has emerged as one of the less toxic polysulfonated polysaccharides that inhibit PrP^{Sc} formation in cell culture and delays the onset of disease transmitted to mice and hamsters (127-129). Size and charge of the polyanion regulated the efficient diffusion of these molecules across the blood-brain barrier, requiring sustained intraventricular cannulation to achieve meaningful concentrations of PPS in brain parenchyma. Such studies involved complications from the surgical procedures used to insert the canula (130) while neither this observational study nor a controlled trial established significant benefit of PPS therapy in humans (131).

Low permeability and transporter efflux have complicated advancement of PrP^{Sc} inhibitors, including polyanions (132), antibodies (133), and some small molecules including quinacrine (134, 135). Unfortunately,

two clinical trials with quinacrine failed to extend the lives of CJD patients (136, 137). In contrast, high-throughput screens in cell-based assays and iterative optimization campaigns executed with consideration of physical properties have produced an expanding set of novel, drug-like small molecules including anle138b, IND24, and compound B (138-142). These compounds and related analogs readily traverse the blood-brain barrier when administered orally and extend the lives of mice inoculated with scrapie or CWD prions (143). Unfortunately, compounds in the IND series have proven ineffective against human CJD prions (139), and the lack of a cell culture assay for CJD prions has prevented high-throughput screens and iterative lead compound improvements. Meanwhile, a first-in-human safety trial of anle138b recently began enrollment albeit in the reported interest of supporting further clinical trials for MSA (144).

To date, no biologic or small molecule agent has proven effective in altering the course of a human ND. In the case of PrP^{Sc} diseases, even quantifiable changes in disease-related pharmacodynamic markers in response to treatment have remained elusive. Clinical studies of amyloid-targeting therapies for AD, however, have demonstrated quantifiable changes in the concentration of monomers in the cerebrospinal fluid and/or amyloid accumulation in the brain, but without reduction of cognitive decline. In fact, verubecestat, a brain-penetrant small molecule BACE1 inhibitor, may have accelerated decline in patients despite its robust effects on biomarkers (145), possibly ending one of the most ardently pursued

investigational avenues into a therapy for AD. Similarly, studies of seven different Aβ antibodies have failed to produce compelling benefits to patients, even after researchers developed strategies to overcome treatment-related edemas (146).

As described throughout this review, research into the underlying causes of the familial forms of many NDs led to the identification and understanding that these mutant proteins, and their wild-type counterparts, are capable of acquiring a toxic gain-of-function. Although conversion of the normal functional protein might be expected to contribute to a toxic loss-of-function phenotype, *in vivo* data do not presently support this hypothesis: knockout mouse models lacking the endogenous cellular form of relevant proteins generally remain free of overt neurological signs (147-149). As such, the overwhelming majority of anti-prion research efforts have remained focused on inhibition and elimination of the toxic prion species, rather than addressing the deficits caused by the loss-of-function of the depleted cellular protein.

CONCLUSION

The studies discussed here have led the scientific community to accept the protein-only hypothesis and have solidified the understanding that prions are proteins that acquire self-propagating alternative conformations, some of which cause neurodegeneration. Originally limited to the PrP protein in

scrapie, kuru, and CJD, the mechanism of prion pathogenesis can now be attributed to other proteins, including Aβ, tau, and α-synuclein. In mammals, more than a dozen prions have been identified, and a similar number of fungal prions have been found. There are even some prions that are beneficial and perform cellular functions (150, 151). The list of disease-causing prions continues to grow as our understanding of NDs expands and our technological tools become more advanced. Putative therapeutics continue to undergo clinical trials for these diseases, but, unfortunately, there is still not a single therapy that prevents formation of nascent prions. Once a therapy is developed that successfully inhibits prion disease progression, it might make ND therapeutics discovery more rapid.

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CONFLICT OF INTEREST

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