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Implications of prion adaptation and evolution paradigm for human neurodegenerative diseases

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Abbreviations: AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; *APOE,* apolipoprotein E gene; CDI, conformation-dependent immunoassay; MM, MV, VV common polymorphisms in codon 129 of *PRNP* gene coding for methionine (M) or valine (V); MM1 sCJD, sporadic Creutzfeldt-Jakob disease homozygous for methionine (M) in polymorphic codon 129 of *PRNP* gene and 21 kDa fragment (Type 1) of unglycosylated $rPrP^{Sc}$ on WBs; MM2 sCJD, sporadic Creutzfeldt-Jakob disease homozygous for methionine (M) in polymorphic codon 129 of *PRNP* gene and 19 kDa fragment (Type 2) of unglycosylated $rPrP^{Sc}$ on WBs; PD, Parkinson disease*; PRNP,* prion protein gene; PrP, prion protein; PrP^{C}, normal or cellular prion protein; PrP^{Sc}, misfolded pathogenic prion protein; $rPrP^{Sc}$, protease-resistant conformers of pathogenic prion protein (PrP 27-30); sPMCA, serial Protein Misfolding Cyclic Amplification; $sPrP^{Sc}$, protease-sensitive conformers of pathogenic prion protein

There is a growing body of evidence indicating that number of human neurodegenerative diseases, including Alzheimer disease, Parkinson disease, fronto-temporal dementias, and amyotrophic lateral sclerosis, propagate in the brain via prion-like intercellular induction of protein misfolding. Prions cause lethal neurodegenerative diseases in humans, the most prevalent being sporadic Creutzfeldt-Jakob disease (sCJD); they self-replicate and spread by converting the cellular form of prion protein (PrP^{C}) to a misfolded pathogenic conformer (PrP^{Sc}). The extensive phenotypic heterogeneity of human prion diseases is determined by polymorphisms in the prion protein gene, and by prion strain-specific conformation of PrP^{Sc}. Remarkably, even though informative nucleic acid is absent, prions may undergo rapid adaptation and evolution in cloned cells and upon crossing the species barrier. In the course of our investigation of this process, we isolated distinct populations of PrP^{Sc} particles that frequently co-exist in sCJD. The human prion particles replicate independently and undergo competitive selection of those with lower initial conformational stability. Exposed to mutant substrate, the winning PrP^{Sc} conformers are subject to further evolution by natural selection of the subpopulation with the highest replication rate due to the lowest stability. Thus, the evolution and adaptation of human prions is enabled by a dynamic collection of distinct populations of particles, whose evolution is governed by the selection of progressively less stable, faster replicating PrP^{Sc} conformers. This fundamental biological mechanism may explain the drug resistance that some prions gained after exposure to compounds targeting PrP^{Sc}. Whether the phenotypic heterogeneity of other neurodegenerative diseases caused by protein misfolding is determined by the spectrum of misfolded conformers (strains) remains to be established. However, the prospect that these conformers may evolve and adapt by a prion-like mechanism calls for the reevaluation of therapeutic strategies that target aggregates of misfolded proteins, and argues for new therapeutic approaches that will focus on prior pathogenetic steps.

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Broad Range of Human Prions

The human prion diseases are unique in that a single pathologic process may present as a sporadic, genetic, or infectious disease. The most common form of human prion disease, originally described as transmissible spongiform encephalopathy (TSEs), is sporadic Creutzfeldt-Jakob disease (sCJD), accounting for ~85% of cases. First shown to be transmissible to non-human primates in 1967,[1,2] its origin and pathogenesis remain enigmatic. Now, the generally accepted model posits that the infectious pathogen responsible for TSEs is a misfolded protein, designated PrP^{Sc}.[3] This protein is a pathogenic conformational isoform of the normal cellular prion protein,[4-8] PrP^{C}, that is encoded by the host's *PRNP* gene and expressed at different levels in all mammalian cells.[9] The discovery that misfolded proteins may be infectious represents a new biological paradigm, and although originally deemed heretical, this protein-only model is now supported by a wealth of biochemical, genetic, and animal studies,[5,6,10-13] including recent success in generating infectious prions in vitro.[14-20] The PrP^{Sc} conformer is believed to self-replicate by binding to monomers of PrP^{C} that have predominantly α-helical secondary structure; this causes the protein to convert to the oligomeric, amyloid-forming

PrP^{Sc} state with predominantly β sheet secondary structure. However, the exact structural intermediate steps remain poorly understood.[21,22] Compared with PrP^{C}, the β sheet secondary structure of brain-derived PrP^{Sc} increases from ~3% to ~45%, and this conformational transition leads to its insolubility in non-denaturing detergents and increased resistance to proteolysis.[7,8] Consequently, the half-life of the protein increases from physiological ~18 h for PrP^{C} to 36 h for PrP^{Sc},[23] leading to the progressive accumulation of PrP^{Sc} in the infected brain. The lasting mystery surrounding replication of the PrP^{Sc} conformer is one of the fundamental problems of biology that remains to be solved. Two fundamental characteristics of human prion diseases are (1) the age dependency of their occurrence and (2) the extraordinary heterogeneity of the clinico-pathological phenotype.[24,25]

Human Prion Strains and Prion Coexistence

The human prion diseases are probably the most phenotypically diverse neurodegenerative disorders. The broad phenotypic heterogeneity of sporadic Creutzfeldt-Jakob disease (sCJD)[24] is currently understood as a complex interplay between polymorphisms in codon 129 of the *PRNP* gene translated to either methionine (M) or valine (V), and different PrP^{Sc} conformers coding for distinct strains of prions.[24,26] On serial passages in the same host, distinct prion strains propagate and replicate unique phenotypes of the diseases with remarkable reproducibility, including incubation time, symptoms, distribution of pathology in the brain, and major molecular characteristics of PrP^{Sc}. Experiments in transgenic mice expressing PrP^{C} of different species led to the conclusion that species of prion is dictated by the amino acid sequence of the host's prion protein, and the mismatch between amino acid sequences of infecting prion and host PrP^{C} is responsible for the so-called species barrier, which may restrict prion replication and cause a change in prion characteristics.[26] Variations within the same species of prion, which cause remarkably different disease phenotypes in the same host, are referred to as prion strains.[26,27] The existence of distinct prion strains that can be passaged indefinitely was long offered as an argument for the existence of a prion-specific genome and has divided the scientific community. Subsequently, rapid progress in the past decade has produced multiple lines of evidence convincingly demonstrating that prion strain characteristics are encoded in the unique self-replicating conformation of PrP^{Sc}.[28-31]

In contrast with other mammalian prions, proteinase K digestion in sCJD prions leads to a more complex pattern with either Type 1 or Type 2 $rPrP^{Sc}$. These two types differ in the proteinase K cleavage site at residues ~82 (Type 1) or ~97 (Type 2), and respectively, lead to 21 or 19 kDa mass of unglycosylated fragment of protease-resistant pathogenic prion protein ($rPrP^{Sc}$). Using sensitive biophysical techniques, we recently discovered a broad spectrum of distinct PrP^{Sc} conformers in 20 cases of sCJD with the same codon 129 polymorphism (MM) and the same Type of $rPrP^{Sc}$. These data implied that sCJD is caused by a broad array of distinct prions.[27,32,33] Subsequent experiments with sedimentation velocity separation using high speed centrifugation in sucrose gradient revealed that sCJD prions exist in the continuum of particles composed of <20 to >600 PrP^{Sc} molecules. The Type 1 PrP^{Sc} particles sedimented significantly more slowly than Type 2, indicating that Type 2 PrP^{Sc} formed larger assemblies.[32] These findings suggest that the packing of PrP^{Sc} monomers with different conformations in distinct particles is responsible for the peptide fragmentation pattern, consisting of predominantly 19 kDa fragments in Type 2 $rPrP^{Sc}$, or 21 kDa in Type 1 $rPrP^{Sc}$, after proteinase K treatment.[34] Remarkably, progression rates of the disease correlate with the replication rate of human prions in vitro, which is in turn governed by the size and conformational instability of particles formed by PrP^{Sc}.[26,32,33] Cumulatively, smaller prions particles composed of less stable conformers of PrP^{Sc} replicate faster in vitro as well as in vivo.

Our subsequent experiments with highly sensitive conformation-dependent immunoassay (CDI)[35-37] also demonstrated frequent, and perhaps universal, presence of both 21 kDa (Type 1) and 19 kDa (Type 2) unglycosylated fragments of protease-resistant (r) PrP^{Sc} in the same sCJD brain.[34] The fragments were present at different ratios, and indicate co-occurrence of markedly different PrP^{Sc} conformers, often in the same anatomical structure in the same brain. These quantitative findings extended previous qualitative observations with diverse antibodies and western blot techniques.[38-42] Apart from challenging the validity of the clinicopathological classification of sCJD based on PRNP gene polymorphism and western blot patterns of Type 1 or Type 2 $rPrP^{Sc}$ [24], these findings raised some fundamental questions: (1) Do the coexistent Type 1 and Type 2 $rPrP^{Sc}$ form distinct or hybrid particles composed of both types of PrP^{Sc}? (2) What is the impact of coexistence of distinct PrP^{Sc} conformers? (3) Do they replicate independently and thus imply co-existence of different sCJD prions?

Although the possible coexistence of different prions in naturally prion-infected sheep, goat, and mink has been suspected early on,[28,43-47] the early experiments could not differentiate between two possibilities: (1) strain adaptation caused by a switch from the primary sequence of the original host's PrP^{Sc} to a different PrP sequence in the new host, or (2) selection of strains from a co-existing pool in the natural host.[48,49] Since Type 1 and Type 2 prion particles can be separated by sedimentation velocity,[32] we investigated whether coexistent Type 1 and Type 2 $rPrP^{Sc}$ form distinct particles. Using high-speed centrifugation in sucrose gradient and sedimentation velocity separation in tandem with CDI and conformational stability assay, we isolated two populations of prion particles, each composed of a relatively homogenous population of conformers that had either Type 1 or Type 2 N-terminus proteolytic cleavage sites and different conformational stability (**Fig. 1**). Since we did not observe a change in the sedimentation velocity nor the formation of hybrids after mixing isolated "pure" MM1 and MM2 $rPrP^{Sc}$ in vitro, we concluded that they do not interact. Thus, our findings indicate that the coexistence of distinct prion particles with

different conformational structure, or packing of the monomers of PrP^{Sc}, is a common feature of sCJD.

Mechanism of adaptation, evolution, and competition of prions

Change in biological characteristics of prions observed upon crossing the species barrier and in experiments with subcloned cell lines indicate that prions may undergo evolution and adaptation, but the exact molecular mechanism of this effect has remained speculative.[31,49,51] To investigate the impact of the coexistent prion particle types on this process, we recently used a modified protein misfolding cyclic amplification (PMCA)[52] with homologous, as well as mutant, unglycosylated $PrP^{C(N181,197Q)}$ substrate carrying methionine in codon 129.[34] The serial PMCA of "pure" Type MM1 and mixed Type MM1+2 sCJD seeds underwent two distinct phases. In the first adaptation phase, the amplification was limited, and detectable only with CDI. In the second replication phase, we observed an abrupt increase in the amplification rate. We selected for these experiments sCJD samples that had approximately equal concentrations of Type 1 and Type 2 $rPrP^{Sc}$. Remarkably, within the Type 1+2 particle mixture, Type 2 $rPrP^{Sc}$ gradually disappeared, even though "pure" Type 2 sCJD amplified very well. This effect resulted in the uniform selection of Type 1 $rPrP^{Sc}$ in mixed Type 1+2 cases and a progressive drop in the stability of the amplified $rPrP^{Sc}$. These findings provided the first experimental evidence for an evolutionary process within the Type 1+2 prion mixture, with selection that favors Type 1 conformers with the lowest stability.[34] The initial preferential amplification rate of Type 1 PrP^{Sc} is not surprising, since there is typically a higher percentage of less stable protease sensitive oligomers in Type 1 PrP^{Sc}, as found in our recent studies. This may explain why this $rPrP^{Sc}$ subtype represents ~70% of all sCJD cases.[27,32] Our data also correlate with the superior transmissibility and short incubation times of MM1 sCJD prions, and with incomplete transmissions and extended incubations times of MM2 sCJD prions observed in transgenic mice that overexpress homologous or chimeric human PrP^{C}.[53,54]

The inhibition of Type 2 amplification that occurs in the Type 1+2 mixture contrasted sharply with the very efficient replication of "pure" Type 2 sCJD (with less than 5% of Type 1 present). These findings indicate that the unglycosylated $PrP^{C(N181,197Q)}$ is not per se a preferential substrate for amplification of Type 1 PrP^{Sc}, and suggest interference between Type 1 and Type 2 conformers during replication. The prion interference has been observed in vivo in mice and Syrian hamsters that were inoculated simultaneously or sequentially with two distinct strains of prions.[55-58] Since we observed no direct interaction between different conformers of PrP^{Sc} in our in vitro mixing experiments with "pure" MM1 and MM2 sCJD prions, we concluded that the most likely explanation is competition for PrP^{C} substrate or auxiliary molecule; however, the exact molecular mechanism of this phenomenon remains to be fully elucidated.[34]

Cumulatively, the distinct particle size, conformational stability, and amplification rate of these prion subtypes argue for the frequent coexistence of different sCJD prions in the same host (**Fig. 1**). Under favorable conditions with compatible PrP^{C} substrate, the mixture of human PrP^{Sc} conformers may undergo an evolution that selects a subset with the highest replication rate, due to the lowest stability (**Fig. 1**). Notably, the adaptation phase and prion strain evolution inferred from experiments with cloned cells[31] and Tg mice,[48,49] has been shown in our experiments to be a conformational process. Thus, the selection of a relatively narrow population of conformers with similar conformational stability during passage in experimental animals or cells, together with high activation energy barriers preventing conversion to different prion strains, is likely responsible for the exceptional stability of the biological characteristics of laboratory prion strains, as long as they are propagated in the same host or cells. However, many important questions remain unanswered, specifically, (1) how the initial ratio between Type 1 and Type 2 would influence the outcome, and (2) whether the first adaptation phase is due to the requirement for the critical threshold stoichiometry between seed PrP^{Sc} and mutant substrate $PrP^{C(N181,197Q)}$ needed for optimum replication, or (3) whether adaptation is due to the absence of sugar chains on $PrP^{C(N181,197Q)}$ substrate and double N→Q mutation, or (4) whether this first phase is caused by the difference between mouse and human auxiliary molecules. Since all our PMCA experiments were performed with PrPs carrying methionine in codon 129 of the *PRNP* gene, it also remains to be established if valine in the same position will impact the process. However, the evolutionary conformational selection mechanism of PrP^{Sc} may explain the recently observed drug-induced evolution of mammalian prions.[59] In these experiments, Oelschlegel and Weissmann exposed different prion-infected cell sub-lines to the drug (swainsonine) and observed not only drug-resistant,

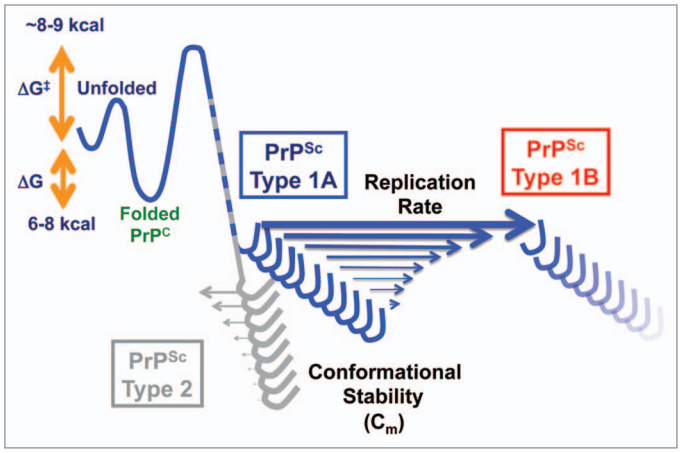

Figure 1. Schematic reaction coordinates of conformational transition from less stable PrP^{C} to more stable PrP^{Sc}, and conformational evolution of sCJD PrP^{Sc}. The isolates of sCJD prions homozygous for methionine in codon 129 are composed of two populations of PrP^{Sc} conformers: less stable Type 1A PrP^{Sc} and more stable Type 2. Their replication with unglycosylated mutant $PrP^{C(N181,197Q)}$ substrate leads to initial preferential amplification of less stable Type 1A PrP^{Sc} and continuing selection of progressively less stable Type 1B PrP^{Sc}. The ΔG is the energy difference between unfolded and folded state of PrP^{C}; $\Delta G^{\#}$ is the activation energy necessary for conformational transition from PrP^{C} to PrP^{Sc} state.[50]

but also drug-dependent prion populations, which propagated more rapidly in the presence rather than the absence of the drug. Moreover, their data demonstrated that new, initially drug-dependent prions became new stable prion variants after drug withdrawal. These prion adaptations are most likely driven by the conformational selection mechanism we observed in our experiment in vitro and call for the reevaluation of different therapeutic strategies that target amyloid-forming aggregates of PrP^{Sc}. High-resolution structural tools and research into the role of PrP^{Sc} ligands must address the apparent conformational plasticity of PrP^{Sc}, which is likely responsible for the coexistent spectrum of prion conformers, and enables the prion evolution that results in extensive phenotypic diversity (**Fig. 1**).

Implications for neurodegenerative diseases caused by protein misfolding

The advanced understanding of clinicopathological heterogeneity and pathogenesis of late onset Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), and other diseases linked to protein misfolding, demands that we identify the factors that lead to a spectrum of different phenotypes and different progression rates. The most frequent form of dementia is late-onset (>65 y of age) Alzheimer diseases (AD).[60] However, both early and late onset forms are pathologically characterized by the presence of amyloid β peptides (Aβ) plaques, and intraneuronal tangles of hyperphosphorylated forms of microtubule associated protein tau (MAPT).[61] The causal mutations in amyloid precursor protein (*APP)*, presenilin 1 (*PSEN1),* and presenilin 2 (*PSEN2*) genes, which have been identified in early-onset forms, established the central role of amyloid β (Aβ) and its processing in AD.[62] However, the role of the amyloid deposits and tangles in the cognitive decline and pathogenesis of late-onset sporadic AD is more difficult to define. A major determinant in the risk of late-onset AD is the polymorphism of the *APOE* gene, in which a single e4 allele increases the risk by a factor of 4, and two e4 alleles increases the risk by a factor of 13. Additional polymorphisms in several recently identified genes may moderately increase the risk of disease.[63] Thus, the extensive phenotypic variability of AD with variable progression rates,[64,65] clinical symptomatology,[66] and pathological findings[67,68] suggests a complex impact of different structural forms of misfolded Aβ and tau proteins,[69] variable genetic backgrounds,[63] and compensatory mechanisms ("cognitive reserves").[70]

While the genetic and environmental factors linked to the risk of developing AD are well recognized, the factors leading to variable progression rates of late-onset AD are unknown.[63] Recently, the Prion Surveillance Centers in the US and Europe independently described a novel subgroup of patients who have rapidly progressive dementia that clinically imitates prion diseases, and which, after exhausting neuropathological investigation and prion protein gene sequencing, is concluded to be rapidly progressive late-onset AD (rpAD).[64,71-74] The data collected from multiple Prion Centers uniformly demonstrate the absence of positive family history or comorbidity, the presence of distinctive clinical characteristics, and a frequency of e4 alleles in the *APOE* gene that corresponds to the general population. A systematic investigation of the genetics and molecular pathology of Aβ and tau in those patients should lead to the identification of biological factors responsible for the variable progression rates of AD. These findings will be crucial in developing new therapeutic targets for AD, for preclinical diagnostics, and for individualized therapeutic approaches.[60]

Investigating the conformational structure of brain Aβ and tau is critical for deciphering their role in the variable progression rates and phenotypes of AD. Extensive analysis of aging brain samples indicates that the pathological process underlying AD starts early in isolated brain anatomical structures and then spreads through neuronal projections.[67] This process can be accelerated in transgenic mice models of AD and tauopathy by intracerebral injection of preformed misfolded Aβ or tau, and different structural conformers of misfolded proteins have varying potency to accelerate the pathology.[75,76] These data suggest a prion-like mechanism, and since synthetic Aβ is significantly less active in this "seeding" effect than Aβ of brain origin, the data imply a conformational and biological plasticity, which is the basis for prion subtypes (strains).[26,77,78] These findings have raised some fundamental questions, specifically, whether different conformers of Aβ or tau contribute to varying progression rates of the disease, and whether subtle differences in the conformation of Aβ or tau may be responsible for the distinct disease phenotypes. Additionally, there is a large body of literature on the conformational characteristics and folding pathways of synthetic and recombinant Aβ and tau, which raises the question of how these variable structures are relevant to the structure of brain Aβ and tau and to the pathogenesis of the disease.

Several therapeutic trials targeting amyloid deposits in AD have failed. These disappointing results triggered a reexamination of the pathogenetic assumptions that lead to their development, and exposed a critical need for new therapeutic targets and earlier diagnostic detection of the disease.[79] This goal is especially important in light of our investigations of prion adaptation and evolution, which imply that misfolded proteins, including those causing AD and PD may evolve, and thus gain resistance to the therapeutic ligand that originally targeted them. Equally important is to advance our understanding of phenotypic heterogeneity in AD, and the essential requirement for the identification of genetic and conformational proteomic markers that would differentiate distinct subgroups of patients, who may respond differently to administered therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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