Wen-Quan Zou ·
Pierluigi Gambetti *Editors*

Prions and Diseases

Second Edition

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Preface

Almost 2 years ago, Mr. William Lamsback, editor from Springer, reached out to us about the possibility to work on a second edition of our book *Prions and Diseases*, whose first edition was published by Springer in 2013. We were told that the printed book sold well and had nearly 20,000 downloads online. Indeed, studies on prions and prion diseases have been popular since 2008 according to PubMed, and there is a strong demand for top experts in the field to report and discuss these new developments and advances.

Prions apparently have become the prototype of other misfolded proteins associated with more common neurodegenerative diseases including Alzheimer's and Parkinson's diseases. Many principles and technologies developed originally from the investigation of prions and prion diseases have been widely applied to prion-like neurodegenerative diseases, such as cell-to-cell transmission, animal transmission studies, protein misfolding cyclic amplification (PMCA), and real-time quaking-induced conversion (RT-QuIC) assays. Because of these similarities, Stanley Prusiner, the Nobel laureate who discovered scrapie pathogens and coined term "prion" in 1982, redefined prions as "proteins that acquire alternative conformations that become self-propagating" in 2013: a characterization that underlines the applicability of the prion concept to all conformational degenerative diseases. As a result, more researchers and clinicians are becoming interested in the past and current prion research.

The first edition updated human and animal prion diseases from basic science to clinical diagnosis and possible treatments. The second edition, besides preserving the strengths of our first edition, adds major changes. First, the two volumes are combined into a single one. Second, the 37 chapters are grouped into 10 sections including history, general aspects of prions, conversion and strain of prions, environment and transmission of prions, modeling of prions, human prion disease and other pathogenies, animal prion diseases, yeast prions, diagnosis and human prion surveillance, and treatment. Third, new topics have been added including stem cell models, genetic prion diseases, new human prion diseases, skin biomarkers, protective role of cellular prion protein in tissue ischemic reperfusion injury, human prion disease surveillance, and gene therapy.

To date, two Nobel prizes (actually, maybe two and a half, if the Nobel prize in Chemistry to Kurt Wütrick, who worked extensively on the prion protein, is considered) have been awarded for research on prions. Given that many unsolved issues remain, it is likely that additional Nobel prizes will be awarded for new discoveries related to prions. Hopefully, this book will be useful to our future Nobel laureates.

Cleveland, OH, USA

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Prions are proteins that acquire alternative conformations that become self-propagating… Some prions are beneficial and perform cellular functions, whereas others cause neurodegeneration.
Stanley B. Prusiner, “Biology and Genetics of Prions Causing Neurodegeneration” Annual Review of Genetics, 47:601–623, 2013

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Part I
History

Chapter 1
Transmissible Spongiform Encephalopathy: From Its Beginnings to Daniel Carleton Gajdusek

Paul Brown

Abstract Scrapie was the original member of what has become a family of both animal and human spongiform encephalopathies. Described clearly in the eighteenth century in both England and Germany as a fatal contagious disease of sheep, it was not experimentally transmitted until 1936 and became the subject of wide-ranging research in a number of laboratories in Great Britain. The human analog was first described in 1920 by the German neurologists Creutzfeldt and Jakob and experimentally transmitted by Gajdusek in 1968, following similar success in transmitting another analogous human disease (kuru) 2 years earlier. The evolving story of these and other members of the transmissible spongiform encephalopathy family (including "mad cow" disease) has led through a maze of studies involving many unexpected twists and turns, eventually culminating in the discovery of a new category of infectious disease caused by the misfolding of a normal host protein (PrP^{TSE} or "Prion").

Keywords Transmissible spongiform encephalopathy (TSE) · Prion disease, Scrapie · Kuru · Creutzfeldt–Jakob disease (CJD) · Transmissible mink encephalopathy · Chronic wasting disease

1.1 In the Beginning …

… there was scrapie. How far back in time is unknown, but it is thought to have originated somewhere in Europe during the late Middle Ages. Whatever the historic beginnings, we know that by the eighteenth century, it was prevalent in both England and Germany and that its introduction into England probably came from the

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importation of Spanish merino sheep that, although highly susceptible to scrapie, had wool of exceptional quality.

At least two centuries elapsed between the recognition of the disease and the first attempts to investigate it scientifically. Veterinary literature was limited to its incidence, clinical features, and speculation about its cause until the end of the nineteenth century, when Charles Besnoit and Charles Morel, colleagues in veterinary medicine at Toulouse, France, recognized the regular presence of spongiform change in the spinal cord and adjacent nerves but considered it to be part of a wider pathology that most likely represented a toxic peripheral neuropathy (Besnoit and Morel 1898). Besnoit also directed a number of transmission experiments in sheep that, unfortunately, were destined to fail because of a surveillance period limited to 9 months (Besnoit 1899), an oversight that a half-century later was also to delay recognition of the transmissibility of the human disease, kuru. Among the younger faculty members at that time was Jean Cuillé, who would later recognize this need for an extended period of postinfection observation, and publish with Paul-Louis Chelle a superb set of experiments between 1936 and 1938 that established beyond any doubt that scrapie was indeed a transmissible disease (Cuillé and Chelle 1936, 1938) (Fig. 1.1).

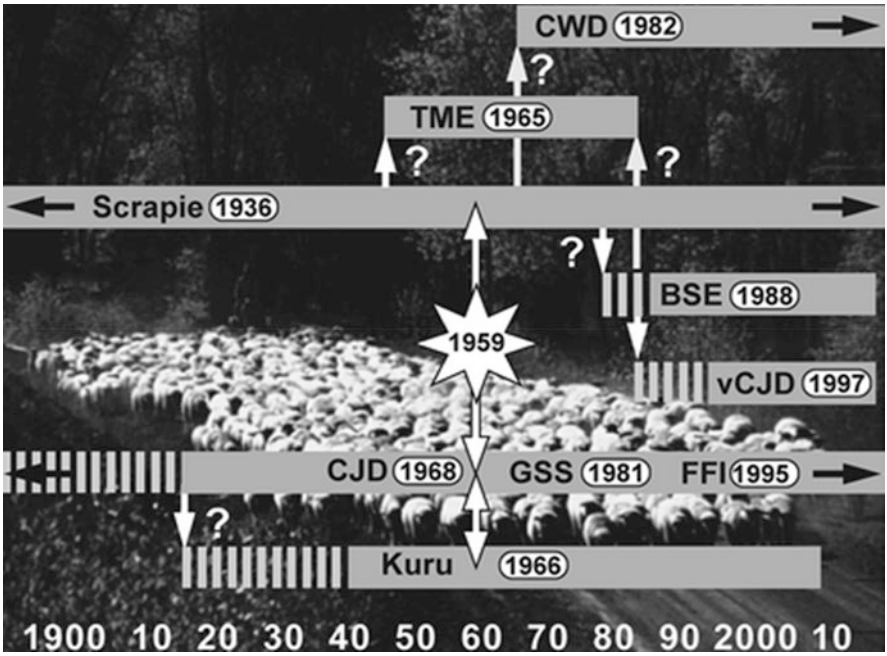

Fig. 1.1 The chronology of TSE. The position and length of the *bars* are keyed to the timeline at the *bottom of the figure*. *Striped regions* represent the possible or probable (but unproven) preexistence of the disease. The date of the first reported experimental transmission of each disease is shown within the *bars*. The year 1959 is emphasized to draw attention to its importance as the year in which the kuru–scrapie–CJD connection was made

About the same time that Cuillé and Chelle published their studies, transmissibility was accidentally confirmed when a formalinized louping ill vaccine prepared from sheep central nervous system (CNS) tissues was identified as the cause of a mini-epidemic of scrapie in Scotland (Gordon 1946). Investigation of the outbreak revealed that one batch of vaccine had included material from Cheviot lambs born of ewes that subsequently developed scrapie. These observations laid the groundwork for a flowering of experimental research that was mostly concentrated in Great Britain during the next 30 years, although scrapie was also under study in Iceland, where it had the name "Rida," and in the USA, where it became a growing concern following its diagnosis in Suffolk sheep imported from Great Britain via Canada in 1947.

1.2 Working Out the Biology (in Sheep)

All of the early work on scrapie was conducted in sheep, an extremely inconvenient bioassay animal requiring observation periods of several years in carefully monitored farms, which meant that research remained limited to the very few facilities capable of performing such experiments. Worse still, the unpredictable response of sheep to the same experimental inoculum made it difficult and at times impossible to conduct quantitative titration studies.

Thus, the pioneering work of David R. Wilson at the Moredun Institute in Edinburgh during the 1940s, largely overshadowed by the personalities and careers of the many researchers who followed him, was a remarkable achievement. Conducting experiments almost single-handedly in sheep that had only a 25% transmission rate, he added transmissibility via intradermal and intravenous routes to those reported by Cuillé and Chelle; studied the pathogen's filtration and sedimentation behavior; and discovered its surprising resistance to a variety of chemical and physical treatments, including heat (100 °C for 30 min), exposure to phenol, chloroform, and formaldehyde, and UV irradiation (in retrospect the most interesting finding). He also documented the survival of infectivity in dried brain tissue after 2-year storage. A great deal of experimental work published during the next several decades built upon the foundation laid down by Wilson.

The fact that scrapie was of lesser concern to the sheep industry than several other diseases, and was not known (then or now) to be a human pathogen, resulted in little governmental interest in the disease. That indifference changed when, in the early 1950s, North America, Australia, and New Zealand placed embargos on the importation of British sheep in response to the discovery of undiagnosed scrapie in their exported sheep. (Never underestimate the power of commercial interests on the funding of scientific research, which recently surfaced again when "mad cow disease" appeared on the scene.) Increased funding from the UK expanded the program at Moredun under the continuing direction of Wilson, and later John Stamp, and at the Agriculture Research Council (ARC) facility at Compton, England, under the direction of William Gordon.

Gordon conceived and executed a massive study using over 1000 sheep to investigate the breed susceptibility to scrapie (the "24 breed experiment"), leading to the selection for experimental purposes of two flocks of the Herdwick breed: one highly susceptible and the other relatively resistant. He also put together a very active group of scientists, including Gordon Hunter, Geoffrey Millson, Richard Kimberlin, Carol Walker, and Iain Pattison, who produced a flood of research papers during the 1960s to the 1980s dealing with genetic susceptibility, pathogenesis, and the nature of the scrapie agent.

Meanwhile, at Moredun, Stamp and Alan Dickinson began a wide-ranging study of scrapie strains in Cheviot sheep, producing, for the first time, sound experimental evidence for the maternal transmission of infection and spread of disease through close contact, and in a remarkable set of classical genetic analyses established that a single gene (*Sip*) with two alleles controlled the incubation period in sheep. Dickinson later became the founding Director of the ARC and MRC Neuropathogenesis Unit, also in Edinburgh, where he was soon joined by Kimberlin, Hugh Fraser, Moira Bruce, and David Taylor (and later by Jim Hope, Nora Hunter, and Jean Manson)—who as a group with wide-ranging expertise in pathogenesis, disinfection, molecular biology, and molecular genetics would advance knowledge in each of these areas in the years that followed.

1.3 The Mouse That Roared

In 1961, at Compton, Richard Chandler succeeded in adapting sheep scrapie to the mouse (Chandler 1961). This accomplishment immediately opened the door to studies that would have been prohibitive if limited to bioassays in sheep and later made possible all of the genetic engineering that is crucial to so much work being done today. Pattison describes the event with his customary flair (Pattison 1972):

> I still feel the urge to genuflect as I pass the spot at our Institute (Compton) beside the boiler house, where my colleague R.L. Chandler paused 1 day in 1960 to suggest to me that he might inoculate three strains of mice (C57, CBA and Swiss) with brain material from two clinical types of goat scrapie (drowsy and scratching). Chandler had already found that the three strains of mice had different susceptibilities to *M. johnei*. He subsequently injected the two strains of scrapie i/c and he transmitted the drowsy strain in 7 months in the Swiss strain and to the other two strains a few weeks later. These mouse strains of scrapie bred true with an incubation period of 4 months. Thus occurred the greatest single advance in scrapie research since experimental transmission of the disease by Cuillé and Chelle in 1936.

This technical advance nearly, but not quite, extinguished all further experimental studies in sheep: the exceptions being studies in which non-rodent species are used to confirm the results in mice, or where there is a need for large amounts of tissues or fluids (e.g., blood), or most recently, in studies designed to explore the behavior of bovine spongiform encephalopathy (BSE) infection in sheep. Three of the most important early studies in mice were conducted at the following laboratories:

– At the NIH Rocky Mountain laboratory in Montana, Carl Ecklund and William Hadlow initiated an exhaustive study of the distribution and level of infectivity in a wide variety of tissues and fluids in Chandler's strain of mouse-adapted scrapie, and in mice inoculated with material from naturally and experimentally infected sheep and goats.
– At Compton, Kimberlin and Walker extended these pathogenesis studies to the dynamics of peripheral infection, implicating lymph nodes and spleen along a pathway through visceral sympathetic nerves to the thoracic spinal cord and thence to the brain.
– At the ARC unit in Edinburgh, Dickinson's group applied the same classical genetic approach they had used in sheep, discovering that a similar gene (*Sinc*) controlled the incubation period in mice. They also showed that distinctive patterns of brain lesion distribution were reproducibly associated with different scrapie strains. The conjunction of these two observations led to a method of TSE strain identification that would later serve as the most persuasive evidence for a close strain similarity between BSE and vCJD (Bruce et al. 1997).

1.4 The Nature of the Beast

Amidst all of this work, two crucial questions stood out: what was the relative importance of an infectious versus genetic origin of the naturally occurring disease and, assuming the existence of an infectious agent, what were its biochemical components? The first question was a major topic of discussion at a 1964 meeting convened by the USDA in Washington, DC. After listening to 3 days of heated debate, novitiates in the audience were left wondering if all medical meetings were going to be similarly confrontational (they would not be disappointed). Two participants were in almost diametrical opposition: H.B. (James) Parry, an Oxford veterinarian who argued for genetics as the exclusive cause of the naturally occurring disease, and Dickinson, who argued that scrapie was caused by an infectious agent that was influenced by genetic susceptibility. In due course, Dickinson's position would be fully validated. In fact, the *Sip* and *Sinc* genes that Dickinson had identified by classical genetics were none other than the prion-encoding *Prnp* alleles later identified by molecular genetics.

The other question—biochemical characterization of the infectious agent—was (and continues to be) a subject of intense research interest and importance. Although the burden of evidence for different strains of the scrapie agent clearly implied the existence of a nucleic acid genome, there were indications as early as the 1960s that nucleic acid was not only unlikely to be the sole constituent of the scrapie pathogen but, based on radiation resistance data, unlikely even to be present. The first clue came from the early inactivation studies by Wilson, noted above, that included resistance to standard sterilizing doses of UV radiation. Then came the set of inactivation studies by Hunter, Millson, and Kimberlin that, in conjunction with their demonstration of a firm association of infectivity with cell membranes, led Gibbons

and Hunter to propose that the infective entity was a modified glycoprotein subunit of membranes that multiplied by inducing similar chemical or conformation changes in newly "infected" cell membranes (Millson et al. 1976).

The "coup de grace" came from a set of rigorously controlled irradiation studies published by Tikvah Alper and colleagues between 1966 and 1971, in which both the resistance of scrapie brain extracts to very high doses of ionizing and UV radiation and the UV inactivation profile were inconsistent with any known virus or nucleic acid. One paper, in particular, began with the following point-blank abstract: "Scrapie is a slowly developing disease of the nervous system. Experiments on the effects of ultra-violet irradiation of suspensions of infected mouse brain extracts confirm that the agent responsible for it does not depend on a nucleic acid for its ability to replicate. No evidence is obtained, however, to indicate whether the agent is associated with a protein" (Alper et al. 1967).

No one doubted the validity of Alper's radiation resistance work, but no one knew how to deal with it—in other words, how to accommodate a clear indication of the absence of nucleic acid in the pathogenic agent and still satisfy the dogma of nucleic acid-directed replication. Explanations invoking protection or repair of nucleic acid eased acceptance of her data, but her conclusions remained in a kind of limbo for years.

1.5 The Transition from Biology to Molecular Biology

In 1967, the mathematician John Stanley Griffith suggested three ways by which a protein might self-replicate, remarking that "there is no reason to fear that the existence of a protein agent would cause the whole theoretical structure of molecular biology to come tumbling down" (Griffith 1967). He presented free energy equations for the polymerization of protein subunits on preexisting dimerized molecules, that is, a template mechanism, as had been suggested by Gibbons and Hunter. He went on to say that "there is an obvious analogy between the idea presented here and the idea that a gas can only condense on nuclei which are already present: many of the more general schemes could be summed up by saying that the subunits can only polymerize by utilizing condensation nuclei of polymers which are already there." He concluded that scrapie could be "a protein or a set of proteins which the animal is genetically equipped to make, but which it either does not normally make or does not make in that form. It may be passed between animals but actually be a different protein in different species. Finally, in either case, there is the possibility of spontaneous appearance of the disease in previously healthy animals."

Credit for the discovery of the first disease-specific structure in a transmissible spongiform encephalopathy (TSE) goes to Patricia Merz, working at the Institute for Basic Research in Developmental Disabilities in Staten Island, New York, who in the late 1970s began to study extracts of scrapie-infected mouse brains under the electron microscope. She identified fibrillar structures very similar to those seen in Alzheimer's disease, which she named "scrapie-associated fibrils" (SAF), and in

further studies also found them in the brains of humans and experimental animals infected with CJD (Merz et al. 1981; Merz and Somerville 1983).

What all of these experiments lacked was a molecule that specifically co-purified with infectivity, but this was finally rectified by 1982 in Stanley Prusiner's laboratory, using the 263 K hamster model of scrapie that had been developed by Kimberlin and Walker in 1977 (Kimberlin and Walker 1977). This model proved to have exceptionally high concentrations of infectivity in the brain (10^{10} LD_{50}/g) after an incubation period of only 2 months, a fortuitous combination that made it possible to undertake the purification of a sufficiently large amount of highly infectious fibrils (renamed "prion rods" by Prusiner) to isolate a peptide subunit that could then be subjected to the tools of modern molecular biology.

The overall contribution of scrapie to the field of TSE was aptly summarized by Pattison (1972), who concluded his reflections with the statement that "Scrapie is one of four closely similar diseases, the others being kuru, Jakob–Creutzfeldt disease, and transmissible mink encephalopathy. Research on scrapie was responsible for the recognition of this group of diseases, to which others may be added in due course, and knowledge of the vagaries of scrapie has been of great value in planning research on them all, for in planning a complicated journey it is reassuring to know that similar ground has already been covered."

1.6 The Discovery of Kuru

In the mid-1950s, a young pediatrician turned research scientist named Carleton Gajdusek was stationed at the Walter Reed Army Medical Center, where, in 1954, he was assigned to spend a year in Australia to study the immunology of liver disease in the laboratory of Sir MacFarlane Burnet. Ever the explorer, he traveled widely during his stay, including a trip to Papua New Guinea to satisfy what would become a lifelong interest in primitive cultures, and there met Vincent Zigas, a charming if somewhat eccentric Lithuanian physician who was working as a Medical Officer in the Eastern Highlands. Zigas told him about a strange neurological disease (kuru) that was decimating the Foré-speaking peoples in his area of practice and invited him to the Highlands to see for himself. He did so and was intrigued by the high incidence, age and sex distribution, and neurological characteristics of the disease (Gajdusek and Zigas 1957). His journals and letters detail the heroic efforts needed to establish a beachhead in Okapa, the administrative center of the Foré region, including a dedicated hospital that for many years operated under the direction of Dr. Michael Alpers, and a native personnel network to identify and transport the continuing stream of new patients to and from Okapa.

He experienced many difficulties with the Australian colonial authorities (Papua New Guinea was then a dependency of Australia), who sometimes resented his dramatic intrusion into their territory. He once remarked that the US government would not be pleased in the converse situation of an Australian research team studying a new disease on an Indian reservation. In fact, one of Gajdusek's most remarkable

and generous traits was, with a single exception, his acceptance of people and events that would depress or anger almost anyone else, as part of the "comédie humaine." He was simply incapable of feeling offended or bitter and never looked back.

He was also an authentic genius, whose interests spanned physics, anthropology, medicine, music, and literature, and his early career was spent in the laboratories of a number of Nobel Laureates. It did not take him long to join their ranks: in 1976, he was awarded a Nobel Prize for his demonstration that kuru, a neurodegenerative disease, had an infectious cause. Kuru had been recognized for decades by the affected population (who considered it to be due to sorcery) and by European locals—everyone from missionaries to bush pilots—who attributed the disease to cannibalism. The difficulty was proving it, as is evident from the innumerable failures to find the cause of toxic, hormonal, nutritional, and infectious causes during the first several years of study.

1.7 The Kuru–CJD–Scrapie Triangle

The year 1959 was a banner year for TSE (Fig. 1.1). Since his encounter with kuru, Gajdusek had been spending a good part of each year in the field, establishing a kuru hospital in Okapa, the administrative center of the region, organizing the care of kuru patients, doing autopsies, trying to discover the cause of the disease, and conducting preliminary therapeutic trials based on all the possible causes under study. During this time, he sent brains from a dozen kuru cases to Igor Klatzo, a neuropathologist working at the NIH. In 1959 he published his findings, noting widespread neuronal degeneration (including vacuolation), myelin loss, astroglial and microglial proliferation, scattered perivascular cuffing, and, in half the cases, a predominantly cerebellar location of amyloid plaques. He did not mention spongiform change and attributed the neuronal vacuolation to postmortem artifact. However, in his discussion comparing kuru to other diseases, he concluded that "Creutzfeldt–Jakob disease appears to be closest in resemblance" (Klatzo et al. 1959).

This astute observation by Klatzo was all the more remarkable because the diagnostic criteria for Creutzfeldt–Jakob disease (CJD) had been in disarray since its initial description in 1920 and remained so through the late 1960s. Creutzfeldt's original case was described as a "new and unusual type of neurological disease" in a 22-year-old woman with a 1-year illness characterized by tremors, spasticity, pyramidal signs, nystagmus, ataxia, myoclonus, and dementia (Creutzfeldt 1920). Neuropathology showed diffuse neuronal loss and astrogliosis, but vacuolation was neither mentioned nor illustrated. A year later, in 1922, Jakob reported four cases that he thought resembled Creutzfeldt's case (Jakob 1921). A review of the slides from Jakob's cases was undertaken by Colin Masters in 1982 (Creutzfeldt's slides had not survived), who concluded that only one of the cases (a 42-year-old male) satisfied the criteria for what we now call Creutzfeldt–Jakob disease: the

histopathology included neuronal loss, astrogliosis and a diffuse spongiform change throughout the cerebrum and cerebellum (Masters and Gajdusek 1982).

Over a period of several years, Jakob and his students gradually acquired a fuller appreciation of spongiform encephalopathy as a pathological entity, including the first case of familial CJD, and somewhat later, in the mid-1930s, Gerstmann, Straüssler, and Scheinker reported the first family with the disease that now carries their names (GSS) (Gerstmann et al. 1936). Nevertheless, the clinical and neuropathological characteristics of CJD remained elusive until the bedrock criterion of transmissibility allowed its clear separation from a host of other neurodegenerative diseases of unknown etiology.

Hadlow's recollection of events that led him to make the kuru–scrapie connection was recounted in a reminiscence published in 2008:

> The unlikely linkage of these two diseases came about fortuitously while I was an employee of the USDA studying the pathology of scrapie at Compton. William Jellison, a friend and colleague from Rocky Mountain Laboratory, Hamilton, Montana, where I had worked before coming to England visited me in Compton and casually mentioned an exhibit he saw the previous day at the Wellcome Medical Museum in London. It had to do with a strange brain disease affecting the primitive people in Papua New Guinea. He thought I might like to see it owing to my interest in neuropathology. Five days later I saw the exhibit in London. Neuronal degeneration and intense astrocytosis likened kuru to scrapie. The likeness was made even more so by the single and multilocular vacuoles in the perikaryon of large neurons. From the start I was drawn to them for they were so much like those in scrapie (Hadlow 2008).

Earlier in the 1950s, Bjorn Sigurdsson, working in Iceland, had set out criteria for "slow infections" that included species specificity. In his letter to Lancet, Hadlow recalled this observation, noting that "scrapie can be induced experimentally in the sheep and in the closely related goat but not in other species so far tested." He went on to conclude that "It might be profitable, in view of veterinary experience with scrapie, to examine the possibility of the experimental induction of kuru in a laboratory primate, for one might surmise that the pathogenetic mechanisms involved in scrapie—however unusual they may be—are unlikely to be unique in the province of animal pathology" (Hadlow 1959). He had recognized the twin needs for extended observation periods and the use of a species closely related to humans.

1.8 Experimental Transmission of Kuru

At the NIH, brain tissue had already been inoculated into numerous laboratory rodents that were observed for periods of up to several months, with negative results, but now Gajdusek went about organizing a primate colony at the Patuxent Wildlife Center in Laurel, MD, under the able direction of Clarence J. (Joe) Gibbs, Jr., who had served with him at the Walter Reed Army Medical Center. By 1963 all was in readiness, but Gajdusek decided to wait until new autopsy specimens could be obtained under optimal conditions for the survival of any infectious agent before

initiating a chimpanzee inoculation program. The author well remembers being sent to New Guinea only a few months after joining the laboratory in July 1963 with instructions to get autopsies on any kuru patients who died during his month-long stay. Only one patient died, and in a hut under the flickering light of a hurricane lantern, with the deceased woman's husband hovering nearby, it was necessary to barter for each organ that was taken (coffee, canned goods, flashlights, knives, etc.), and also satisfy his very sharp eye for reassembling the body to its pre-autopsy condition. Gajdusek had set up an elaborate logistical system to preserve the viability of any infectious agent that might be present, including canisters of liquid nitrogen at the autopsy site, Land Rovers and Piper Cubs on call, and way-station reservoirs of additional liquid nitrogen at each airport between the middle of New Guinea and Washington DC. As it turned out, the brain from this case was among the first three to transmit kuru to chimpanzees (the two others having been collected by Gajdusek himself). Little did we then know that the transmissible agent could have withstood boiling, standard sterilizing chemicals, and burial in the ground for 3 years and still have remained infectious.

The publication in 1966 (Gajdusek et al. 1966) of the first experimental transmission of kuru from three of seven patients, whose brain tissue homogenates had been inoculated intracerebrally into chimpanzees 18–21 months earlier, was followed by an explosive decade of activity in Gajdusek's NIH laboratory, and as Pattison had said, the earlier studies of scrapie provided a valuable road map for this new exploration of kuru. The first order of business was to validate the transmissibility of the disease and, if successful, begin to characterize the properties of what appeared to be a "slow" or "unconventional" virus. Chimpanzee-to-chimpanzee passage of kuru was accomplished in 1967 (Gajdusek et al. 1967), and a large series of experiments in a variety of primate species was carried out to determine the physical/chemical resistance, filtration size, host range, and pathogenesis of this new "virus" (Table 1.1).

1.9 The Expanding Horizon of Transmissible Spongiform Encephalopathy

The other pressing need, in view of Klatzo's observation of the neuropathological similarities between kuru and CJD, was to find a case of CJD to inoculate, which was not an easy task considering the rarity of the disease and its confusion with other dementia syndromes. However, a fully typical neuropathologically verified case was soon provided by Peter Daniel and Elizabeth Beck at the Maudsley Hospital in London, England, which transmitted disease to a chimpanzee 13 months after intracerebral inoculation, in 1968 (Gibbs Jr et al. 1968). Ironically, that same year Kirschbaum published a comprehensive review of all known cases of CJD, favoring an etiology of vascular origin (Kirschbaum 1968).

Table 1.1 Animal species used in TSE experiments (The most frequently used species are shown in bold type)

Primates	
Apes	**Chimpanzee**, Gibbon
Prosimians	Bushbaby, lemur, gibbon, slow Loris
Old World monkeys	**African green**, baboon, bonnet, **Cynomolgus**, langur, Mangabey, Patas, **rhesus**, pig-tailed, stump-tailed, Talapoin, Vervet
New World monkeys	**Capuchin**, marmoset, owl, **spider**, **squirrel**, wooly
Non-primates	
Rodents	**Guinea pig**, **hamster**, **mouse**
Carnivores	Mink, ferret
Ungulates	Horse
Felines	**Domesticated cat**
Avians	Chicken, duck, Turkey
Suidae	Domesticated pig
Caprinae	Sheep, goat

Although interest shifted dramatically from scrapie to CJD in the years following its experimental transmission, two animal diseases, transmissible mink encephalopathy (TME) and chronic wasting disease (CWD) of deer and elk, were recognized as belonging to the TSE family by Dieter Burger and Hartsough (1965) and by Williams and Young (1980), respectively (Burger and Hartsough 1965; Williams and Young 1980; Williams et al. 1982). Both diseases may have originated from exposure to scrapie-infected sheep that had been present in the USA since the late 1940s, but that epidemiologically plausible hypothesis will never be proven. In fact, one of the more interesting features of TME is its association with the consumption of cattle rather than sheep carcasses on two US mink ranches in 1963 and 1985, leading to speculation about an early undetected occurrence of BSE in the USA (Marsh et al. 1991). No further incidents have occurred in the USA since the second outbreak (TME has also been diagnosed in Canada, Finland, and Russia as late as 1986). In contrast, CWD has assumed more and more importance as it spreads from its origin in Colorado mule deer to cervids in North America that now include the Midwest and both US coastlines, as well as in Canada, South Korea, and most recently, Norway and Finland. It poses an obvious risk to the comparatively small number of humans who hunt and/or consume venison and other vital organs, and a potentially greater future threat via cross-contamination of wild predators (the cat family is highly susceptible), and eventually to captive animals and livestock. The unique attribute of CWD that makes it important is its presence in free-ranging animals that cannot be subjected to the kinds of preventive or destructive measures applied to animals in captivity.

The most recent addition to the TSE family—BSE—appeared on the scene in 1986 in the UK, as a new disease of cattle, and spread through most European and a few non-European countries within the next few years. Strictly speaking, it qualifies for discussion in this historical account, but as its occurrence extends well

beyond the era when Gajdusek was actively engaged in the field, and it is sufficiently important to deserve a detailed discussion in a chapter of its own, we will instead return to the human diseases with which Gajdusek was most involved.

As news of the transmissibility of CJD spread through the neurological community, the NIH laboratory became a global clearinghouse of case referrals including hundreds of cases of possible or suspected CJD, all of which were inoculated into primates. The early use of chimpanzees rapidly gave way to a variety of monkeys (Table 1.1), and as features of the disease came to be defined in each species, the squirrel monkey became the preferred assay animal because of a susceptibility greater than 90% (nearly equal to the chimpanzee) combined with a comparatively short mean incubation period of 24 months (Table 1.2 and Fig. 1.2). However, the observation that the same inoculum could sometimes produce disease after widely spaced incubation periods in replicate monkeys signaled caution in accepting incubation periods as a measure of the minimum infective dose in any experiment using only two or three animals, a point that is often forgotten in current research studies (Fig. 1.3).

The search for additional cases suspected of having CJD or diagnoses of other neurodegenerative diseases, and the laborious task of characterizing the transmissible agent, including its host range and pathogenesis, consumed a much larger number of animals and a much longer period of time, lasting well into the 1980s. Consider the simple matter of estimating the mean lethal dose (LD_{50}) of infectivity in a given tissue. Working with mice or other rodents, the usual technique would be to inoculate groups of 5–6 animals with a spread of dilutions large enough to bracket an unknown end point, typically totaling 40–50 animals, which would be unthinkable when using primates. Even a "stripped down" titration using pairs of animals at successive 100-fold dilutions would require at least eight animals. Add to this the need for observation periods of at least 5 years, and the difficulty of obtaining even the most basic information becomes formidable.

Over the years, the NIH laboratory bought, bred, and housed thousands of monkeys and hundreds of apes used in primary isolation and passage attempts, species susceptibility experiments, and pathogenesis bioassays, located at various sites in California, Hawaii, Louisiana, New Mexico, New York, Texas, and Virginia, as well as overseas in Paris and Marseille. Eventually, all primate research was consolidated to Gulf South in the middle of Louisiana Cajun country, and Fort Detrick,

Table 1.2 Characteristics of CJD transmissions in the most frequently used primate species

	Chimpanzee	New world monkeys			Old world monkeys	
		Squirrel	Spider	Capuchin	Rhesus	Cynomolgus
No. animals inoculated	29	211	31	45	28	23
Transmission rate (%)	97	93	97	80	68	22
Mean incubation period (months)	1.7	25	32	40	64	61
Mean duration of illness (months)	1.7	1.3	1.6	2.4	3.2	2.1

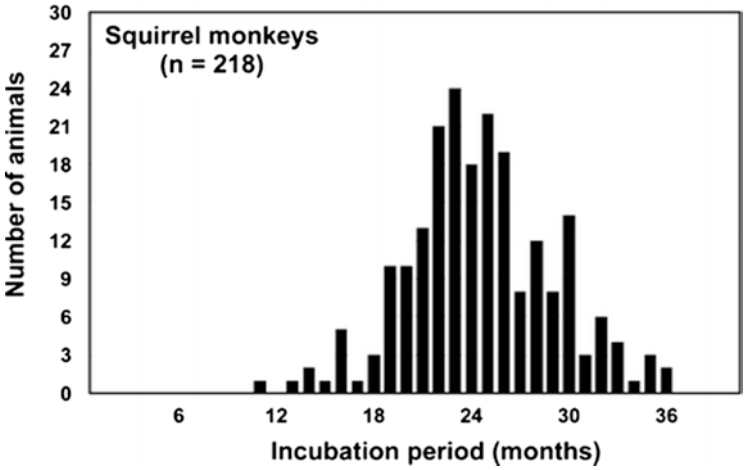

Fig. 1.2 Incubation periods in 218 squirrel monkeys inoculated intracerebrally with human CJD brain homogenates

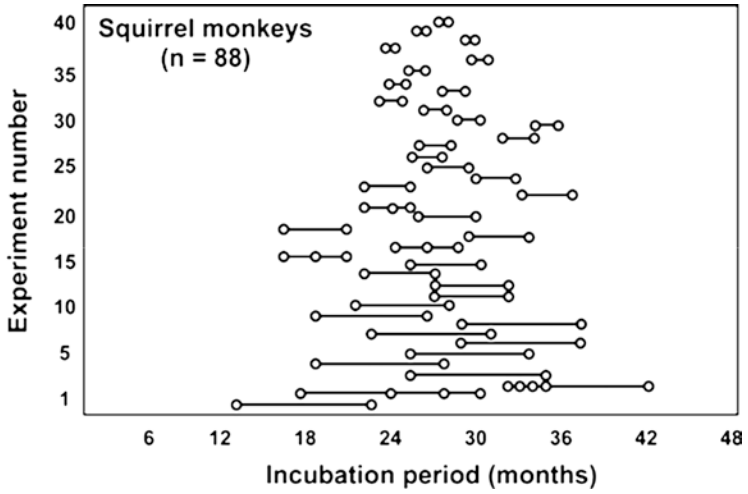

Fig. 1.3 Incubation periods in 40 experiments in which replicate (or in a few cases, more than two) squirrel monkeys were inoculated intracerebrally with the same human CJD brain homogenate

about 30 miles north of the NIH in Frederick, MD. Transmission experiments on non-primate species were mostly conducted at a spacious farm-like facility in Otisville in southern New York State. It is to the everlasting credit of Dr. Joseph Smadel, NIH Associate Director who had earlier been Gajdusek's chief at the Walter Reed Army Institute of Research, and Dr. Richard Masland, Director of the NIH Institute of Neurological Diseases and Blindness, to have at its inception approved and assisted in this gigantic undertaking.

1.10 Clinical and Epidemiological Precisions

During the 1970s, the unassailable criterion of transmissibility led to an appreciation of the range of clinical syndromes associated with CJD and made it possible, finally, to define the essential features with a precision that had hitherto been impossible. This evolving understanding was recorded in several papers based on larger and larger numbers of cases culminating in a synthesis based on 300 transmitted cases of transmissible spongiform encephalopathy published in 1994 (Brown et al. 1994a). During this period, the two remaining members of the quartet of human spongiform encephalopathies were also found to be transmissible: GSS in 1981 (Masters et al. 1981) and fatal familial insomnia (FFI) in 1995 (Tateishi et al. 1995). However, the need for diagnostic verification of cases by transmission studies was, in most instances, abolished by the twin discoveries of a high level of protein kinase inhibitor (14–3–3) in the spinal fluid with a diagnostic specificity >90%, and of a specific pathognomonic amyloid protein (PrP^{TSE}) in brain tissue that could be detected by ELISA or Western blot.

In stark contrast to the multiple transmissions of each of the spongiform encephalopathies, not a single transmission followed similar inoculations of any non-spongiform neurological disease (including Alzheimer's disease, Pick's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis) or a wide variety of non-neurological diseases of unknown etiology like sarcoidosis, lupus erythematosus, Crohn's disease, and rheumatoid arthritis (Table 1.3). It is sometimes forgotten in the present-day impulse to demonstrate the transmissibility of Alzheimer's disease, using various "seeding" techniques and genetically altered susceptible mice, that over 100 cases of neuropathologically verified Alzheimer's disease have been inoculated into primates with uniformly negative results (Brown et al. 1994a). Thus, whatever the similarities between the two diseases (and there are many), inoculation of host species closely related to humans under conditions typically used to demonstrate infectivity simply does not transmit disease, and any claim that Alzheimer's disease is infectious must contend with these consistently negative results. Stated another way, facilitating or

Table 1.3 Disease categories of referrals to the NIH laboratory for transmission studies

Disease category	Number of cases	Number of animals	Observation period (years)	Number of transmissions
TSE	440	1914	1–21	291
Alzheimer's disease	105	240	1–24	0
Other neurodegenerations	115	224	1–30	0
Other neurological diseases	453	1040	1–26	0
Non-neurological diseases	53	76	1–30	0
Total	1166	3494	–	291

accelerating disease in animal models of Alzheimer's disease should not be confused with causing disease in humans.

Given the experimental transmissibility of sporadic CJD and the increasing repertory of cases referred to the NIH, it was not long before the question of human contagion arose, which led to a burgeoning series of epidemiological studies beginning in 1971 with Giovanni Alemà's search for cases of CJD in Italy (Alemà 1971). This was really only a "sketch" that served to inaugurate the much larger canvases to come, but Alemà deserves credit for first recognizing the need to look at epidemiology, a fact that is almost never cited. Brian Matthews and Robert Will substantially extended the epidemiological exploration of CJD in a systematic 5-year retrospective study in England and Wales (Will and Matthews 1986), and Françoise Cathala and the author followed with an even more intensive 10-year investigation of CJD in France (Brown et al. 1987). With the appearance of variant CJD (vCJD) in 1996, the entire European community, together with individual countries elsewhere in the world (e.g., Argentina, Australia, Canada, and Japan), established a coordinated program of surveillance that continues to this day. The most important results with respect to sporadic CJD are that it occurs worldwide in a random distribution at an average annual incidence of about one to two cases per million population, with additional outbreaks of iatrogenic and bovine-sourced disease.

Beginning in the 1970s, some cases of apparently sporadic disease began to be recognized as having an iatrogenic origin, at first in operative procedures involving contamination of a corneal graft and a neurosurgical stereotactic electrode, and later on a much larger scale from cadaveric sources of human growth hormone and dura mater grafts. A more recent cause of iatrogenic disease has come from secondary infections in recipients of packed red cell donations from individuals incubating vCJD in a pre-symptomatic stage of the disease. Three primate transmission experiments contributed to knowledge about iatrogenic disease by demonstrating infectivity (1) on the "sterilized" stereotactic EEG needle; (2) in one lot of human growth hormone; and (3) in leukocytes during the preclinical phase of disease of an experimentally inoculated chimpanzee.

1.11 Therapeutic Essays

Studies having potential relevance to therapy may be said to have begun with early experiments on the resistance of the scrapie agent to physical and chemical treatments. Unfortunately, the pathogen was far more resistant than its host to heat, radiation, and chemicals, and the most effective treatments (now used for ex vivo disinfection) such as autoclaving, or exposure to strong solutions of NaOH (lye) or NaOCl (bleach), although obviously irrelevant for therapeutic considerations, provided a clue to the challenges that lay ahead. Furthermore, in the era of pre-molecular biology, when the etiology of TSE was thought to be an unconventional virus, all such trials were mere shots in the dark, in the hopes that something that worked on viruses might work equally well on TSE infections. All failed to qualify as practical

therapeutic agents, although two categories—polyanionic compounds and polyene antibiotics—were found to prolong the incubation period of scrapie-infected laboratory rodents when given at or near the time of infection. Because this is almost never known in human infections, even the occasional promising results in experimental animals could not be realized in humans (Brown 2010).

With the development of ex vivo infectivity assays, a few such chemical agents were found to reverse or even abolish cell culture infections, and it became tempting to move from these successes directly to human trials without the intermediate step of animal experiments. The recent experience with quinacrine dramatically illustrates the error of this haste, as subsequent experiments in animals confirmed its failure to affect the disease in humans.

Some elegant strategies involving genetic manipulations or prophylactic vaccines in mice are unfortunately either impractical or of limited use in humans. Another conceptual approach of targeting chaperones rather than the prion protein itself is in its infancy. Whatever the prospective treatment, it is first going to have to pass the stringent test of efficacy in already symptomatic experimental animals before any human therapeutic trial is undertaken, unless a reliable assay for preclinical infection in humans becomes available. The topic is thoroughly reviewed in another chapter of this book (Knight this volume).

1.12 The End of an Era

If the 1970s were about biology, and the 1980s saw a transition to molecular biology, the 1990s can be considered the decade of molecular genetics. Since the time of Jakob and Gerstmann, it had been known that CJD could in rare cases also assume a familial form and that the even rarer occurrence of GSS was always restricted to families. With the discovery in the 1980s of a host gene that encodes the normal "prion" protein, the time had come to search for mutations responsible for familial forms of human spongiform encephalopathy. Identification of the first such mutation was reported by Karen Hsiao et al. in 1989—at codon 102 in a family with GSS (Hsiao et al. 1989).

By the turn of the century, over 30 different mutations had been identified (there are now more than 60), and here again, Gajdusek played a major role because of his extensive global contacts and the efforts of a small research team led by Lev Goldfarb, which first identified the polymorphism at codon 129 (Goldfarb et al. 1989), then what were to become the two most common PRNP mutations worldwide at codons 200 and 178 (Goldfarb et al. 1991a, 1992), as well as several other more restricted mutations among the many being identified in other laboratories. In collaboration with Robert Petersen in Pierluigi Gambetti's laboratory, they also discovered the determining influence of codon 129 on whether the codon 178 mutation would result in the clinical syndromes of CJD or FFI (Goldfarb et al. 1991b) and, of historical interest, identified the codon 178 mutation in the original CJD family

reported by Jakob (Brown et al. 1994b), and the codon 102 mutation in the original GSS family reported by Gerstmann (Hainfellner et al. 1995).

As the decade progressed, and the NIH primate program wound down, molecular research—both biological and genetic—overtook the dwindling number of "classical" transmission experiments in both quantity and importance, and genetically embellished mice came to be the preferred method for exploring a number of remaining issues related to host susceptibility and pathogenesis. There is currently an understandable tendency to equate the detection of PrP^{TSE} by protein amplification methods, or transmissibility in humanized transgenic mice, with a risk of "real-life" transmission. Until this assumption is confirmed by transmission to normal animals under natural experimental conditions, this risk remains speculative, and the most appropriate animals for such confirmatory experiments are primates.

Gajdusek retired from the NIH in 1996, and most of the laboratory staff either found other employment or retired. Gibbs stayed on until his death in 2001, and the author remained until 2004, bringing to a close the largest, longest, costliest, and possibly most fruitful experimental animal study ever undertaken in the field of medical science. Gajdusek died sometime after 4 p.m. on December 11, 2008, at the age of 86. The last page of his journal contains the following two entries

December 11, 2008

10 a.m. Psychology and Law Library, University of Tromsø

I am at my library office trying to sort out my life. I'm much better placed than at my crowded hotel room desk. I have most of my mailing done. Now I can concentrate on getting a recorder to play my CDs. What luxury I live in!

To bring 2008 to a close is my current goal. I dare not contemplate much further. I would like to finish some further journals, but that is appearing unlikely. To have lived into my 86th year is much more than I ever anticipated or planned on. Now, I wonder what I should do. My life is essentially finished.

I've mailed a check to Yavine and hope all is well with him. The only outstanding payment is my lost check to Magame. I will attend to that shortly. Now, to get off these mundane matters, and back to serious thoughts. To start listening to the Gregorian Chants and early Baroque music I have on hand is my first priority. That should bring me back to this world.

4 p.m. Clarion Hotel, Bryggen Tromsø

Returned from the University where I copied pp. 120–164 of ledger XVIII for the last 11 of the individual archivists, which is a prodigious sharing of my current journal with 38 individuals.

These last "mundane matters" nevertheless bear witness to an abiding generosity towards the Oceanic family he had nurtured, an undiminished range of intellectual and esthetic sensibility, and a clear presentiment of mortality, aware of what he had accomplished and what he was leaving behind. His journal, begun during childhood, grew to more than 70 volumes containing over 10 million words and was still growing at the time of his death, bringing to a close the daily record of one of the most distinguished scientific careers of the twentieth century.

Acknowledgments The author apologizes to the many scientists whose names and contributions were omitted from this review due to the constraints of covering a very large subject in a very small space. However, he cannot omit special thanks to Drs. R. Bradley and R. Kimberlin for their careful critical reviews.

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Part II
General Aspects of Prions

Chapter 2
The Rich Chemistry of the Copper and Zinc Sites in PrP^C

Glenn L. Millhauser

Abstract Research over the last two decades demonstrates clearly that the function of the cellular form of the prion protein, PrP^C, is related to its ability to bind copper and zinc. Zinc (Zn^{2+}) coordination is homogeneous and localized to the octarepeat domain, with participation of the histidine side chains. In contrast, copper uptake is complex and dependent on the oxidation state of the metal ion (Cu^+ or Cu^{2+}) and its concentration. This chapter will cover a brief history of PrP^C–metal interactions leading to the current structural models, Cu^{2+}-promoted structural features that protect against PrP^C neurotoxicity, a recently recognized relationship between Cu^{2+} coordination and inherited prion disease arising from octarepeat inserts, assessment of PrP-copper electrochemical features, with insight into the basis of PrP^C neuroprotection and transmembrane signaling, and recent findings of how copper participates in the regulation of PrP^C proteolysis.

Keywords Prion · Zinc · Copper · Nuclear magnetic resonance · Electron paramagnetic resonance · Proteolysis · Protein structure · Neurotoxicity · Electrochemistry · Electrophysiology

2.1 Introduction

Research over the last two decades continues to find remarkable functional roles for the normal cellular form of the prion protein (PrP^C). PrP^C supports myelin development (Bremer et al. 2010), influences sleep-wake cycles (Tobler et al. 1996), is upregulated at sites of ischemic injury (McLennan et al. 2004), promotes neuron

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development (Kanaani et al. 2005), protects nerve cells against chemical and oxidative assaults (Klamt et al. 2001; Rachidi et al. 2003), and modulates select transmembrane proteins (Watt et al. 2012; Kuffer et al. 2016; Evans and Millhauser 2017; Salzano et al. 2019). Although one cannot yet assign a sole function to PrP^C as, say, a signaling molecule, enzyme, or transporter, it is clear that the protein is required for normal neurological function. Most functional investigations link PrP^C to metal ion binding, specifically to copper and zinc. This link was emphasized in an elegant X-ray fluorescence study that examined the spatial location and relative levels of iron, copper, and zinc in mouse brain (Pushie et al. 2011). Comparison of wild-type, PrP knockouts (KO), and 20× overexpressers revealed remarkable differences in specific brain regions, with each metal ion exhibiting a unique PrP-dependent profile. For example, PrP appears to drive copper levels near the ventricles and thalamus, whereas zinc is upregulated in cortical regions. And while there is scant evidence suggesting that PrP^C directly binds iron, its levels are nevertheless influenced by PrP expression, perhaps suggesting a relationship between distinct metal transporters, as established in yeast (Bleackley and Macgillivray 2011).

This chapter will begin with a brief historical review of the PrP metal ion literature, with emphasis on works that frame current thinking. Next, I will describe the biophysical features of the copper and zinc sites in PrP^C. Unlike most other metal binding proteins that present a single, well-defined high-affinity site, PrP responds dynamically with a rich variation of coordination modes that depend on metal concentration and the presence of competing species. Recognition of these distinct coordination modes provides new insight into inherited disease resulting from octarepeat inserts. I will also describe electrochemical work that not only provides a detailed characterization of PrP-copper redox properties but also suggests a mechanism for PrP-mediated signaling. This chapter will conclude with new findings that reveal the role of metal ions in PrP^C proteolysis.

2.2 Brief History

PrP^C is able to bind both copper and zinc, but most studies emphasize the specific interaction with Cu^{2+}. (Note: Copper possesses two common, biologically relevant oxidation states: Cu^+ and Cu^{2+}.) Hornshaw et al. recognized that the histidine-rich octarepeat domain, containing four tandem PHGGGWGQ segments, would likely bind Cu^{2+}, and demonstrated this directly with mass spectrometry (Hornshaw et al. 1995a, b). Moreover, they showed a persistent 1:1 complex, although it was also noted that the OR region could take up additional equivalents. Next, using circular dichroism (CD), which detects conformational changes, and fluorescence quenching, they estimated a Cu^{2+} dissociation constant in the low micromolar range (Hornshaw et al. 1995a, b).

In 1997, Brown et al. published a landmark study that clearly identified a physiological connection between PrP and copper (Brown et al. 1997). First, using a peptide corresponding to the PrP N-terminal domain, PrP(23-98), they showed that

the protein takes up multiple Cu^{2+} equivalents with positive cooperativity, described by an unusually high Hill coefficient. Estimated affinity was higher than initially found by CD, as reflected in a low, submicromolar dissociation constant. Brown and colleagues further compared brain copper levels between wild-type and KO mice and reported a severe reduction in brain copper in the transgenics. Many aspects of this work have been revisited in the last 20+ years, but there is little doubt that this initial publication firmly established PrP^C as a copper metalloprotein.

The lowered copper content in the mouse KO suggested that perhaps PrP^C functions as a transporter. PrP^C is attached to membrane surfaces through a GPI anchor and is cycled from the extracellular space to early endosomes through endocytosis, with approximately 90% of the protein returned to the surface by exocytosis. As monitored in N2a mouse neuroblastoma cells, Pauly and Harris showed that addition of 200 μM copper stimulated rapid PrP^C internalization, while removal of the metal ion allowed the protein to redistribute back to the membrane surface (Pauly and Harris 1998). Elimination of the octarepeats, or the His residues within the repeats, fully disrupts these copper-dependent processes (Perera and Hooper 2001). Similarly, certain mutations in the octarepeat domain that give rise to familial prion disease also interfere with copper-stimulated endocytosis (Perera and Hooper 2001). Collectively, these findings suggest that PrP^C may play a key role in copper trafficking. However, early examinations of tissue copper, and copper protein activity, in brain fractions derived from wild-type and transgenic mice possessing different levels of PrP^C failed to find a correlation between PrP^C expression and copper levels (Waggoner et al. 2000). Consequently, this promising line of research did not progress. However, the X-ray fluorescence imaging work described in the "Introduction" section, certainly motivated a renewed look at the role of PrP^C in neuronal copper distribution.

In parallel to cellular assays were several notable structural and biophysical investigations (Stöckel et al. 1998; Viles et al. 1999; Aronoff-Spencer et al. 2000; Van Doorslaer et al. 2001; Burns et al. 2002, 2003; Garnett and Viles 2003; Valensin et al. 2004; Chattopadhyay et al. 2005). Early work focused primarily on the octarepeat domain, although newer research finds copper sites outside of this region. Viles et al. performed a wide array of spectroscopic experiments including CD, nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR) (Viles et al. 1999). This work demonstrated a 1:1 stoichiometry between each histidine (His) containing repeat segment and Cu^{2+} and suggested a micromolar dissociation constant. Moreover, they identified a strong pH dependence, with tight copper binding only at pH 6.0 and above. These findings have endured many follow-up studies. To account for cooperative uptake, they proposed a ring-like structure of alternating His imidazole side chains and Cu^{2+} ions. While there is precedence for this type of structure in the inorganic chemistry literature, it is now considered unlikely to be a significant biological conformation.

Most copper-binding proteins exhibit a very high affinity, reflected by a low dissociation constant (K_d). For example, the K_d for copper at the active site of superoxide dismutase is approximately 10^{-14} M. Early work with PrP N-terminal peptides pointed to a much weaker affinity, suggesting that perhaps PrP might not take up

copper in vivo. This was addressed with detailed MS and fluorescence assays to carefully assess copper binding thermodynamics in full-length PrP (Kramer et al. 2001). Analysis of the observed fluorescence quenching revealed both affinity and detailed stoichiometry, with five Cu^{2+} per protein. Copper uptake showed positive cooperativity with the last equivalent exhibiting a K_d of ~2 μM, well below the level of Cu^{2+} in blood estimated at 18 μM. It is not clear, though, how relevant the comparison to blood copper levels is, given that high levels of PrP are localized to extracellular pre-synaptic surfaces in the CNS (Herms et al. 1999). As will be discussed, more recent analyses find specific binding modes that display very high affinity, below 1.0 nM, and thus further establishing that PrP takes up Cu^{2+} in vivo.

Several recent investigations point to the role of PrP^C as a metal-ion-dependent modulator of signal transduction. For example, Watt et al. demonstrated that Zn^{2+} binding to PrP^C enhances zinc transmembrane transport through the AMPA receptor, a member of the multi-subunit glutamate receptor family (Watt et al. 2012, 2013). PrP^C has also been found to modulate transmembrane currents through NMDA receptors in a copper-dependent fashion. Specifically, copper-occupied PrP^C reduces the NMDA receptor's sensitivity to glycine, a ligand that otherwise promotes persistent cationic currents (Stys et al. 2012; You et al. 2012). Legname and coworkers find that PrP^C exhibits neuronal growth factor activity controlling direction and rate of neurite projections (Kanaani et al. 2005; Nguyen et al. 2019). This function is abolished by mutagenesis of the histidine residues required for copper and zinc coordination.

2.3 Features of Cu^{2+} and Zn^{2+} Coordination in PrP

Copper binds within PrP's N-terminal region, with the relevant segment from the human sequence shown below:

PrP (51-111) PQGGGGWGQ(P**H**GGGWGQ)4GGGT**H**SQWNKPSKPKTNMK**H**

There are five tandem eight-residue repeats, each with the canonical sequence PXGGGWGQ, but in the first repeat, a Gln fills the X position. Since the imidazole side chain of histidine is required for copper uptake, the first repeat does not participate in copper coordination. Thus, from a sequence or genetics perspective, there are five N-terminal octarepeats, but from a metal ion coordination perspective, there are four. Beyond the octarepeat domain, copper also interacts with high affinity at the His residues at positions 96 and 111 (Jones et al. 2005, Walter et al. 2009). The current consensus is that all copper coordination is within the segment PrP(61-111) (human) bounded by the histidines (His, bold H) in the sequence shown above.

A number of early investigations used peptide design, NMR, mass spectrometry, circular dichroism, Raman spectroscopy, molecular modeling, and related biophysical approaches to develop insight into the structure of the Cu^{2+}-octarepeat complex. Ultimately, though, EPR provided the essential insights leading to the current models. EPR is sensitive to the chemical environment at paramagnetic Cu^{2+} centers and,

through hyperfine couplings to copper's unpaired electron, can directly reveal nearby nuclei and atomic features of the coordination environment. Details of the relevant EPR techniques have been reviewed elsewhere (Millhauser 2004, 2007); a summary of the coordination features is given in Fig. 2.1. The copper coordination environment depends critically on the ratio of copper to protein. At low copper concentrations, the four octarepeat His imidazole side chains bind simultaneously to a single Cu^{2+}, as shown in the figure and inset (Chattopadhyay et al. 2005). This is often referred to as the low occupancy binding mode or "component 3," based on component analysis of the EPR spectra. The affinity for this mode is very high, with a dissociation constant of approximately 0.10 nM (Walter et al. 2006).

At intermediate Cu^{2+} concentration, the octarepeats take up two copper equivalents, with each coordinated by two His side chains (not shown) (Chattopadhyay et al. 2005). At high copper concentrations, the octarepeat domain saturates at four equivalents, with each His binding to a single Cu^{2+}, as shown in Fig. 2.1 (Aronoff-Spencer et al. 2000; Burns et al. 2002, 2003; Chattopadhyay et al. 2005). This high

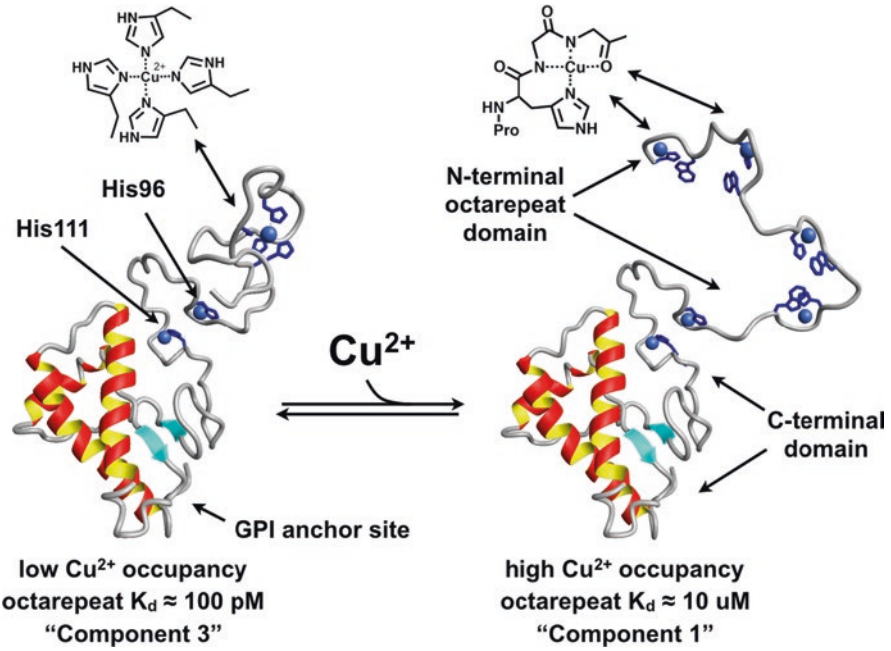

Fig. 2.1 Structural features of PrP^C at low and high Cu^{2+} concentrations. The C-terminal domain is helical, whereas the N-terminal domain is flexible and able to restructure to accommodate different copper coordination modes. At low [Cu^{2+}], the metal ion coordinates to sites localized to His96 and His111. In addition, a single equivalent of Cu^{2+} binds within the octarepeat domain, coordinated by the four His imidazole side chains ("component 3," details shown in the inset). The affinity in the octarepeat domain is high, as characterized by a low K_d of approximately 100 pM. At high [Cu^{2+}], the octarepeat domain restructures to take up four copper equivalents, each coordinated to single His side chain and backbone nitrogens ("component 1," inset). The affinity for this coordination mode is lower than that of component 3

occupancy binding mode is referred to as "component 1." The copper affinity for this state is lower than that of component 3, with a dissociation constant of approximately 10 μM (Walter et al. 2006). The specific coordination features of this high occupancy site, shown in the inset, were determined by isotopic labeling, in combination with a range of EPR techniques (Aronoff-Spencer et al. 2000), and confirmed by X-ray crystallography of the Cu^{2+}-HGGGW complex (Burns et al. 2002).

The specific features of the component 1 site are unusual compared to previously characterized protein copper sites. In most copper metalloproteins, the metal ion is coordinated to His or Cys side chains. For example, copper superoxide dismutase contains the metal ion with four tetrahedrally placed His imidazoles. As seen in the Fig. 2.1 inset, the Cu^{2+} ion coordinates to the His side chain, the deprotonated amide nitrogens of the two Gly residues that immediately follow the His, and a Gly carbonyl. In addition, there is an axially coordinated water molecule that hydrogen bonds to the Trp indole hydrogen (not shown). A coordination sphere with deprotonated amides has been seen previously with the N-terminal copper binding segment of albumin (Harford and Sarkar 1997), and also in peptides, but not in the interior polypeptide segments of a protein. The involvement of amide nitrogens confers significant pH sensitivity since an increase in the H^+ concentration (lower pH) protonates at the nitrogen and competes with copper complexation. Consequently, high occupancy copper binding is unstable below pH ~ 6.0. It has been proposed that this might provide a chemical mechanism for release of Cu^{2+} in the endosomal compartments (Burns et al. 2002).

In addition to Cu^{2+} uptake in the octarepeats, there are two additional binding sites localized to His96 and His111 (human PrP numbering), and these also exhibit sub-nanomolar affinity. These two sites are often referred to as the "5th sites," since early studies suggested that only the involvement of His96, beyond that of the four sites in the octarepeat domain (Burns et al. 2003). We prefer to label these as "non-octarepeat" coordination sites, thus underscoring their distinct location and chemical properties (Walter et al. 2009). At both of these non-octarepeat sites, copper coordinates to the imidazole side chain, the His backbone nitrogen, and two additional backbone nitrogens from the residues on the N-terminal side of the His (Burns et al. 2003). Affinity at these sites is high with a K_d that is similar to that found for the multi-His component 3 mode in the octarepeat domain. Titration studies show that these non-octarepeat sites take up copper simultaneously with component 3 (Walter et al. 2009). Once PrP^C is saturated with Cu^{2+}, the octarepeat domain restructures to component 1 coordination, thus enabling additional binding equivalents, as shown in Fig. 2.1.

Like copper, zinc also binds to PrP^C and stimulates endocytosis (Pauly and Harris 1998). Because this metal ion is found only as diamagnetic Zn^{2+}, EPR is of limited use in directly evaluating its coordination features. To address this, we applied several complementary approaches. First, using an octarepeat peptide, as well as full-length PrP^C, we competed Zn^{2+} against Cu^{2+} and monitored by copper EPR. Interestingly, we found that regardless of concentration, Zn^{2+} was not able to displace Cu^{2+}, which shows that copper has a much higher affinity than zinc (Walter et al. 2007). However, Zn^{2+} was able to influence the Cu^{2+} coordination mode,

shifting the distribution to favor component 1 binding. Next, we tested Zn^{2+} coordination to a range of octarepeat-derived peptides and monitored binding with the reagent diethylpyrocarbonate (DEPC) (Walter et al. 2007), which chemically modifies free imidazole groups, but only if they are not involved in metal ion coordination. Analysis by mass spectrometry showed protection against DEPC modification only with the full octarepeat domain. Collectively, these experiments demonstrate that Zn^{2+} coordinates to the four octarepeat His imidazoles, equivalent to that observed for Cu^{2+} in its low occupancy mode. With a K_d of approximately 200 μM, the affinity is substantially lower than any of the coordination modes found for Cu^{2+}. However, because Zn^{2+} competes with Cu^{2+}, it is able to influence copper coordination in a concentration-dependent fashion. These results, summarized in the scheme in Fig. 2.2, show that when copper levels are low, PrP can simultaneously bind both copper and zinc. At higher copper levels, the protein accommodates the zinc by shifting to the high occupancy binding mode that minimizes the ratio of histidines to copper. However, when no rearrangement can accommodate both zinc and the available copper, it is the zinc that is displaced, not the copper. Finally, Markham et al. used ^{113}Cd (nuclear spin = ½) as a Zn surrogate for NMR studies (Markham et al. 2019). Cd, like Zn, is in group 12 of the periodic table and therefore forms a stable divalent cation. Analysis of the ^{113}Cd chemical shifts and $^2J_{NH}$ scalar couplings confirmed the expected octarepeat His coordination through the ε2 nitrogen.

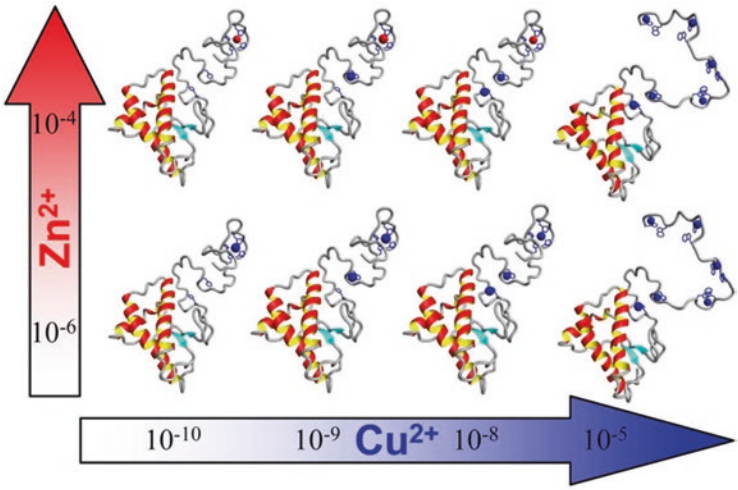

Fig. 2.2 Models representing metal binding in the N-terminal domain of PrP. Top row (High Zinc); Zinc (red) is bound by the octarepeat region (left) while non-octarepeat sites (H96 and H111) are available for copper binding (blue, middle). Copper at high concentrations will displace zinc from octarepeats to form up to four equivalents of component 1 (right). Bottom row (low zinc); copper (blue) is bound by the octarepeats in component 3 when copper is low (left), with increasing copper loads the non-octarepeat sites (middle). High copper (right column) results in component 1 copper binding by the octarepeats. Approximate molar metal concentrations are shown in the arrows

Companion isothermal titration calorimetry (ITC) experiments performed with Zn^{2+} gave a measured K_d between 17 μM and 40 μM, suggesting a somewhat higher affinity than that previously measured by DEPC competition experiments. Interestingly, NMR chemical shifts, binding assays, mutagenesis, and companion molecular dynamics studies implicated the C-terminal residue E199 (E200 in the human sequence) as participating in the Cd second coordination sphere through a salt-bridge with a His imidazole δ1 NH.

It was originally thought that the PrP^C N-terminal and C-terminal domains were structurally independent of each other. Consequently, it was expected that both copper and zinc would interact solely with the octarepeat domain and, in the case of copper, the non-octarepeat segments surrounding His96 and His111, as well. However, the independence of these two protein domains was brought into question by Sonati et al. who showed that C-terminally directed monoclonal antibodies (mAbs) modulated N-terminus-driven toxicity, as demonstrated in both cerebellar organotypic murine brain slices and in mice (Sonati et al. 2013). This led to a functional model of PrP^C, which describes the protein as possessing an N-terminal toxic effector domain and a C-terminal regulatory domain. These results pointed to a physical interaction between the PrP^C N- and C-terminal domains that are responsible for arresting inherent N-terminus-promoted toxicity. Coincident with these findings, Spevacek et al. reported magnetic resonance investigations into potential, Zn^{2+}-mediated higher-order structure in PrP^C (Spevacek et al. 2013). ^1H-^{15}N-HSQC experiments in the presence of Zn^{2+}, which binds solely to the octarepeat domain, found that the presence of the metal ion led to significant line broadening of cross-peak signals from C-terminal residues. Moreover, the affected residues were localized to a well-defined patch on C-terminal helices 2 and 3. Double Electron-Electron Resonance (DEER) EPR of PrP^C with nitroxide labels engineered into the N- and C-terminal domains confirmed that Zn^{2+} addition brings these two protein segments into close proximity. Together, these experiments suggest that the surfaces of C-terminal helices 2 and 3 form a critical patch to which the Zn^{2+} occupied octarepeat binds, in turn suppressing N-terminal PrP^C toxicity. Interestingly, the implicated patch is negatively charged, thus providing an electrostatic driving force for interaction with the Zn^{2+}-occupied octarepeat, and also carries the majority of missense mutations (>60%) that confer inherited prion disease (Spevacek et al. 2013).

It was subsequently shown that copper binding to PrP^C also drives a strong interaction between the protein's N- and C-terminal domains, as shown in Fig. 2.3 (Evans et al. 2016; Wu et al. 2017; McDonald et al. 2019; Schilling et al. 2020). EPR, NMR, and mass spectrometric characterization of this Cu^{2+}-promoted *cis* interaction further supports involvement of the C-terminal patch identified by the prior studies with zinc. Moreover, the epitope of the POM1 mAb, identified as highly toxic by Sonati et al. (2013), overlaps the C-terminal surface that would otherwise contact the copper-occupied octarepeat domain (Evans et al. 2016).

A number of PrP mutants with polypeptide deletions in the central region, between the copper/zinc-binding octarepeat domain and the globular C-terminal domain, are found to be remarkably toxic, producing a neonatal lethal phenotype in transgenic mice (Shmerling et al. 1998). In addition, whole-cell patch clamp

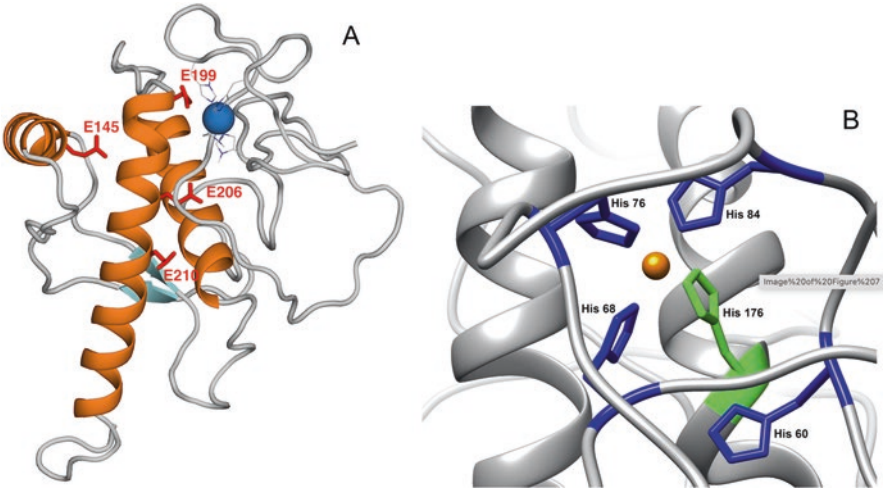

Fig. 2.3 Copper stabilizes a neuroprotective interaction between the N-terminal and C-terminal PrP^C domains. (**a**) Representative structure from an MD trajectory of MoPrP(54–230), with Cu^{2+} bound as multi-His (component 3) in the N-terminal OR. The interdomain structure is stabilized by interaction between the OR-bound Cu^{2+} ion and acidic residues on helix 3 (E199, E206, and E210). (**b**) Detailed EPR analysis finds that Cu^{2+} coordination arises from three octarepeat His residues (blue) and one C-terminal His at position 176 (green)

electrophysiological measurements find that transfection of these mutants in various cell lines and cultured neurons produces large, spontaneous, transmembrane cationic currents, mediated by the polybasic PrP N-terminus (residues 23-31) (Solomon et al. 2010). Rescue of these currents is achieved by co-transfection with wild-type PrP. Of the various deletion mutants studied thus far, Δ105-125 (ΔCR), is particularly toxic, requiring the largest amount of wild-type PrP for rescue. A central hypothesis arising from these studies is that the metal ion-promoted *cis* interaction holds the N-terminal residues away from the plasma membrane, thus restricting the formation of transmembrane pores. This was tested directly with both biophysical and electrophysiological approaches. NMR showed that ΔCR-PrP^C exhibited a substantially reduced Cu^{2+}-promoted *cis* interaction, as indicated by a loss of line-broadened residues in ^{1}H-^{15}N-HSQC spectra (Wu et al. 2017). Cross-linking mass spectrometry showed that Cu^{2+} organizes the N-terminal domain in a conformation that would sequester residues 23-31 away from the plasma membrane (McDonald et al. 2019). In parallel, spontaneous currents from ΔCR-PrP transfected into the N2a neuroblastoma cells were suppressed by the addition of Cu^{2+} in the form of copper-pentaglycine (Wu et al. 2017); however, deletion of the octarepeat domain eliminated current suppression by copper. Given that ΔCR-PrP produces a phenotype consistent with aspects of genuine prion disease, these findings provide compelling evidence that the copper/zinc-promoted *cis* interaction stabilizes PrP^C in its proper, non-neurodegenerative conformational state.

Molecular details of the Cu^{2+}-promoted *cis* interaction are only now beginning to emerge. With component 3 copper coordination (Cu^{2+}:PrP = 1:1), the copper center retains its formal 2+ charge and consequent electrostatic interaction with the negatively charged patch on the regulatory C-terminal domain. Schilling et al. noticed that this patch also possesses two conserved His residues, H139 and H176 (mouse sequence), that might offer further stabilization by direct coordination with the copper center (Schilling et al. 2020). NMR experiments performed on PrP with these residues mutated to Tyr reveal a clear weakening of *cis* interaction, yet, a suite of pulsed EPR experiments find conservation of the four-His coordination shell. Together, these observations demonstrate that the component 3 copper site in full-length PrP^C is comprised of three octarepeat histidines and one C-terminal histidine, as shown in Fig. 2.3. Moreover, whole-cell patch clamp experiments find that elimination of these two His residues in PrP-expressing neuroblastoma (N2a) cells leads to enhanced spontaneous currents. We therefore conclude that copper acts as a bridge linking the PrP effector and regulatory domains and that this interaction is further stabilized by complementary electrostatic forces.

2.4 A Role for Altered Copper Coordination in Octarepeat Expansion Disease

Approximately 10–15% of human TSE cases are inherited and arise from mutations in the open reading frame of the *PRNP* gene (Prusiner 2004). Of these, most are missense mutations in the folded C-terminal domain. For example, the E200K mutation causes midlife development of CJD with most patients dying 6–24 months after onset (Colombo 2000). In addition to these, point mutations are insertional mutations of one to nine PHGGGWGQ segments in the octarepeat domain (Goldfarb et al. 1991). This class of mutations is enigmatic insofar that they modify a region of the protein that is not essential for propagating prion disease. Treatment of PrP^{Sc} with proteinase K cleaves the protein at approximately residue 90, thereby removing the octarepeat domain, but the remaining protease-resistant aggregate retains infectivity. Despite these results, early studies with transgenic mice showed that the PrP octarepeats modulate the disease process. Specifically, inoculated mice expressing a modified PrP^C lacking residues 32–93 develop disease but with longer incubation times than wild-type, produce tissues with lower prion titers and a reduced presentation of prion plaques (Flechsig et al. 2000).

Disease progression in individuals with octarepeat expansions depends on the number of inserts. Individuals with one to four extra octarepeats develop disease with an average onset age of 64 years, whereas five to nine extra octarepeats result in an average onset age of 38 years, a difference of almost three decades (Croes et al. 2004; Kong et al. 2004). A number of previous studies examined the biophysical properties of expanded octarepeat domains with emphasis on either the rate of amyloid production or its uncomplexed backbone conformation (Leliveld et al.

2006, 2008; Dong et al. 2007). However, none of these identified a quantitative link between octarepeat length and age of disease onset.

Given the profound influence of octarepeat domain length on expansion disease, we explored whether the domain's response to copper is altered by insertion number (Stevens et al. 2009). We also reevaluated all known cases of human prion disease resulting from octapeptide insertions and compared the findings to biophysical studies that examined the balance between component 1 and component 3 coordination, as a function of octarepeat domain length. Beginning with statistical data from two existing studies (Croes et al. 2004; Kong et al. 2004), we surveyed the clinical literature, pooled the data, and established a new data set covering approximately 30 families and 108 individuals. Onset age for individual cases are shown in Fig. 2.4a. The red line is drawn at 55.5 years. All cases of up to four octarepeat inserts (eight repeats total) are above this line, and 96% of the cases of five or more octarepeat inserts are below the line. Although there is significant scatter in reported onset age for each specific octarepeat length, the dramatic shift to early onset disease between four and five inserts is apparent. A detailed statistical analysis shows that the results are indeed consistent with the presence of two groups, one composed of individuals with 1 to 4 OR inserts and another of individuals with 5 to 8 inserts (Stevens et al. 2009).

We then performed EPR analysis on a series of PrP-derived constructs from four to nine repeats, corresponding to zero to five insertions. The experiments showed that domains with 4–7 repeats (i.e., zero to three insertions) behave much like the wild-type. However, constructs of 8 or 9 repeats exhibit persistent component 3 coordination. Moreover, these constructs take up approximately twice as much copper as the wild type. Equivalent trends were observed with full-length recombinant

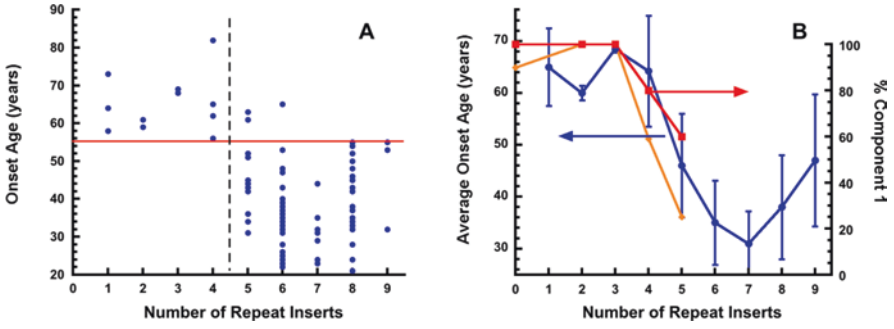

Fig. 2.4 The relationship between onset age for familial prion disease resulting from octarepeat inserts and copper coordination modes. (**a**) Onset age for individual cases as a function of extra octarepeat inserts. Note that wild-type corresponds to four repeats, so three inserts correspond to seven total repeat segments. The horizontal red line is at 55.5 years and represents a statistically defined separation between late and early onset. (**b**) Average onset age, with standard deviation (blue circles, left axis), and component 1 coordination (orange diamonds and red squares, right axis, for 3.0 and 4.0 equivalents Cu^{2+}, respectively) as a function of extra octarepeat inserts. At both copper concentrations, component 1 coordination drops suddenly at approximately the same OR length threshold as average onset age

protein, where we compared wild-type with mutant PrP^C containing 5 repeat inserts. To underscore these findings, we compared the average onset age and standard deviation, as a function of octarepeat length, to Cu^{2+} binding properties. The longest OR expansions favor component 3 coordination and resist component 1. Thus, component 1 coordination serves as a convenient measure of altered Cu^{2+} binding properties. Figure 2.4b shows the relative population of component 1 coordination for each OR construct superimposed on the average age of onset. For wild-type and expansions involving up to seven repeats (three inserts beyond wild-type), component 1 coordination is dominant for both 3.0 and 4.0 equivalents Cu^{2+}. However, at eight and nine ORs (four and five inserts, respectively), the population of component 1 coordination drops precipitously.

These data reveal a remarkable relationship, where decreased onset age and persistent component 3 coordination take place at threshold of eight or more total repeats. It is possible, therefore, that our findings suggest an important protective role for component 1 coordination that may be lost in cases of octarepeat expansion disease with four or more inserts. However, the recent work by Schilling et al provides a different perspective (Schilling et al. 2020). In their analysis of how C-terminal His residues stabilize the protective N-term—C-term *cis* interaction through a bridging copper ion, they recognized that expansion of the N-terminal octarepeats could diminish this otherwise protective interdomain contact. NMR analysis of PrP^C with octarepeat insertions found that up to three additional octarepeat segments did not weaken the observed *cis* interaction. However, at four or five insertions, which marks the transition to early onset prion disease, NMR evidence of the interaction was essentially eliminated. Consequently, with four or more insertions, both component 1 binding *and* the protective interdomain *cis* interaction are reduced. Together, these findings motivate a careful examination of the distinct chemical properties and reactivity of component 1 vs component 3 copper coordination, and further strengthen the hypothesis that the copper-mediated *cis* interaction is critical for arresting inherent PrP^C neurotoxicity.

2.5 Electrochemical Properties of the PrP Copper Sites

Copper's ability to cycle between the Cu^+ and Cu^{2+} oxidation sites is essential for life. For example, cellular respiration relies on cytochrome c oxidase, a copper-dependent enzyme that converts molecular oxygen to water ultimately leading to the production of ATP. Since the earliest studies connecting PrP^C to copper uptake, there has been interest in understanding reduction-oxidation (redox) cycling at the copper sites. One line of inquiry suggests that PrP^C functions as a superoxide dismutase (SOD), which inactivates toxic O_2^- converting it to the more benign hydrogen peroxide (H_2O_2). This hypothesis has been controversial and is reviewed elsewhere (Daniels and Brown 2002; Brown 2009). The connection between copper coordination mode and onset age for octarepeat expansion disease, discussed above, certainly motivates an evaluation as to whether component 1 and component 3 coordination sites give rise to distinct redox properties.

Initial electrochemical studies used cyclic voltammetry to evaluate short single repeat peptides as models of component 1 coordination (Bonomo et al. 2000). Reduction of Cu^{2+} to Cu^{+} was found to be energetically unfavorable, leading to the possibility that PrP^C may stabilize copper in its oxidized form. From a neuroprotective perspective, this could be important since weakly complexed copper readily cycles between oxidation states resulting in the production of reactive oxygen species (ROS) that are often cytotoxic. By stabilizing copper in a single oxidation state, PrP^C may quench this deleterious chemistry.

Component 3 coordination, with four His residues, appears somewhat similar to the active site in SOD and initially suggested that it might readily undergo redox cycling. Redox kinetics, as measured by bathocuproine absorbance, suggested that indeed component 3 was more easily reduced than component 1 (Miura et al. 2005). Building from these results, it was proposed that PrP^C might function in concert with endocytosis as a copper reductase. In this scenario, extracellular Cu^{2+} binds to PrP^C with component 1 coordination, and the complex is internalized by endocytosis. Next, the low pH drives rearrangement in the octarepeat domain to favor component 3 coordination, leading to reduction to Cu^{+}. Finally, the copper is released and internalized through a copper transporter.

In collaborative work with Zhou and coworkers, we revisited the detailed electrochemical features of component 1 and component 3 coordination modes (Liu et al. 2011). The full octarepeat domain with one equivalent of Cu^{2+} served as a model for component 3 coordination. Cyclic voltammetry performed in the presence of ascorbate, with and without oxygen, and under nearly reversible conditions showed facile reduction to Cu^{+}, along with a significant increase in affinity. Thus, as opposed to cycling copper, these data suggest that Cu^{+} is very stable in this low occupancy mode, and unlikely to be reoxidized back to Cu^{2+}. Next, we used the same conditions to examine component 1 coordination and found reduction potentials consistent with a copper center that supports cycling between its oxidation states. However, when we compared the findings to free copper or simple copper-peptide complexes like those found in blood or cerebral spinal fluid, we observed that the reaction was controlled and less likely to produce cytotoxic species such as hydroxyl radicals. Additional assays demonstrated that copper bound to PrP with component 1 coordination, under reducing conditions by ascorbate, gently converts dissolved oxygen to hydrogen peroxide. A summary of these findings is shown in Fig. 2.5.

The ability to bind copper and facilitate redox cycling is shared with the Aβ peptide and α-synuclein, which are causative in Alzheimer's and Parkinson's diseases, respectively. Unlike PrP^C, however, these species exhibit only a single binding mode and, therefore, a single profile for producing hydrogen peroxide. Comparing coordination modes identified for these two neurodegenerative species with those for PrP^C, we find that component 3 in PrP^C is by far the least reactive, producing hydrogen peroxide at the lowest rate, whereas component 1 is the most reactive (Liu et al. 2011). Thus, PrP^C exhibits vastly different electrochemical profiles, depending on copper occupancy. Both modes are neuroprotective, with component 3 coordination completely inhibiting copper redox activity and component 1 regulating activity with the controlled formation of hydrogen peroxide.

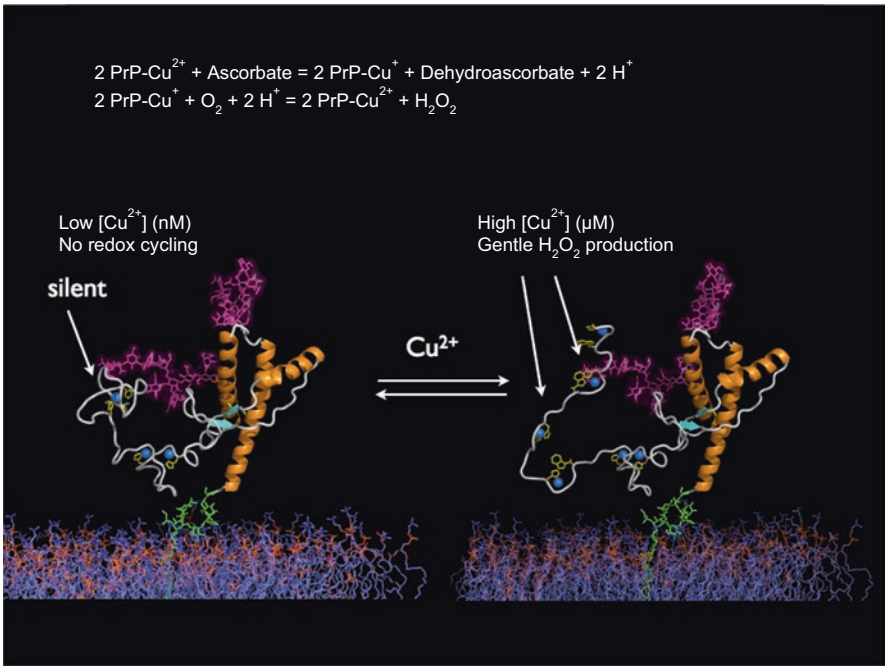

Fig. 2.5 Schematic representation of the possible roles of PrP^C–Cu^{2+} complexes in quenching the Cu^{2+} redox cycling or gradual production of H_2O_2 for signal transduction. PrP is tethered to cell membrane via the GPI anchor (green) with its α-helices in the C terminus shown in orange, N-linked carbohydrates in purple, and the N-terminal copper binding segment depicted in white. When [Cu^{2+}] is at a low level (nM or lower), Cu^{2+} (blue sphere) remains bound in the component 3 mode (left), quenching the Cu^{2+} redox cycling. At higher [Cu^{2+}] (μM) the binding mode transitions to component 1 (right), leading to a gradual and controlled production of H_2O_2

Together, these findings support a role for PrP^C in suppressing copper's inherent redox activity that would otherwise be very damaging to cellular components. However, the discovery that high copper occupancy PrP^C produces hydrogen peroxide suggests additional biochemical control. Similar to nitric oxide, hydrogen peroxide is now considered a signaling species of particular importance in the immune system and also in protein localization (Veal et al. 2007). There are likely several possible mechanisms for H_2O_2 action. For example, PrP^C has been linked to transmembrane signaling (Mouillet-Richard et al. 2000) and it is noteworthy that hydrogen peroxide readily crosses membrane bilayers and inactivates phosphatase and kinase active sites by reaction with catalytic residues.

2.6 Copper Regulation of PrP^C Proteolytic Cleavage

PrP^C undergoes enzymatic cleavage at two well-defined sites leading to detectable truncated forms in vivo. One proteolysis site resides between K109-H110 (mouse sequence), termed α-cleavage, and produces the N-terminal and C-terminal fragments, N1 and C1, respectively. The preponderance of recent evidence suggests that α-cleavage, which separates most of the flexible PrP N-terminus from the folded C-terminus, is due to action from one or more members of the ADAM (A Disintegrin And Metalloproteinase) family of enzymes, specifically ADAM8, ADAM10, and ADAM17. Among these, ADAM8 is established as responsible for α-cleavage in skeletal muscle tissue (Liang et al. 2012). The domains released by α-cleavage exhibit potent activities. The N1 fragment is antiapoptotic, possibly acting through the inhibition of caspase-3 (Guillot-Sestier et al. 2009). Conversely, the C1 fragment promotes apoptosis through p53-dependent caspase-3 activity, although it appears as though the protective effects of N1 significantly outweigh the pro-apoptotic effects of C1 (Sunyach et al. 2007). Perhaps more importantly, substoichiometric levels of C1 protect against PrP^{Sc} propagation.

PrP^C also undergoes β-cleavage, which takes place at multiple sites within and immediately following the octarepeat domain, producing N2 and C2 fragments (Chen et al. 1995). Experiments with different cell lines expressing PrP^C find that levels of C2 are greatly enhanced upon the addition of peroxide, suggesting proteolysis by reactive oxygen species (ROS) generated by intrinsic copper (McMahon et al. 2001; Watt and Hooper 2005). A separate pathway to β-cleavage of PrP^{Sc} is enzymatic, produced by calpains (Yadavalli et al. 2004) and cathepsin (Dron et al. 2010) proteases. In general, β-cleavage is observed in normal brain tissue, but C2 is enriched in prion infection. Unlike the N1 and C1 fragments, N2 and C2 do not show any bioactivity or neuroprotection, although β-cleavage's production of N2 and C2 may indirectly assert a biological effect by prohibiting the formation of N1.

The prevailing paradigm of PrP^C cleavage posits that α-cleavage is enzymatically driven and constitutes normal processing, while β-cleavage results from aberrant copper redox activity and is associated with the development of prion disease. But the identification of several ADAM family enzymes producing α-cleavage, along with the structural features promoted by copper and zinc, motivated a reassessment of PrP^C proteolysis. Interestingly, detailed analysis of the resulting proteolytic products found that α-cleavage does not take place at a single site but, instead, may take place at one of three proximal sites, termed α1, α2 and α3, depending on the specific ADAM enzyme and added metal ion (McDonald et al. 2013). Importantly, both Cu^{2+} and Zn^{2+} suppress β-cleavage, in turn favoring α-cleavage, thereby providing yet an additional mechanism by which these physiologic metal ions inhibit aberrant, neurotoxic signaling of the prion protein (McDonald et al. 2013).

The cumulative findings reviewed here emphasize the complex connection between zinc and copper uptake and the variability in copper binding as controlled by concentration. The relationship between copper coordination modes and the observed onset age for prion disease, which is associated with octarepeat expansion,

suggests that metal ion regulation may also factor into the development of disease. New electrochemical findings provide a foundation for understanding how PrP^C protects cells against oxidative assaults and also reveal a possible mechanism for transmembrane signaling, while detailed studies of PrP^C proteolysis find that metal ions may be crucial for inhibiting deleterious protein degradation pathways. Further refinement of these concepts is sure to lead to a precise function for PrP^C and perhaps new insights into how the loss of function contributes to neurodegenerative disease.

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Chapter 3
PrP Prion Structures

Byron Caughey, Efrosini Artikis, and Allison Kraus

Abstract The biophysical properties of authentic infectious prion protein (PrP)-based mammalian prions have long impeded determination of their detailed structures. However, considerable recent progress has been made using cryo-electron microscopy. Three near-atomic resolution structures of ex vivo prions have now been reported, one of hamster 263K scrapie and the others of wildtype and glycophosphatidylinositol (GPI)-deficient forms of the mouse RML strain. Each of these highly infectious prion fibrils have ordered cores with parallel in-register intermolecular β-stack (PIRIBS) architectures that share major structural motifs. However, the 263K fibril differs from the RML structures in the detailed conformations of those motifs and the overall shapes of the fibril cross-sections. Such motif variations likely contribute to the strain-dependent templating that underpins conformationally faithful prion propagation. In the wild-type prion structures, N-linked glycans and GPI anchors project outward from the fibril surface. The wildtype and anchorless (and severely glycan deficient) RML fibrils have similar folds, indicating that these post-translational modifications do not substantially alter the core structure of this strain. However, in the wild-type structures, the GPI anchors follow the twisting fibril axis and are likely to bind cellular membranes. This binding may contribute to the pathognomonic membrane distortions of wild-type prion diseases. Analysis of the 263K structure with molecular dynamics simulations has suggested a mechanism for the hamster-to-mouse transmission barrier. These initial high-resolution structures provide foundations for understanding prion molecular pathogenesis, but given the multitude of mammalian prion strains, much further work will be required to characterize the full range of prion structures.

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3.1 Introduction

As early as the 1960s, researchers were perplexed by the unusual properties of the scrapie agent and proposed that they might be self-propagating states of proteins (Griffith 1967; Pattison and Jones 1967). In the ensuing decades, the infectious scrapie agent and related pathogens of the transmissible spongiform encephalopathies (TSEs) were dubbed prions (Prusiner 1982), and the protein involved became prion protein or PrP, with the infectious form often called PrP^{Sc} (Prusiner 1998). For nearly seven decades after the first proposals of protein structure-based pathogens, the 3D structures that allow prions to replicate as deadly infectious agents remained enigmatic. One of the key mysteries was how distinct strains can be propagated faithfully, passage after passage, in a single host genotype if prions carried no agent-specific nucleic acid genome. Another question was what, mechanistically, controls transmission barriers when prions are passed from one host genotype to another. In other words, why do some PrP sequence mismatches between hosts matter so much more than others? These mysteries have been difficult to explain with any clarity without detailed knowledge of prion structures. However, near-atomic cryo-EM structures of highly infectious brain-derived prions (Kraus et al. 2021a, b; Hoyt et al. 2021; Manka et al. 2021), as well as much more innocuous synthetic recombinant PrP fibrils (Gallagher-Jones et al. 2018; Glynn et al. 2020; Wang et al. 2020; Li and Jaroniec 2021), have begun to emerge. Here we review those structures and their new mechanistic implications for prion replication, strain fidelity, species barriers, and pathogenesis. We focus on overtly fibrillar forms of prions because those are the only ones for which highly resolved structures are available.

3.2 Development of Initial Parallel In-Register and 4-Rung β-Solenoid Models for PrP^{Sc} Fibrils

The accumulation in the literature of a variety of coarse empirical descriptors of prion fibrils allowed increasingly grounded structural models to be proposed (Groveman et al. 2014; Spagnolli et al. 2019). Ultrastructural imaging indicated that prions could be fibrillar, with properties of amyloids (Merz et al. 1981; Prusiner et al. 1983; Gabizon et al. 1987; Hope et al. 1988; Silveira et al. 2005) while other studies have described infectious units that are smaller than elongated fibrils (Silveira et al. 2005; Tzaban et al. 2002; Sajnani et al. 2012; Vanni et al. 2020;

Cortez et al. 2021). Diffraction studies of ex vivo prion fibrils showed that, as is typical of amyloid fibrils, PrP polypeptide chains run perpendicular to the fibril axis with spacings of ~4.9 Å. Measurements of the intermolecular distances between specific labeled residues in synthetic recombinant PrP fibrils using electron paramagnetic resonance (EPR) (Cobb et al. 2008; Cobb et al. 2007) and solid-state NMR (Tycko et al. 2010; Helmus et al. 2011; Groveman et al. 2014; Theint et al. 2017, 2018; Shannon et al. 2019) provided strong evidence that such fibrils could assemble with parallel in-register intermolecular β-sheet or stack (PIRIBS) architectures. In PIRIBS structures, residues in one molecule are aligned along the fibril axis with the corresponding residues of adjacent molecules in the stack, that is, in-register (Fig. 3.1a). Although these types of studies established that certain PrP residues were within PIRIBS structures in synthetic PrP fibrils, they did not establish the overall folds of the polypeptides.

Nonetheless, based on such initial findings, Groveman and colleagues envisioned PIRIBS-based models for infectious prion fibrils, which typically have much larger proteinase K (PK)-resistant cores and are much more infectious than the synthetic fibrils studied in the EPR and NMR studies (Groveman et al. 2014). More recently, a quite distinct 4-rung β-solenoid (4RβS) model was proposed for the GPI-anchorless RML (aRML) prion fibril based on brain-derived prion fiber diffraction patterns, low-resolution cryo-EM imaging, and H/D-exchange data (Spagnolli et al. 2019) (Fig. 3.1b). Among the key arguments for the 4RβS model were meridional diffraction signals at 9.6, 6.4, and 4.8 Å, corresponding to second-, third-, and fourth-order diffraction of a β-sheet with a 19.2 Å spacing of features along the fibril axis (Wille and Requena 2018). In the proposed 4RβS model, single PrP molecules provide four successive, distinct rungs along the axis of a protofilament (Spagnolli et al. 2019), in contrast to a single rung in PIRIBS models. In such an arrangement, the interfaces between monomers in the stack, for example, would be ~19 to 20 Å and would be consistent with a 19.2 Å diffraction. This model also postulates that two intertwined protofilaments comprise the overall fibril, whereas, in the PIRIBS models, a single PrP molecule spans the entire fibril cross-section. In any case, when these widely divergent PIRIBS and 4RβS models were proposed, there was insufficient empirical data on ex vivo prions to discriminate between these architectures.

3.3 Cryo-EM of Synthetic PrP Fibrils

In the last couple of years, cryo-EM combined with single particle analysis and helical reconstruction (Scheres 2020) has revealed near-atomic resolution structures for recombinant human PrP PrP94-178 (rhu94-178) (Glynn et al. 2020), human PrP23-144 (rhu23-144) (Li and Jaroniec 2021), full-length human PrP23-231 (Wang et al. 2020), mutant full-length human E196K PrP23-231 (rhu23-231 E196K) (Wang et al. 2021), and a much shorter synthetic peptide (residues 168–176) of bank vole PrP (Gallagher-Jones et al. 2018). Importantly, as is true of most of the

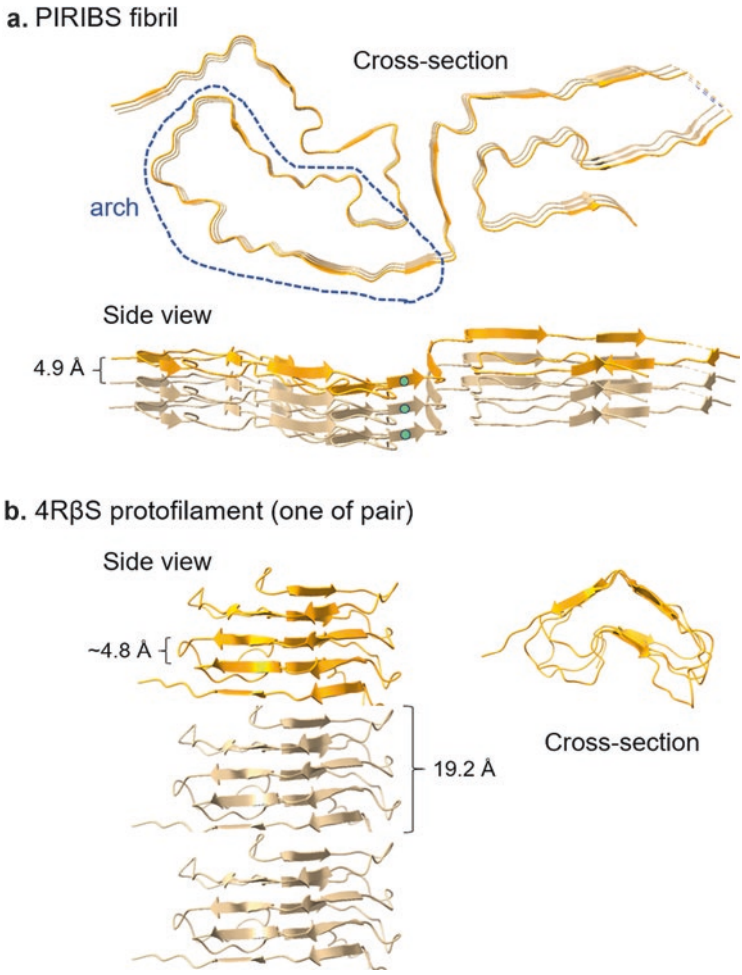

Fig. 3.1 Parallel in-register intermolecular β-sheet/stack (PIRIBS) versus 4-rung β-solenoid (4RβS) models for prion fibrils. (**a**) Trimeric segment of PIRIBS-based fibril as determined by high-resolution cryo-EM of 263K prions (Kraus et al. 2021b). A single monomeric unit is highlighted in orange. In PIRIBS (and not 4RβS) architectures, each amino acid residue in one monomer is aligned with the corresponding residue in the adjacent monomers (aqua blue circles). A dashed line circumscribes a representative arch (the middle arch), by which we mean a loop that bends back on itself. We have previously referred to these motifs as β-arches, but now simply call them arches because some do not meet all of the criteria of β-arches in which sidechains within β-strands on the opposing flanks of the arch interact directly. (**b**) Trimeric stack assembled from a 4RβS protofilament model proposed for GPI-anchorless RML prion based on lower resolution data (Spagnolli et al. 2019). These models were each drawn using PDB coordinates as reported in (Kraus et al. 2021b; Spagnolli et al. 2019) using ChimeraX (Pettersen et al. 2021). In the case of the 4RβS illustration, the published coordinates of the monomer were used and stacked manually using in Powerpoint to depict the concept of a 4RβS protofilament without intending to accurately represent any proposed interfaces between monomers

synthetic fibrils mentioned above, the PK-resistant cores of these fibrils are much smaller than those found in bona fide tissue-derived infectious PrP^{Sc} fibrils. Such synthetic fibrils are likely to be either non-infectious or many orders of magnitude less infectious per unit protein (Li et al. 2018; Kraus et al. 2017; Groveman et al. 2017; Caughey and Kraus 2019). Nonetheless, these studies provided important initial clues to how various recombinant PrP constructs can assemble into fibrils in vitro.

Each of these synthetic PrP fibrils has a PIRIBS architecture. However, their ordered fibrillar cores are comprised of different sequences. Fibrils of the N- and C-terminally truncated rhu94-178 fibrils have two closely packed, symmetrical protofilaments (Glynn et al. 2020). The core of each protofilament contains a β-arch of residues 106–145 (Fig. 3.2). These same residues comprise the ordered core of fibrils formed from rhu23-144, but with a quite distinct conformation and a fibril cross-section comprising four identical protofilaments (Li and Jaroniec 2021). The human PrP23-144 sequence corresponds to that expressed in humans with a form of

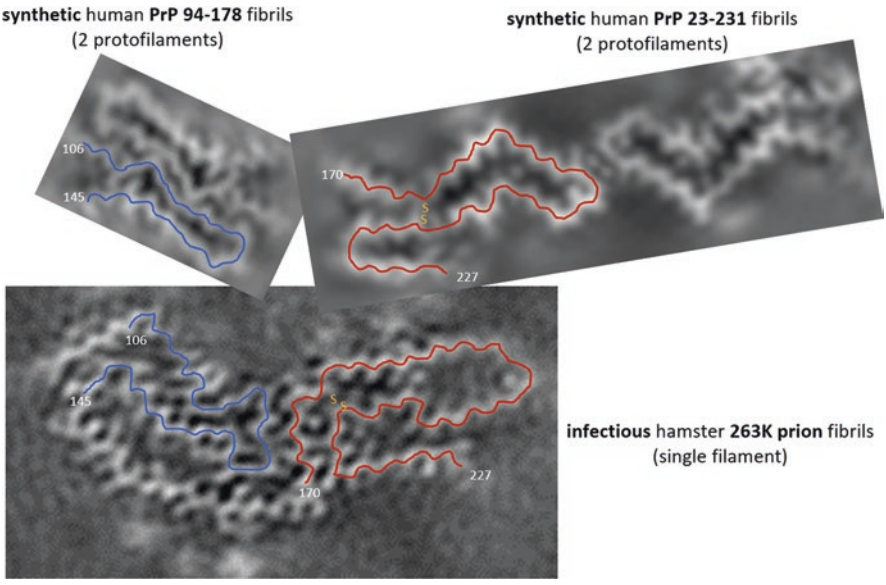

Fig. 3.2 Cross-sections of infectious brain-derived hamster 263K prion fibrils and likely non-infectious synthetic recombinant human PrP fibrils. The underlying images are taken and adapted with permission from projections of density maps derived from single-particle cryo-EM analyses of fibrils of 263K prions (Kraus et al. 2021a, b), synthetic rhuPrP94-178 (Glynn et al. 2020), and rhuPrP23-231 (Wang et al. 2020). Note that, the synthetic fibrils have two identical symmetrically arranged protofilaments, whereas the 263K fibril core is comprised of a single filament. Blue lines trace the polypeptide backbones of residues 106–145 in one of the protofilaments (top left) that, in the 263K structure, form the N arch (bottom panel, also see Fig. 3.3d). Red lines trace backbones of the respective disulfide arches and additional C-terminal strands within residues 170–227 as it occurs in synthetic PrP 23-231 fibrils (top right) and 263K prion fibrils (bottom panel)

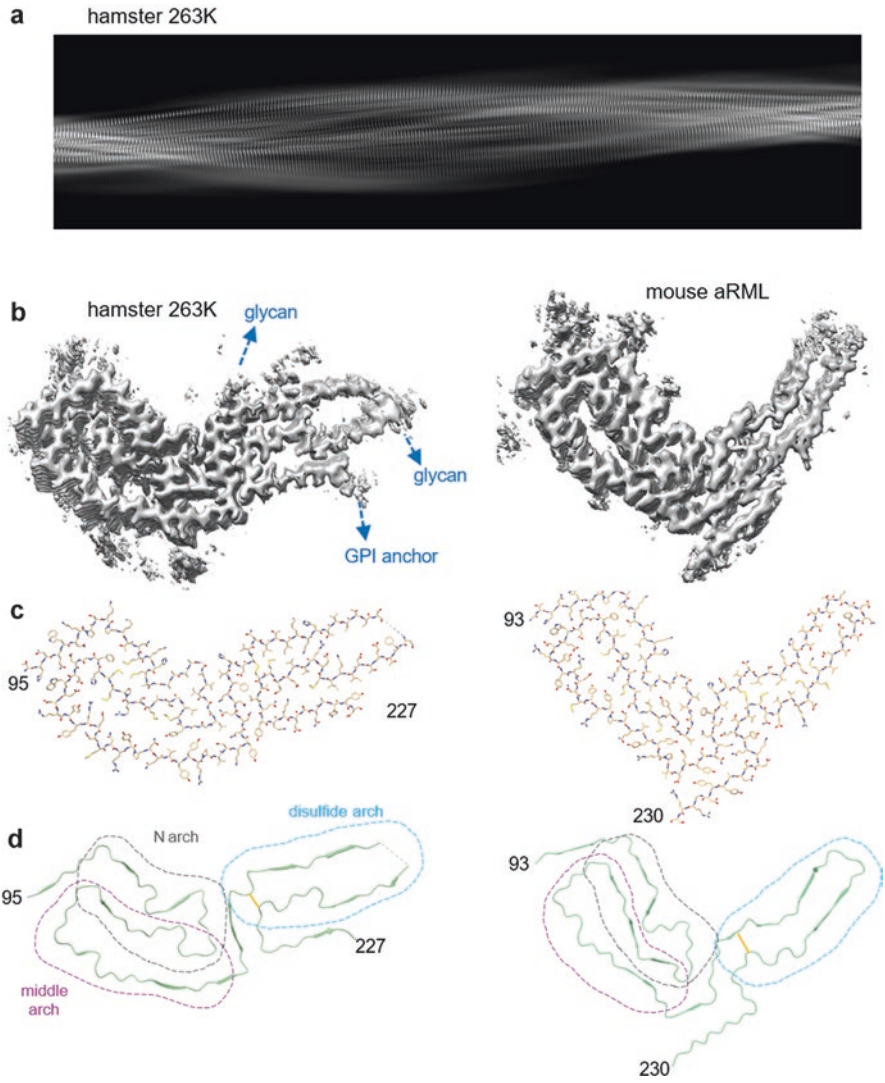

Fig. 3.3 Structures of hamster 263K and mouse aRML prion strains (Kraus et al. 2021b; Hoyt et al. 2021). (**a**) Lateral view of hamster 263K fibril (density map projection). (**b**) Enlarged cross-sectional views of fibril density maps. Presumed positions of the structurally variable and mostly unresolved N-linked glycans and GPI anchor are indicated on the 263K map. aRML is deficient in these post-translational modifications. (**c**) Atomic models (monomeric subunits). (**d**) Ribbon diagrams with structural motifs outlined in the 263K model that are analogous to, but distinct from, those in aRML. Panel **a** adapted with permission from (Kraus et al. 2021b)

Gerstmann–Sträussler–Scheinker (GSS) syndrome linked to expression of PrP with the rare Y145Stop mutation (Ghetti et al. 2018), but whether the respective conformations of the synthetic and in vivo fibrils are similar remains to be determined.

Fibrils derived from rhuPrP23-231 have two protofilaments, with the ordered cores being formed by C-terminal residues 170–229 (Fig. 3.2). In contrast to the rhu94-178 (Glynn et al. 2020) or rhu23-144 (Li and Jaroniec 2021) fibrils, these protofilament cores feature a arch linked at the base by the natural disulfide bond formed between Cys179 and Cys214. This disulfide arch is related to disulfide arches suggested previously by multiple EPR and ssNMR studies of synthetic human and rodent PrP fibrils containing this C-terminal domain (Groveman et al. 2014; Cobb et al. 2007, 2008; Tycko et al. 2010). A disulfide arch also dominates the PIRIBS core of fibrils of the familial human prion disease-linked E196K mutant of rhu23-231 PrP (Wang et al. 2021). However, this arch has a distinct conformation, showing that the disulfide arches can differ between fibrils formed from mutant versus wild-type human PrP sequences.

3.4 Near-Atomic Cryo-EM Structures of Infectious Tissue-Derived Prions

As of this writing, three high-resolution cryo-EM structures of fully infectious, ex vivo prion fibrils have been reported, including those of the hamster 263K scrapie strain (Kraus et al. 2021a, b) and both wildtype (wt) and GPI-anchorless (a) forms of the mouse RML scrapie strain (Hoyt et al. 2021; Manka et al. 2021). Each of these bona fide protease-resistant PrP^{Sc} (PrP^{Res}) preparations was shown to contain approximately 10^9 50% lethal doses (LD_{50}) per mg protein. The wildtype 263K and RML prions also have GPI-anchors and abundant N-linked glycans, whereas the aRML strain is deficient in these post-translational modifications (Chesebro et al. 2005). As noted above, these ex vivo prions have much larger proteinase K-resistant cores than those of the synthetic PrP fibrils described above. Indeed, this span of ~140 to 150 residues of the highly infectious prion fibrils is also larger than those of most, if not all, other neuropathologic protein amyloids.

That said, and consistent with what has been seen so far with synthetic PrP amyloids, the ex vivo prion fibril structures also have PIRIBS cores (Kraus et al. 2021a, b; Hoyt et al. 2021; Manka et al. 2021) (Fig. 3.3) with single monomers comprising the entire cross-sections of these fibrils. Occasionally, laterally aligned duplexes of fibrils can be seen (Hoyt et al. 2021; Manka et al. 2021), but not regularly enough to be resolved as discrete subpopulations by single-particle cryo-EM analysis. Importantly, the aRML and wtRML fibril cross-sections are strikingly similar (compare refs (Hoyt et al. 2021; Manka et al. 2021)), but each is distinct from the 263K cross-section in overall shape as well as conformational detail (Fig. 3.3b–d).

Among the key features of the 263K (Kraus et al. 2021a, b) and RML (Hoyt et al. 2021; Manka et al. 2021) prions are 3 arches. These include two types of arch motifs that are seen, albeit with conformational variation, in synthetic fibrils, that is, those spanning ~113 to 131 and ~170 to 229 (Fig. 3.3d). As we expected from our initial modeling of a PIRIBS architecture for infectious prions (Groveman et al. 2014), the

much larger cores of ex vivo prions have both of these arches at once, whereas the synthetic fibrils have only one or the other. We now refer to the more N-terminal of these arches as the "N arch." As with the synthetic fibrils, we refer to the C-terminal arch as the "disulfide arch" (Fig. 3.3d). An additional feature of 263K and RML prions is another arch, namely the middle arch, that occurs between the N- and disulfide arches. The middle arch shares its N-terminal flank with the N arch. Another shared feature of the 263K and RML prions is a steric zipper between the extreme N-terminal residues of the core against the head of the middle arch.

Although the 263K and RML prion structures share these key structural motifs, their conformational details are substantially different between these strains (Kraus et al. 2021a, b; Hoyt et al. 2021; Manka et al. 2021). Notably, the N arches of 263K and RML fibrils have strikingly different heads or tips despite having identical glycine- and hydrophobic amino acid sequences spanning residues 113–138 (hamster numbering). Possibly, the conformational options of these head regions are influenced by the sequence differences that are immediately N- and C-terminal to the shared stretch of residues in the loop. The C-terminal half of the prion fibril cores of these strains also have marked conformational differences. For example, whereas in 263K the disulfide arch is nearly aligned with the N arch, these β-arches in the RML strains are almost perpendicular to one another, giving the cross-section a V-shape (Fig. 3.3c, d). The extreme C-terminal residues, where the GPI anchors are attached in the wild-type structures, project in opposite directions. In 263K, residues 219–227 flank the disulfide arch, whereas in the RML structures, the analogous residues flank residues 166–171. The otherwise similar aRML and wtRML structures differ in the C-terminal residues that could be assigned in the resolved map, with the ordered cores of aRML and wtRML extending to residues 230 and 225, respectively. This may be due to presence of structurally heterogeneous GPI anchors on the latter, which may compromise the resolution of the adjacent residues. Similarly, the resolved amyloid core for 263K prions (95–227) did not include the extreme C-terminal residues linked most closely to the glycolipid.

With respect to the mechanism by which these prions grow, the cross-sectional differences between the 263K and the RML prion fibrils clearly give them distinct templates on the fibril tips where the incorporation of new monomers occurs (Kraus et al. 2021a, b; Hoyt et al. 2021; Manka et al. 2021). Presumably contributing to these distinct templates is the difference in sequence between the hamster and mouse PrP sequences at 8 positions within the fibril core (e.g., see Figure S8 of (Kraus et al. 2021b)). The purely conformational, as opposed to sequence, determinants of prion strain should be clarified by analyses of strains isolated from hosts of the same genotype.

3.5 PrP^{C} to PrP^{Sc} Conversion

Given the respective structures of PrP^{C} and PrP^{Sc} that are now known, it is clear that complete refolding of the secondary and tertiary structures of PrP^{C} is required (Fig. 3.4) (Kraus et al. 2021a, b). The steps involved, and the involvement of monomeric or oligomeric intermediates, remain unclear. Among the major conformational changes that must occur are dissociation of the PrP^{C}'s β1-Helix 1-β2 loop from Helices 2 and 3 (Kraus et al. 2021a, b; Hoyt et al. 2021; Manka et al. 2021), a process that has been predicted and described previously as a "banana-peeling model" (Adrover et al. 2010). The α-helices also must be rearranged into extended chains, and the small intramolecular β1-β2 sheet dissociated. Contributing to the complexity of the conversion process is the polarity of the 263K and RML fibrils with opposite ends that are not equivalent (Kraus et al. 2021a, b; Hoyt et al. 2021). Notably, deviations from planarity of each monomer within the prion fibril stack mean that, for example, the hydrophobic heads of the N β-arches protrude at one end and recede at the other. This might affect the initial points of contact of the PrP^{Sc} template with incoming PrP molecules, and consequently, the sequence of events

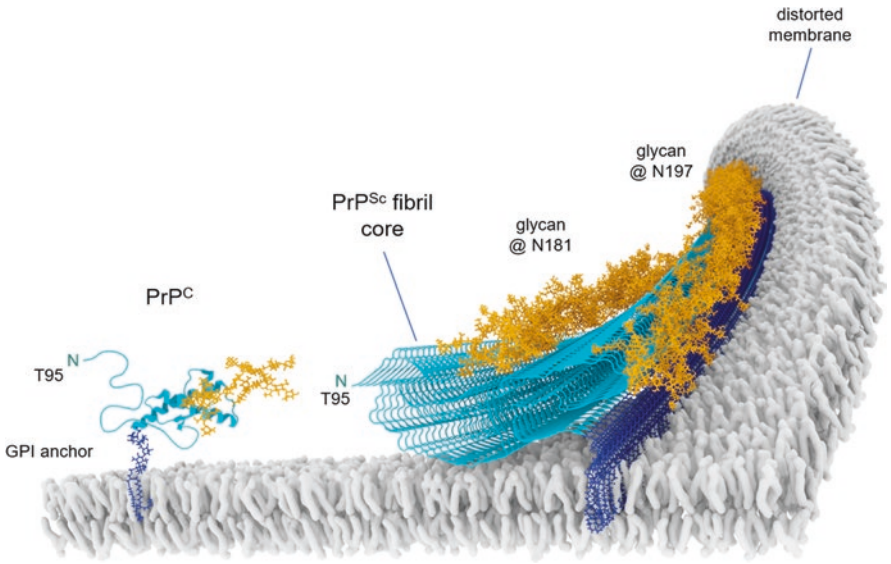

Fig. 3.4 Hypothetical depictions of the membrane-bound hamster PrP^{C} monomer (residues 95–231) and corresponding residues in each monomer of the 263K prion multimer. Polypeptides (aqua blue) are shown with N-linked glycans (yellow) and GPI anchors (blue) imbedded in a phospholipid membrane. The PrP^{C} and 263K structures were drawn using PDB coordinates referenced in (James et al. 1997; Kraus et al. 2021b), respectively. In the PrP^{C} structure, the serpentine line at the N-terminus represents residues 95–124 that are disordered in the NMR-based PrP^{C} structure. The GPI and N-linked glycan illustrations show single representative structures that, in actuality, are heterogeneous (Rudd et al. 1999; Stahl et al. 1992). Adapted with permission from (Kraus et al. 2021b). Graphics by Austin Athman

and kinetics of growth at each end. Given the in-register polypeptide alignment within the PrP^{Sc} end product, it seems likely that certain residues of an incoming monomer initiate contact with the analogous residues on the PrP^{Sc} template. Then adjacent residues might "zip" onto the polypeptide track of the template, forming periodic intermolecular β-sheets and loops along the way. Whatever the actual conversion mechanism, it will also likely be influenced crucially by interactions with anionic cofactors. Such cofactors have been shown to be important in conversion (Wong et al. 2001) and the assembly of infectious prions in vitro (Shaked et al. 2001; Deleault et al. 2003, 2005, 2007, 2010, 2012a, b; Supattapone 2020; Geoghegan et al. 2007; Wang et al. 2010; Miller et al. 2013) and are likely to electrostatically compensate for the stacking of positively charged and mutually repulsive, residues along the axis of the fibril (Kraus et al. 2021b; Groveman et al. 2014, 2015). Interactions with membranes can also affect conversion reactions (Baron et al. 2002, 2006; Baron and Caughey 2003; Rouvinski et al. 2014; Wegmann et al. 2008). In the membrane-bound context of wild-type forms of PrP^{C} and PrP^{Sc}, their relative topologies and modes of contact should be constrained by C-terminal tethering of each to the same phospholipid bilayer (Fig. 3.4).

3.6 Impacts of Glycans and GPI Anchors

Multiple studies have documented profound effects of GPI anchors and N-linked glycans, or lack thereof, on prion disease pathogenesis [e.g. (Chesebro et al. 2005, 2010; Sevillano et al. 2020; Bett et al. 2013; Cancellotti et al. 2010; Klingeborn et al. 2011; Wiseman et al. 2015; Race et al. 2018; Makarava et al. 2020)]. For example, in hosts expressing PrP without the GPI anchor signal sequence, PrP^{Sc} accumulates in the large extracellular amyloid plaques. Such hosts include genetically engineered transgenic mice (Chesebro et al. 2005, 2010; Klingeborn et al. 2011; Raymond et al. 2012; Rangel et al. 2013) or humans expressing anchorless PrP mutants such as Y145X 163X, Y226X, Q227X, and G131V (Ghetti et al. 2018). Genetic manipulations of the N-linked glycosylation of the host's PrP molecules can also markedly affect prion disease phenotypes [e.g., (Sevillano et al. 2020; Cancellotti et al. 2010; Wiseman et al. 2015)]. However, these post-translational modifications do not seem to substantially alter the core structures of at least 3 murine prions (RML, ME7, and 22L) strains, as probed by infrared spectroscopy (Baron et al. 2011). This conclusion is confirmed in much greater detail by the new cryo-EM structures of the wtRML and aRML fibril cores, which, as noted above, are quite similar (Hoyt et al. 2021; Manka et al. 2021). Also, fundamental RML strain phenotypes including incubation period and neuropathological lesion profile are maintained through passages from wildtype mice into anchorless PrP mice and back again (Chesebro et al. 2010), although more subtle long-term effects on inhibitor sensitivity have been reported (Mahal et al. 2012). Still, the overall similarity of the aRML and wtRML core structures, together with their divergence from the 263K structure, are consistent with polypeptide core structures "encoding" the

fundamental self-replicative properties of strains as postulated previously (Bessen et al. 1995; Bessen and Marsh 1994; Telling et al. 1996). Nonetheless, the phenotypes of those strains can be affected profoundly by the GPI anchors and glycans available in a given type of host or tissue (e.g. (Chesebro et al. 2005, 2010; Sevillano et al. 2020; Bett et al. 2013; Cancellotti et al. 2010; Klingeborn et al. 2011; Wiseman et al. 2015; Race et al. 2018; Makarava et al. 2020)).

Such phenotypic effects are likely due to different interactions of wildtype and anchorless prion fibrils with their tissue environments, as mediated by the glycans and GPI anchors on their surfaces (Fig. 3.4). With prion fibrils tethered to the membrane, glycans and bound membranes would blanket the C-terminal half of the fibril cores and restrict the access of other macromolecules to the polypeptide. Presumably, this would slow, or even preclude, easy access of proteostatic or innate immune macromolecules that might be involved in prion clearance or fragmentation. Access to PrP^{Sc} might be particularly limited within distorted membrane invaginations that are pathognomonic lesions of prion disease (Rouvinski et al. 2014; Wegmann et al. 2008; Caughey et al. 2009; Jeffrey et al. 2011, 2017; Jeffrey 2013). Among the more intriguing of those lesions are spiral twisted membrane inclusions (Jeffrey 2013; Jeffrey et al. 2017). As these spiral structures can be immunogold-stained for PrP^{d}, it is tempting to speculate that PrP^{Sc} fibrils lie at their cores, with the spiraling GPI anchors of the fibril(s) pulling and distorting cocoon-like membranes that wrap them. Membrane attachments might also enhance prion replication by promoting fragmentation due to stresses imposed by membrane dynamics. Fragmentation is thought to be key in prion replication in vivo (Meisl et al. 2021). Also, cell-to-cell spreading might be facilitated via prion binding to membranous particles such as exosomes and tunneling nanotubes (Caughey et al. 2009; Gousset et al. 2009; Vassileff et al. 2020). Such mechanisms, as well as toxic effects of GPI-mediated membrane distortions, might help to account for more the rapid disease progression that has been observed in wild-type hosts (Chesebro et al. 2005, 2010; Klingeborn et al. 2011). Ultimately, however, like wild-type prions, anchorless prions can be highly infectious and lethal for the host (Chesebro et al. 2010).

3.7 Structure-Based Modeling of Transmission Barriers

When prions are transmitted between hosts of different PRNP genotypes, profound inefficiencies, that is, transmission barriers, can be observed [e.g., (Prusiner et al. 1990)]. For an infection to take hold, the incoming PrP^{Sc} must be able to convert and recruit the heterologous PrP^{C} of the new host. Although there is considerable sequence homology between the PrP sequences of different mammalian hosts, mismatches of as little as a single residue can inhibit such heterologous conversions (e.g., (Prusiner et al. 1990; Scott et al. 1993; Goldmann et al. 1994; Kocisko et al. 1995; Priola et al. 1994; Priola and Chesebro 1995; Bossers et al. 1997; Raymond et al. 1997, 2000; Asante et al. 2015)). Modeling based on the new high-resolution 263K prion structures, together with knowledge of key mismatches controlling the

hamster 263K-to-mouse transmission barrier (Scott et al. 1993; Priola et al. 2001), has suggested a plausible molecular mechanism for this barrier (Kraus et al. 2021b). Specifically, the sequence mismatch at residue 155 (hamster numbering), which is N in hamsters and Y in mice, had been shown to be particularly influential (Scott et al. 1993; Priola et al. 2001). In the 263K structure, the sidechain of N155 is in a tightly packed area, and in silico modeling suggests that attempts to incorporate a bulkier Y sidechain at this position would cause steric clashes and require adjustments in hydrogen bonding and the polypeptide backbone to form a hybrid prion structure (Kraus et al. 2021b). We suspect that these effects slow the kinetics of conversion and/or the stability of the product to an extent that greatly reduces the efficiency of infection. In contrast, several other sequence mismatches between the hamster and mouse PrP sequences are much less inhibitory, presumably due to the positions of those residues on the outside of the fibril core or in less tightly packed interior positions. Given the multitude of prion strains/conformations and the variety of PrP sequence mismatches that influence their transmission efficiencies, we assume that the mechanisms of transmission barriers will be diverse.

3.8 Conclusions

The availability of high-resolution 3D structures of fully infectious prions is now helping us understand how prions replicate with conformational fidelity, how they interact with their tissue environments to cause disease, and how sequence mismatches between hosts can result in transmission barriers. So far, only three such structures are available, and much more work will be needed to characterize the entire spectrum of PrP-based prion structures. Such work will provide important structural foundations for the rational design and discovery of drugs or vaccines that can block propagation, promote clearance, and/or detoxify prions in infected individuals.

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Chapter 4
Insoluble Cellular Prion Protein and Other Neurodegeneration-Related Protein Aggregates in the Brain of Asymptomatic Individuals

Wen-Quan Zou

Abstract The pathological detergent-insoluble prion protein (PrP^{Sc}) is derived from its normal detergent-soluble cellular form (PrP^{C}) through a structural transition from α-helixes into β-sheets, which is associated with a group of transmissible neurodegenerative diseases or prion diseases. According to the prevailing seeding model, PrP^{Sc} formation requires a precursor of PrP^{Sc} or an intermediate form between PrP^{C} and PrP^{Sc}. However, the precursor or intermediate form in the brain remains to be determined. In 2006, we identified in uninfected human and animal brains a novel PrP conformer termed insoluble PrP^{C} ($iPrP^{C}$) that possesses PrP^{Sc}-like properties such as detergent-insolubility, resistance to protease, and tendency to form aggregates. Notably, other common neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) have recently been proposed to share a prion-like seeding mechanism by which the detergent-soluble brain monomeric cellular proteins form the detergent-insoluble misfolded protein aggregates that transmit from cells to cells. This chapter reviews the physiochemical properties of $iPrP^{C}$ and discusses its formation and pathophysiology. It also highlights the findings and implications of other misfolded proteins such as amyloid-β, tau, and α-synuclein associated with AD and PD in the brain of asymptomatic individuals.

Keywords Prion protein · Prion disease · Insoluble prion protein · α-Synuclein · Amyloid-β · Tau · Parkinson's disease · Alzheimer's disease · Variably protease-sensitive prionopathy · Dementia · Memory

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4.1 Introduction

The cellular prion protein (PrP^{C}) is a universally expressed membrane protein present predominantly in the central nervous system (CNS). Deposition in the CNS of its pathologic isoform (PrP^{Sc}) derived from PrP^{C} via a conformational transition is a molecular hallmark of prion diseases (PrDs), a group of fatal transmissible spongiform encephalopathies, neurodegenerative disorders, or prion diseases in humans and animals. Numbers of physiological and pathophysiological functions of PrP^{C} have been reported, involved in copper transportation (Brown et al. 1997), antioxidative stress (Brown et al. 2001), neurotransmission (Ford et al. 2002), cell-cell adhesion (Málaga-Trillo et al. 2009), cell-cell junctions, signalling (Petit et al. 2013), Amyloid-β (Aβ) receptor in Alzheimer disease (AD) (Laurén et al. 2009; Chap. 22), and cancer biology (Liang et al. 2006; Meslin et al. 2007; Antonacopoulous et al.; Li et al. 2009; Chap. 23). It has been proposed that PrP^{C} has beneficial and deleterious effects on cognition (Collinge et al. 1994; Laurén et al. 2009; Linden et al. 2008; Westaway et al. 2011; Das and Zou 2016). Moreover, it has been well demonstrated that the coexistence of PrP^{C} and PrP^{Sc} is the prerequisite for the emergence of PrDs. The two PrP conformers mainly studied so far are believed to be implicated in these diseases. PrP^{C} and PrP^{Sc} share the same primary sequence but have distinct secondary structures (Meyer et al. 1986; Caughey et al. 1991; Pan et al. 1993). PrP^{C} is monomeric, rich in α-helical structure, sensitive to proteinase K (PK) digestion, soluble in non-denaturing detergents, non-infectious, and present in both uninfected and scrapie-infected brains. In contrast, PrP^{Sc} is oligomeric or aggregate, rich in β-sheet structure, partially resistant to PK digestion, insoluble in detergents, infectious, and present only in infected brains. Interestingly, we have previously demonstrated that PrP^{Sc} but not PrP^{C} can be specifically captured by anti-DNA antibodies or DNA-binding proteins, suggesting that the PrP^{Sc} aggregates may bind to DNA or acquire a DNA-like structure (Zou et al. 2004). Soluble PrP^{C} is the only conformer that has been detected in the uninfected mammalian brain. In contrast, insoluble PrP^{Sc} exhibits chameleon-like conformations, which may underlie the distinct prion strains and phenotypes of PrDs identified in animals and humans (Bessen and Marsh 1992; Parchi et al. 1996; Caughey et al. 1998; Safar et al. 1998; Zou and Gambetti 2007; Collinge and Clarke 2007). Our identification of insoluble cellular PrP ($iPrP^{C}$) in the uninfected human and animal brain may raise two possibilities: that the PrP^{C} molecule in the brain also exhibits chameleon-like conformations that are implicated in their beneficial or deleterious effects, and that these species may play a role in the pathogenesis of PrDs and other neurodegenerative disorders (Yuan et al. 2006; Zou 2010; Zou et al. 2011b).

Notably, prion diseases have become a prototype of neurodegenerative diseases including but not limited to Alzheimer's disease (AD) and Parkinson's disease (PD) in terms of pathogenesis as well as related concepts and techniques used for investigating prions and prion diseases. For instance, the misfolded proteins including amyloid-β (Aβ) (Meyer-Luehmann et al. 2006; Stöhr et al. 2012), tau (Clavaguera et al. 2009; Iba et al. 2013; Lasagna-Reeves et al. 2012), α-synuclein (Luk et al.

2012a, b; Masuda-Suzukake et al. 2013), huntingtin with polyQ repeats (Ren et al. 2009), superoxide dismutase 1 (SOD1) (Münch et al. 2011), and TDP-43 (Chen et al. 2010; Nonaka et al. 2013) are also transmissible in vitro and/or in vivo. It has been proposed that neurodegenerative diseases share a prion-like self-propagating mechanism by which the misfolded proteins propagate and spread through cell-cell transmission as do prions (Prusiner 2013; Guo and Lee 2013; Goedert 2015). Like prions, they are derived from their normal cellular counterparts; moreover, insoluble Aβ, tau, and α-synuclein can be observed in the brain of asymptomatic, even very young individuals (Braak and Braak 1991; Savva et al. 2009; Braak and Del Tredici 2011; Braak et al. 2011; Jansen et al. 2015; Crary et al. 2014; Josephs et al. 2017; Braak and Braak 1995; Dickson 1998; Del Tredici et al. 2002; Braak et al. 2003).

4.2 Prion Protein Is Characterized by the Presence of an Intrinsically Chameleon-Like Conformation

Studies using recombinant PrP (rPrP) in vitro have indicated that PrP possesses a highly variable conformation. In aqueous solutions, rPrP could be folded into pH-dependent α-helical conformations, a thermodynamically more stable β-sheet, and various stable or transient intermediates (Zhang et al. 1997). A stopped-flow kinetic study demonstrated that PrP folded by a three-state mechanism involving a monomeric intermediate (Apetri and Surewicz 2002). It was found that the population of this partially structured PrP intermediate increased in the presence of relatively low concentrations of urea and was more stable at acidic pH 4.8, compared to neutral pH 7.0. Moreover, this approach revealed that PrP mutations, linked with naturally occurring familial prion diseases, showed a pronounced stabilization of the folding intermediate (Apetri et al. 2004). These findings suggest that the intermediates play a crucial role in PrP conversion and serve as direct precursors of the pathologic PrP^{Sc} isoform. The existence of a PrP folding intermediate was also indicated by hydrogen exchange experiments (Nicholson et al. 2002), and by studies using high-pressure NMR and fluorescence spectroscopy (Kuwata et al. 2002; Martins et al. 2003). In addition to a β-oligomer and an amyloid fibril (Baskakov et al. 2001; Morillas et al. 2001; Lu and Chang 2002; Sokolowski et al. 2003; Baskakov et al. 2004), two additional polymeric transient intermediates were also identified during fibrillogenesis of rPrP in vitro (Baskakov et al. 2002).

The cellular PrP^{C} molecule is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor. Several experiments have indicated that the PrP conformation is affected by its local conditions. For example, the interaction of the anchorless recombinant PrP with lipids in a membrane-like environment resulted in a conformational transition (Wang et al. 2007; Re et al. 2008). Increasing the local concentration of membrane-anchored PrP^{C} seems to induce a conformational transition accompanied by oligomerization of PrP^{C} (Elfrink et al. 2008). Recently, Faris et al. identified mitochondria PrP^{C} in healthy mice, which is a transmembrane

isoform with the C-terminus facing the mitochondrial matrix and the N-terminus facing the intermembrane space, which is PK-resistant (Faris et al. 2017). Therefore, the tendency of PrP to form multiple nonnative β-sheet-rich isoforms in vitro, as demonstrated in biophysical studies on rPrP, may represent a unique intrinsic feature of this protein.

Most of the N-terminal region of recombinant human and murine PrP has been observed to be disordered by NMR study (Riek et al. 1997; Zahn et al. 2000). The nucleic acid-binding intrinsically disordered proteins (IDPs) have recently been reported to be involved in diseases by driving liquid–liquid phase separation (LLPS) (Elbaum-Garfinkle 2019). Moreover, it is believed that the formation of membraneless organelles in vivo follows the generation of protein-rich condensates or granules by LLPS (Brangwynne et al. 2009; Boeynaems et al. 2018). PrP is able to form liquid-like condensates (Kostylev et al. 2018). PrP interaction with nucleic acids (NAs) undergoes LLPS, modulates phase separation, and promotes PrP fibrillation in a NA structure and concentration-dependent manner (Matos et al. 2020). Interestingly, DNA/RNA-PrP is involved in the formation of dynamic compartments, which may be associated with various functions of PrP^{C} and its misfolding; the condensates have been proposed to be part of the PrP^{Sc} pathway and therefore represent novel targetable structures for therapeutics (do Amaral and Cordeiro 2021).

4.3 Insoluble Cellular Prion Protein Aggregates Are Present in Mammalian Brains Without Prion-Infection

If the tendency of PrP to form multiple conformations in vitro represents a unique intrinsic feature of this protein, it is conceivable that other PrP conformers would be present in the normal brain in addition to the well-characterized PrP^{C}. To test this, we examined uninfected human and animal brains using a combination of biophysical and biochemical approaches to confirm the presence of additional PrP conformers (Yuan et al. 2006). Indeed, we identified a novel conformer that forms insoluble cellular PrP aggregates and protease-resistant PrP species in uninfected human brains (Yuan et al. 2006). Using gel filtration, we revealed that PrP in uninfected human brains is present not only in monomers with molecular weight less than 66 kDa, but also in oligomers between 66 kDa and 200 kDa, and large aggregates greater than 669 kDa, even 2000 kDa (Yuan et al. 2006) (Fig. 4.1). The new PrP conformer, termed insoluble cellular PrP ($iPrP^{C}$), accounts for approximately 5–25% of total PrP including full-length and N-terminally truncated forms, and a portion of $iPrP^{C}$ is resistant to PK digestion even at 50 μg/mL (Yuan et al. 2006). Notably, the PK-resistant $iPrP^{C}$ has immunoreactive behaviour different from that of classic PrP^{Sc} detected in prion-infected brains; its affinity is much lower for 3F4 while higher for 1E4, compared to the affinity of those antibodies for classic PrP^{Sc} (Yuan et al. 2006, 2008; Zou et al. 2010a, 2011a) (Fig. 4.2). In contrast to the gel mobilities of the deglycosylated PrP^{Sc} type 1 and type 2 that are 21 kDa and 19 kDa,

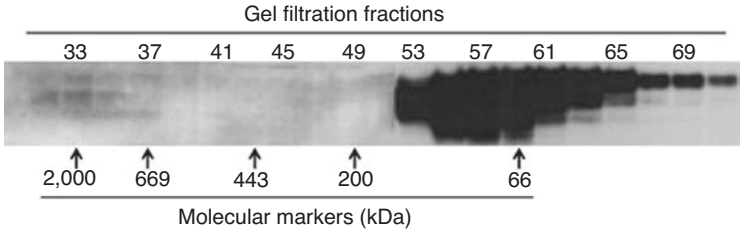

Fig. 4.1 Western blotting of gel filtration fractions of PrP from uninfected human brains. Gel filtration fractions of uninfected brain homogenates were subjected to SDS-PAGE and Western blotting with 3F4. Molecular mass (kDa) of various PrP species recovered in different fractions is indicated by an *arrow* and molecular mass markers used include dextran blue (2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and albumin (66 kDa). PrP was detected not only in fractions with molecular mass less than 66 kDa after fraction 59 but also in fractions with molecular mass greater than 66 kDa before fraction 59 including fraction 33 containing large PrP aggregates (2000 kDa)

respectively, the 1E4-detected PK-resistant deglycosylated PrP has gel mobility at ~20 kDa (Fig. 4.2). The epitopes of the two antibodies 3F4 and 1E4 are adjacent and the C-terminus of the 1E4 epitope between PrP97–105 is connected to the N terminus of the 3F4 epitope between PrP 106–112 (Yuan et al. 2008; Zou et al. 2010a). 3F4 is the most widely used antibody in the detection of human PrP^{C} and PrP^{Sc}, including PrP^{Sc} types 1 and 2 seen in sCJD and inherited CJD, and the internal PrP^{Sc} fragment PrP7–8 seen in GSS. Besides the 1E4-detected 20 kDa band, a PK-resistant PrP band migrating at ~18 kDa is also detectable with an antibody against the C-terminal PrP domain from residues 220–231 (anti-C antibody) (Fig. 4.2) (Yuan et al. 2006). In addition, the new conformer reveals a high affinity for the gene 5 protein (g5p, a single-stranded DNA-binding protein) and sodium phosphotungstate (NaPTA), both of which specifically bind to PrP^{Sc} but not to soluble PrP^{C} (Zou et al. 2004; Yuan et al. 2006; Safar et al. 1998; Wadsworth et al. 2001). By using the g5p enrichment from 500 μL of normal human brain homogenate, two more PK-resistant PrP bands migrating at ~18–19 kDa and ~ 7–8 kDa are detected by 1E4 in the uninfected human brain (Yuan et al. 2006). To rule out the possibility that PrP aggregates detected in the uninfected human brain result from post-mortem autolysis of autopsy tissues or from other neurodegenerative disorders, we also examined frozen uninfected human biopsy brain tissues or normal animal brain tissues from hamsters and cows. We observed that the insoluble PrP^{C} was also detectable in these tissues, a finding which confirmed that $iPrP^{C}$ is a de novo generated PrP conformer (Yuan et al. 2006). Using gel filtration, we recently further demonstrated that not only soluble PrP^{C} monomers, but also soluble PrP^{C} oligomers are present in the uninfected human brain (Xiao et al. 2012).

The presence of additional PrP oligomeric conformers besides the typical soluble PrP^{C} monomers in uninfected brains was also implied in the observations reported by other groups. Consistent with our findings, small amounts of PrP (less than 5% of total PrP^{C}) were also reported to be precipitated by NaPTA from

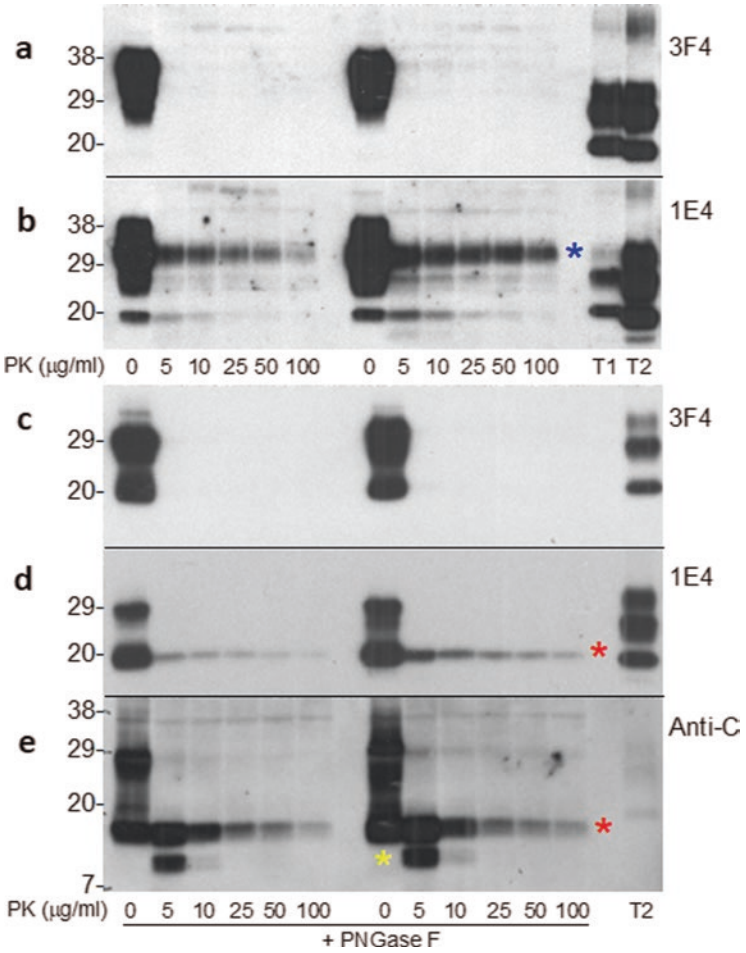

Fig. 4.2 Western blotting of PK-resistance of PrP in uninfected human brains. Brain homogenates from two uninfected human brains received at autopsy were treated with PK at 0, 5, 10, 25, 50, and 100 μg/mL (*upper two panels* ***a*** and ***b***) or PK plus PNGase F (*lower three panels* ***c***, ***d***, and ***e***). The samples were subjected to SDS-PAGE and Western blotting with 3F4, 1E4, and Anti-C antibodies. No PK-resistant PrP was detectable with 3F4 antibody. In contrast, PK-resistant PrP was detected with 1E4 and Anti-C up to 100 μg/mL. With PK alone, three PrP bands migrating at 30–29 kDa, 27–26 kDa, and 21–20 kDa were detected, in which the *upper* band (~30–29 kDa, *blue asterisk*) was predominant while the intensity of the *middle* band was *lowest*, which is apparently different from those of PrP^{Sc} type 1 (T1) and type 2 (T2). After PNGase F treatment, only one band was detected with 1E4 and Anti-C migrating at ~20 kDa and ~ 18 kDa, respectively (PrP^{*20} and PrP^{*18}, *red asterisk*). Interestingly, a band migrating at ~12–13 kDa was also detected with anti-C at low PK concentration (5–10 μg/mL, *yellow asterisk*)

uninfected human brains (Wadsworth et al. 2001). Moreover, by a differential SDS solubility assay, PrP^{C} species with either lower or higher solubility were differentiated in brain homogenates of noninfected humans, sheep, and cattle (Kuczius et al.

2009, 2011). Based on the detergent-solubility, the PrP^C phenotypes in cattle were similar to those in humans but not in sheep (Kuczius and Groschup 2013). Notably, a purified hamster brain PrP^C displayed an unexpectedly high β-sheet component under native conditions (Pergami et al. 1999). This finding provided evidence that the full-length native PrP^C isolated from animal brains exhibited intrinsic conformational plasticity. Moreover, mammalian brain PrP^C from six species was observed to be initially degraded to an intermediate fragment prior to complete proteolysis, suggesting an intrinsic partial PK-resistance (Buschmann et al. 1998). Ward et al. have recently observed that in response to the inoculation of normal brain homogenates, the host brain PrP^C exhibited increased insolubility and protease resistance at 72 h post-inoculation, similar to that of PrP^{Sc} (Ward et al. 2019). The authors proposed that the occurrence of PrP aggregation and protease-resistance results from brain injury due to the inoculation of normal brain homogenates. They believe that these changes were comparable to that observed in the examination of post-mortem human brain tissue (Esiri et al. 2000), in hypoxic human brain tissue from cases of cerebral ischemia (McLennan et al. 2004) and stroke (Mitsios et al. 2007), as well as in brain tissue of sheep with various neurological diseases (Jeffrey et al. 2012). Moreover, the same group has also previously identified a PK-resistant PrP species that is derived from the mitochondria of healthy mouse brain tissues (Faris et al. 2017). Interestingly, PrP aggregates have also been reported in pancreatic beta-cells of uninfected rats in response to hyperglycemia (Strom et al. 2007). In sum, the cumulative evidence shows that insoluble and PK-resistant PrP^C aggregates are present in tissues and organs of uninfected animals and humans.

4.4 Spontaneous Formation of the Insoluble Cellular Prion Protein Has Been Modelled with Cultured Cells and May Result from PrP Cytosolic Accumulation

Lehmann and Harris (1996) modelled the spontaneous formation of PrP^{Sc}-like insoluble PrP in cultured Chinese hamster ovary (CHO) cells expressing wild-type or mutant mouse PrP. Significant amounts of mutant PrP with a point mutation at residue 199 (E199K) (~60%) or six octapeptide repeat insertion mutations between residues 51 and 90 (~90%) linked to inherited human prion disease were detergent-insoluble; notably, approximately 15% wild-type PrP^C was also detergent-insoluble (Lehmann and Harris 1996). While approximately 5% of mutant PrP was resistant to the digestion by PK at 3.3 μg/ml for 20 min, wild-type PrP was completely degraded. Because the two mutant PrP molecules but not wild-type PrP were tightly associated with the plasma membrane, it was hypothesized that the acquisition of PrP^{Sc}-like properties results from an alternation in membrane topology or affinity (Lehmann and Harris 1996). Using the same models, they further identified a three-step endocytic pathway by which mutant PrP forms a PrP^{Sc}-like conformer: initially hydrophobic, then detergent-insoluble, and finally partially PK resistant (Daude

et al. 1997). Using human neuroblastoma cells, Singh et al. also revealed that PrP with Q217R mutation linked to GSS formed a PrP^{Sc}-like form (Singh et al. 1997).

In addition to the above PrP mutations, the two N-linked glycosylation sites located at residue 181, Asn-Ile-Thr residues 181–183, and at residue 197, Asn-Phe-Thr residues 197–199 (Puckett et al. 1991) are believed to play a crucial role in the stabilization of prion protein conformation. The naturally occurring mutations at residue 183, Thr to Ala (PrP^{T183A}), or at residue 198, Phe to Ser (PrP^{F198S}), falling in the two consensus sites, are linked to two distinct familial prion diseases (Nitrini et al. 1997; Tagliavini et al. 1991). Elimination of either site or both by mutagenesis of hamster PrP in CV1 cells, induced intracellular accumulation of mutant proteins (Rogers et al. 1990). Lehmann and Harris observed that mouse PrP mutated at T182 alone, or at both T182 and T198 in CHO cells, failed to reach the cell surface but the PrP with T198 mutation did. Moreover, all three mutant PrP molecules acquired PrP^{Sc}-like physicochemical properties reminiscent of PrP^{Sc}; PrP^{Wt} did so only when synthesized in the presence of N-linked glycosylation inhibitor tunicamycin (Lehmann and Harris 1997). Using human neuroblastoma M17 cells expressing human PrP^{N181G} or PrP^{T183A}, Capellari et al. observed that PrP^{N181G}, but not PrP^{T183A}, reached the cell surface even though both mutations eliminated glycosylation at the first site (Capellari et al. 2000). This observation indicates that the Thr to Ala mutation itself, rather than the elimination of the first glycosylation site, altered the physical properties of the mutant protein (Capellari et al. 2000). Although the F198S mutation falls within the second glycosylation site, Asn-Phe-Thr residues 197–199, PrP^{F198S} slightly increased the efficiency of glycosylation at the first glycosylation site (N181) and greatly increased it at the second site (N197) in cultured cells (Zaidi et al. 2005).

To further investigate the formation of $iPrP^{C}$ and the effect of mutations on the formation of $iPrP^{C}$, we examined $iPrP^{C}$ in cultured M17 cells expressing human wild-type (PrP^{Wt}) and mutant PrP (Yuan et al. 2008; Zou et al. 2011a). We confirmed that the de novo generated iPrP was detectable not only in cells expressing mutant PrP (PrP^{T183A} or PrP^{F198S}) linked to naturally occurring genetic Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker disease, respectively, but also in cells expressing wild-type PrP. Compared to cells expressing wild-type PrP, cells expressing mutant PrP exhibit significantly increased amounts of iPrP forming PrP aggregates and PK-resistant PrP. Most of PrP^{T183A} was composed of oligomers and large aggregates; virtually no monomeric form was present. In PrP^{F198S}, however, monomeric species were still dominant despite an increase in the amounts of aggregates. The enhanced tendency of PrP^{T183A} to form aggregates may result from the intracellular accumulation of the mutant protein. The F198S mutation did not significantly diminish the ability of PrP^{F198S} to reach the cell surface (Zaidi et al. 2005), although the mutation may change the structure around the V14 epitope previously found to be localized between human PrP168-181 (Zou et al. 2011a; Moudjou et al. 2004; Rezaei et al. 2005; Zhang et al. 2021). Therefore, the majority of the $iPrP^{C}$ associated with the T183A mutation may result from PrP intracellular accumulation, raising the possibility that $iPrP^{C}$ is derived predominantly from intracellular PrP species. Immunofluorescence microscopy of tagged PrP also indicated that

PrP^{T183A} accumulates within the cell, whereas PrP^{F198S} was distributed both inside the cell and on the cell surface, consistent with previous observations (Zou et al. 2011a; Capellari et al. 2000; Zaidi et al. 2005).

In uninfected cultured cells, we also confirmed that the PK-resistant $iPrP^{C}$ exhibited higher affinity for 1E4 than for 3F4, which was initially observed in brain tissue samples (Zou et al. 2011a; Yuan et al. 2006, 2008). In Western blotting with cell lysates, 1E4 virtually detected no PrP before PK treatment, and it detected PrP only after PK treatment. However, PrP was stainable by 1E4 in fixed cultured cells treated with or without PK although the PrP signal was weaker in treated than in untreated cells (Zou et al. 2011a). It is worth noting that an antibody against human PrP95-110 (termed 8G8), that actually extends merely two more amino acids toward the N- and C-terminuses of the 1E4 epitope, respectively, stained PrP-expressing cells with a brilliant cytoplasmic fluorescence (Krasemann et al. 1999). However, the number of positive cells was smaller than that of cells stained with antibodies against other PrP regions. Moreover, despite sharing a similar amino acid sequence within the corresponding epitope region, only cattle, but not mouse and hamster PrP, was observed to react with 8G8 (Krasemann et al. 1999). In contrast to 3F4, 1E4 seems to detect intracellular PrP in cultured cells (Zou et al. 2011a). Therefore, like 8G8, 1E4 may recognize a PrP species with a unique conformation in its epitope region.

In the absence of scrapie infection, aggregation of the cellular wild-type PrP in cultured cells was also observed only when proteasome inhibitors were used (Yedidia et al. 2001). It was later reported that PrP^{Wt} accumulated in the cytoplasm of cultured cells under other conditions as well, such as in a reducing environment, or when expressing PrP without both N and C terminal signal peptides (Ma and Lindquist 2001, 2002; Drisaldi et al. 2003; Grenier et al. 2006). Cytosolic PrP forms aggregates that are insoluble in non-ionic detergents and partially resistant to PK (Ma and Lindquist 2001). Accumulated cytosolic PrP aggregates induced by ER stress and inhibition of proteasomal activity were recently observed to travel through the secretory pathway and reach the plasma membrane (Nunziante et al. 2011). Cytosolic PrP was observed not only in cultured cells but also in subpopulations of neurons in the hippocampus, neocortex, and thalamus in uninfected wild-type mice (Mironov Jr et al. 2003). In addition, soluble PrP^{C} in human brain homogenate was observed to switch to insoluble PrP^{C} by treatment with acidic buffers in vitro (Zou and Cashman 2002).

The above observations may suggest that the formation of $iPrP^{C}$ or the aggregation of PrP^{C} is associated not only with mutations of the protein but also with altered cellular conditions that cause abnormal traffic and distribution of PrP in cells including reductive/oxidative stress and low pH.

4.5 Physiology and Pathophysiology of Insoluble PrP^C Aggregates

4.5.1 Long-Term Memory Storage

The $iPrP^C$ with a conformation likely different from soluble PrP^C may have a physiologic function. It has been hypothesized that prion-like conformational changes of related proteins are indispensable for the maintenance of structural synaptic changes required for long-term memory (Si et al. 2003, 2010; Papassotiropoulos et al. 2005; Shorter and Lindquist 2005). Interestingly, 24 h after a word-list learning task, carriers of either PrP polymorphism methionine/methionine (M/M) at residue 129 (129MM) or M/valine (V) (129 MV) genotype were observed to recall 17% more information than did 129VV carriers (Papassotiropoulos et al. 2005). Their further investigation of brain activity with event-related functional magnetic resonance imaging (fMRI) during a word recognition task suggested that the PrP-129 polymorphism affects neural plasticity following learning at a time scale of minutes to hours (Buchmann et al. 2008). The authors proposed that the PrP gene is genetically associated with human long-term memory performance. It is possible that the polymorphism at residue 129 of PrP participates in mediating human memory, in which the 129 M allele has a beneficial effect on long-term memory. Moreover, the impact of a putative PrP conformation rather than pathologic PrP^{Sc} on long-term memory in healthy humans was proposed to be related to physiologically occurring conformational changes (Tompa and Friedrich 1998; Papassotiropoulos et al. 2005).

It would be interesting to determine whether the conversion of soluble PrP^C monomers into insoluble PrP oligomers or aggregates is directly associated with long-term memory storage in the normal human brain (Zou et al. 2011c). The possibility cannot be ruled out that $iPrP^C$ is involved in long-term memory since it is the specific isoform of PrP^C that binds to nucleic acids, an important feature of proteins involved in long-term memory (Sudhakaran and Ramaswami 2017). For instance, the $iPrP^C$ molecule is able to gene five protein (g5p), the single-stranded DNA-binding protein (Yuan et al. 2006, 2008). The binding of recombinant PrP to different types of RNAs has been observed in vitro (Bera and Biring 2018) and the possible binding of $iPrP^C$ to mRNA in vivo cannot be ruled out. RNA has been found to modulate the aggregation of recombinant murine PrP by direct interaction in vitro (Kovachev et al. 2019).

4.5.2 Prion Disease

The in vivo pathway by which PrP^C forms PrP^{Sc} remains poorly understood. Two non-exclusive conversion models were proposed: *refolding* (Griffith 1967; Prusiner 1991) and *seeding* (Jarrett and Lansbury Jr 1993). In the former, the exogenous PrP^{Sc} binds to the PrP^C species that has been partially unfolded and the PrP^{Sc}-bound

PrP^{C} molecule undergoes a refolding process during which the nascent PrP^{Sc} is derived from this PrP^{C} species via a conformational transition. The latter proposes that a small amount of abnormal PrP^{Sc} or PrP^{Sc}-like form (PrP*) is present in the normal brain and is in reversible equilibrium with PrP^{C}. When several monomeric PrP* molecules form a highly ordered nucleus, PrP^{C} is converted to PrP^{Sc} polymers. Obviously, two key elements are required by the seeding model. One is the presence in the uninfected brain of a small amount of endogenous PrP^{Sc} or PrP* and the second is the formation of PrP^{Sc}-derived oligomers. The seeding model, with the two elements, has been recapitulated in vitro using PrP from various fungal and mammalian sources (Ross et al. 2005; Castilla et al. 2005; Tanaka et al. 2005). Indeed, this model well explains the replication pattern of PrP^{Sc} in which a newly recruited polypeptide chain accurately replicates that of a PrP^{Sc} template.

Recent studies also observed that replication of PrP^{Sc} does not always follow the refolding and seeding models, especially in vitro propagation of PrP^{Sc} in the presence of recombinant PrP substrate by serial protein misfolding cyclic amplification (sPMCA). For instance, Baskakov and co-workers have recently proposed an alternative model of PrP^{Sc} replication designated as deformed templating (see Chap. 5; Makarava and Baskakov 2012; Requena 2020; Spagnolli et al. 2020). It appears to involve switching from one cross-β folding pattern present in a template to an altered folding pattern, which undergoes a deformed process.

Given that $iPrP^{C}$ aggregates possess PrP^{Sc}-like physicochemical properties, we propose that $iPrP^{C}$ could represent endogenous PrP^{Sc} (Yuan et al. 2006; Zou et al. 2011a; Das and Zou 2016), an intermediate form (PrP*) between PrP^{C} and PrP^{Sc}, or a silent prion, required for seeding model of PrP^{Sc} formation (Jarrett and Lansbury Jr 1993; Hall and Edskes 2004; Weissmann 2004). Based on the observation that the brain of bigenic mice is capable of clearing prions, it has been proposed that the normal brain contains low levels of PrP^{Sc} (Safar et al. 2005). Under normal circumstances, despite the presence of a small amount of PrP^{Sc}, the brain may maintain an equilibrium between the formation and clearance of PrP^{Sc}. The amount of PrP^{Sc} is expected to be too small to induce a neurodegenerative disorder, which presumably, remains in a silent state. However, prion diseases may be triggered when the levels of the silent prions are significantly increased due to infection, PrP mutation, or unknown causes. Using PMCA, Barria and co-workers generated a new infectious prion without adding exogenous PrP^{Sc} seeds (Barria et al. 2009). This study raises two possibilities (1) PMCA replicates an intermediate PrP^{Sc} that is present in the brain homogenate; or (2) the silent prion is activated by the sonication–incubation cycles during PMCA. Further studies to address these questions will be critical to the understanding of initial molecular events in prion formation.

As mentioned above, $iPrP^{C}$ possesses a unique immunoreactive behaviour of poor affinity for 3F4 and higher affinity for 1E4, compared to other types of human PrP^{Sc} identified so far (Yuan et al. 2006, 2008; Zou et al. 2011a). The two antibodies have adjacent epitopes on PrP (Yuan et al. 2008; Zou et al. 2010b). Thus, the possibility cannot be ruled out that iPrP is a distinct PrP species with an altered conformation and that it may be a conformer which, when it increases, induces an atypical form of prion disease. Some previous observations with experimental animals may

favour this hypothesis. A novel neurologic syndrome was reported in Tg mice overexpressing wild-type PrP and these mice exhibited degeneration of skeletal muscle, peripheral nerves, and the central nervous system (Westaway et al. 1994). The increased amounts of wild-type PrP^C might form aggregates that induce degeneration in those mice. Chiesa et al. observed that homozygous Tg mice overexpressing wild-type PrP at approximately ten-fold but not hemizygous mice overexpressing wild-type PrP at approximately five-fold developed a spontaneous neurodegenerative disorder manifesting tremor and paresis (Chiesa et al. 2008). Nevertheless, abnormal PrP deposits and enlarged synaptic terminals with a dramatic proliferation of membranous structures were found in both types of mice. It was also observed that the overexpressed PrP assembled into insoluble aggregates with mild PK resistance but acquired no infectivity (Chiesa et al. 2008). Misfolding and neurotoxicity of wild-type PrP in transgenic flies were observed to be sequence dependent: Hamster PrP formed large amounts of PrP aggregates with spongiform degeneration, whereas rabbit PrP formed only small amounts of PrP aggregates without spongiform degeneration (Fernandez-Funez et al. 2010). Moreover, the same study also found that although small amounts of PrP aggregates were similarly detected in young flies expressing hamster PrP (day 1), spongiform degeneration was not evident. Therefore, the small amounts of PrP aggregates were unable to induce spongiform degeneration. Interestingly, spongiform degeneration occurred in older flies only when the concentrations of PrP aggregates increased (day 30).

The same unique immunoreactivity behaviour with 1E4 has also been observed in an atypical PrP^{Sc} species we recently identified from variably protease-sensitive prionopathy (VPSPr), a novel human prion disease (Gambetti et al. 2008; Zou et al. 2010b, 2013; Chap. 20). VPSPr exhibits an abnormal PrP species with peculiar glycosylation, enzymatic proteolysis, in vitro seeding activity, and in vivo infectivity (Zou et al. 2010b, 2011c; Wang et al. 2019; Zhang et al. 2021; Notari et al. 2014; Diack et al. 2014; Nonno et al. 2019). The 1E4-detected pathogenetic PK-resistant PrP^{Sc} with a ladder-like electrophoretic profile in the brain is the molecular hallmark of VPSPr. PrP^{Sc} from VPSPr exhibits not only the peculiar immunoreactivity behaviour but also three PK-resistant core fragments, which is similar to $iPrP^C$ (Zou et al. 2010b, 2011c, 2013). These similarities may suggest that they share a common molecular metabolic pathway. Similar to sCJD, VPSPr affects patients regardless of their PrP genotypes defined by 129 MV polymorphism; however, the allelic prevalence is distinct in the two diseases (Zou et al. 2010b; Gambetti et al. 2011a; Notari et al. 2018). Notably, the amounts of PK-resistant PrP^{Sc} in VPSPr seem to be dependent on the polymorphism, a characteristic that has not been observed in sCJD. Recent studies revealed that the infectivity of PrP^{Sc} from VPSPr is incomplete or inefficient in humanized transgenic mice expressing human PrP while it is transmissible in bank voles with attack rates of 5–35% in the first passage and 100% in the second passage (Nonno et al. 2019). Therefore, it is possible that VPSPr characterized by the deposition in the brain of $iPrP^C$-like PrP^{Sc} represents a prion disease, distinct from classical prion diseases and bearing more resemblance to other neurodegenerative diseases such as AD and tauopathies (Gambetti et al. 2011b; Zou et al. 2013; Chap. 20). Because of the similarities between $iPrP^C$ and PrP^{Sc} from VPSPr, the

possibility that VPSPr results from an increase in the amount of iPrP^C cannot be excluded (Zou et al. 2013; Chap. 20).

4.5.3 Alzheimer's Disease

PrP^C has been observed to be the receptor of amyloid-β (Aβ) in AD (Laurén et al. 2009; Balducci et al. 2010; Chap. 22). In 2011, we demonstrated for the first time that the insoluble PrP^C is the main PrP species that interacts with Aβ in the brain of AD patients and transgenic mice expressing human amyloid precursor protein, carrying both the Swedish (K670N and M671L) and Indiana (V717F) mutations (Zou et al. 2011b). This study made the following seven novel findings. First, large PrP and Aβ aggregates are eluted in the same gel filtration fractions from the brains of AD patients and AD mouse models. Second, more than 95% of Aβ co-immunoprecipitated with PrP by 3F4 from these brains is insoluble, while less than 5% of Aβ is soluble. Third, Aβ is co-captured with iPrP^C by gene 5 protein (g5p) from AD brains. Fourth, 6 Aβ42-specific binding regions on the human PrP molecule are identified with a peptide membrane array involving 13-mer human PrP peptides and two Aβ peptides (Aβ42 and Aβ40). Fifth, 4 of 6 Aβ42-specific binding areas are observed in the PrP octapeptide repeat domain of the unstructured N-terminal domain and only one is in the folded C-terminal region between residues 151 and 165. The other Aβ42-specific binding sites are located between the N- and C-terminal domains (residues 119–137). Sixth, compared with its nonspecific binding PrP sites (non-distinguishingly binding to both Aβ42 and Aβ40), the affinity of Aβ42 for its specific binding sites (binding to Aβ42 only) is significantly lower. Finally, the oligomeric state or conformation of Aβ42 and Aβ40 may determine the affinity of the two Aβ peptides for human PrP.

Our findings were largely confirmed by a subsequent study using both Aβ-PrP interaction and co-immunoprecipitation assays in a large AD patient cohort (Dohler et al. 2014). Specifically, they revealed that (1) significant binding of Aβ to PrP^C only occurs in AD, (2) Aβ aggregates bind particularly to the N-terminus of PrP^C, (3) optimal binding of PrP^C to Aβ is observed in the insoluble fraction of AD brain homogenates, and (4) neither expression levels nor PrP-129 polymorphisms of PrP^C influence their binding. The C-terminal PrP^C also has been found to play a role in the interaction between the protein and Aβ. PrP^C inhibits Aβ fibril growth via its C-terminal domain and the proposed binding of Aβ to the N-terminal domain of PrP may cause a conformational change in the C-terminal domain that unmasks additional Aβ-binding sites in that region (Bove-Fenderson et al. 2017). A new study with solid-state MAS NMR spectroscopy revealed that most of the C-terminal domain of PrP is part of the rigid complex with a loss in regular secondary structure in the two C-terminal α-helices (König et al. 2021), which could well explain why the complexes of PrP^C and Aβ are mainly detected in the insoluble fractions (Zou et al. 2011b; Dohler et al. 2014). Notably, the PrP^C-dependent, Aβ oligomers-induced Fyn activation was observed in detergent-insoluble subcellular fractions of

cultured N2A neuroblastoma, suggesting that insoluble PrP^C is involved in Aβ-induced PrP^C-Fyn signalling pathway (Um et al. 2012). Larson et al. revealed that the anti-PrP antibody C20 was able to immunoprecipitate Aβ dimers and activate Fyn, triggering tau aberrant mis-sorting and hyperphosphorylation (Larson et al. 2012). However, they claimed that their results are in contrast with our findings because no Aβ monomers coimmunoprecipitating with PrP^C from AD brains were detected using five anti-PrP antibodies (8B4, C20, 6D11, M20, and 7D11) and four anti-Aβ antibodies (6E10, 4G8, and 40/4-end specific Mab2.1.3 and Mab 13.1.1). The discrepancy between Larson et al. and Zou et al./Dohler et al. remains unknown. One of the possibilities could be due to different antibodies (3F4 antibody used in studies by Zou et al. and Dohler et al.) and lysis buffer. Larson et al. used the RIPA buffer that contains 3% SDS that may dissociate large PrP^C-Aβ assemblies.

The findings that $iPrP^C$ mainly or optimally binds to Aβ aggregates observed by us and Dohler et al. are consistent with other previous observations. For instance, PrP deposits often histologically accompany Aβ-positive plaques in AD brains (Esiri et al. 2000; Ferrer et al. 2001; Kovacs et al. 2002). In addition, Freir et al. displayed that interaction between PrP and toxic Aβ assemblies can be therapeutically targeted at multiple sites (Freir et al. 2011), indicating that their binding sites are not limited only to the internal domain. Remarkably, Kudo et al. showed that not only anti-PrP antibodies but also PrP^C peptides identified in our previous study (Zou et al. 2011b) rescued Aβ oligomer-induced neurotoxicity (Kudo et al. 2012).

Although the exact biological relevance and pathophysiology of the interaction between $iPrP^C$ and Aβ remain unclear, aggregation of one protein was observed to facilitate aggregation of the others (Morales et al. 2010). Moreover, synergistic interactions between other amyloidogenic proteins associated with neurodegeneration have also been reported to promote each other's fibrillization, amyloid deposition, and formation of filamentous inclusions in transgenic mice (Schwarze-Eicker et al. 2005). An increase in the efficiency of Aβ42 aggregation in vitro was dependent on PrP^{Sc} dosage (Morales et al. 2010). Moreover, insoluble PrP^{Sc} aggregates also seemed to facilitate Aβ42 aggregation in vivo; AD mice developed a strikingly higher load of cerebral amyloid plaques that appeared much faster in prion-infected than in uninfected mice (Morales et al. 2010). Our finding that Aβ42 binds to iPrP may suggest that iPrP facilitates the fibrillization of Aβ42 in AD. Similarly, the possibility should be considered that a significant increase in the total number of Aβ plaques observed in bigenic mice overexpressing PrP (Schwarze-Eicker et al. 2005) might result from an increase in the formation of iPrP. Since the less toxic insoluble Aβ42 aggregates constitute the end products of highly toxic soluble Aβ42 oligomers, it is conceivable that the formation of the large aggregates facilitated by $iPrP^C$ may reduce the amount of Aβ42 oligomers. The decrease in the levels of toxic Aβ42 oligomers would then attenuate the cognitive impairment induced by Aβ42 oligomers in AD. If this is the case, $iPrP^C$ may play a protective role in AD. Given that $iPrP^C$ interacts with insoluble Aβ42, whereas soluble PrP^C binds soluble Aβ42 in vivo (Zou et al. 2011b), it is possible that distinct PrP conformers binding to different Aβ42 species thereby function either as receptors for soluble Aβ42 oligomers or as modulators of insoluble Aβ42 deposition. It would be interesting to test this

hypothesis by intracerebrally injecting anti-PrP antibodies against either soluble or insoluble PrP species in AD animal models. This experiment would establish that the multiple conformers of PrP^{C} are coupled with their beneficial and deleterious effects.

4.6 Insoluble Aβ, Tau, and α-Synuclein Aggregates in the Brain of Asymptomatic Individuals

Accumulation and deposition of insoluble Aβ, tau, and α-synuclein aggregates in the brain are the molecular hallmark of AD and PD. These insoluble aggregates are also derived from their soluble monomeric counterparts through a structural transition, a mechanism similar to the conversion of PrP^{C} into PrP^{Sc}. They form extracellular Aβ plaques and intracellular phosphorylated neurofibrillary tangles in AD brains and α-synuclein-containing Lewy bodies in PD brains. Interestingly, examination of brains obtained at autopsy from nondemented and demented cadavers has demonstrated that accumulation of these AD- and PD-pathologies commences before the appearance of clinical symptoms (preclinical phase) (Braak and Braak 1991; Braak and Del Tredici 2011; Braak et al. 2011; Forno 1969; Bloch et al. 2006; Mikolaenko et al. 2005; Del Tredici and Braak 2008). Moreover, in living patients, positron emission tomography (PET) of Aβ revealed that the accumulation of Aβ can be detected approximately 20 years before dementia onset in AD (Gordon et al. 2018; Villemagne et al. 2013; Hansson 2021). Moreover, several lines of evidence also have revealed that they are detectable in the brain of aged individuals who never developed AD and PD clinical symptoms and signs. Positive cortical Aβ-PET was observed in ~10–15% of individuals with normal cognition at age 60 and in ~40% at age 90 (Hansson 2021; Savva et al. 2009; Jansen et al. 2015). Neuropathological examinations have demonstrated that stages A and B of Aβ pathology can be found before clinical dementia (Braak and Braak 1991; Braak et al. 2011). Tau pathology has been observed in primary age-related tauopathy (PART) (Crary et al. 2014; Hansson 2021). It is often confined to the medial temporal lobe area; moreover, PART displays minimal or no Aβ pathology, and seldom has dementia if it has no other primary co-pathology (Crary et al. 2014; Josephs et al. 2017). Lewy bodies and Lewy neurites were also observed in the brain of older individuals without clinical histories of PD (Forno 1969; Bloch et al. 2006; Mikolaenko et al. 2005; Del Tredici and Braak 2008).

4.7 Conclusions

Chameleon-like conformations of PrP^{Sc} are believed to link to transmissible and non-transmissible prion diseases with highly heterogeneous phenotypes (Collinge and Clarke 2007; Zou 2007; Zou and Gambetti 2007). Identification of $iPrP^{C}$ suggests that the normal protein also has chameleon-like conformations. It has been proposed that the variable conformations of PrP^{C} are linked to its beneficial and deleterious effects (Zou et al. 2011c). Demonstration of the presence of insoluble PrP in normal mammalian brains and its potential association with AD and atypical prion disease may open a new avenue in the exploration of prion formation and in the physiology and pathophysiology of the prion protein. Similarly, findings of insoluble misfolded Aβ, tau, and α-synuclein proteins in individuals without AD or PD clinical manifestations such as dementia or motor and non-motor symptoms and signs are also significant because they imply that other co-factors are required for these insoluble misfolded proteins to cause neuronal death. Further investigation on the differences in neurotoxic and non-toxic insoluble misfolded proteins would be critical to our understanding of the pathogenesis of these diseases and developing effective therapeutic compounds for these disorders.

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Part III
Conversion and Strain of Prions

Chapter 5
Prion Conversion and Deformed Templating

Ilia V. Baskakov

Abstract The transmissible agent of prion disease consists of a prion protein in its abnormal, β-sheet-rich state (PrP^{Sc}), which replicates itself according to the template-assisted mechanism. According to this mechanism, the folding pattern of a newly recruited polypeptide chain accurately reproduces that of a PrP^{Sc} template. This chapter introduces an alternative mechanism of prion replication designated as deformed templating that constitutes a switching of the cross-β folding pattern into an alternative pattern. The chapter discusses experimental evidence in support of deformed templating including the work on synthetic prions and illustrations that folding pattern switches within individual amyloid fibrils. The role of deformed templating in prion strain mutations and evolution is reviewed. Changes in the replication environment along with the effects of posttranslational modifications are proposed as driving forces behind deformed templating events. The mechanism of deformed templating is important for a better understanding of the etiology of prion and other neurodegenerative diseases.

Keywords Prion protein · Prion diseases · Neurodegenerative diseases · Deformed templating · Cross-β folding · Amyloid fibrils · Posttranslational modifications · N-linked glycans

5.1 Introduction

Prion diseases, or transmissible spongiform encephalopathies, are fatal neurodegenerative disorders that can be sporadic, inherited, or infectious in origin. Misfolding and aggregation of the normal, cellular form of the prion protein (PrP^{C}) into an

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abnormal β-sheet-rich, disease-related conformation (PrP^{Sc}) underlie the pathogenic mechanisms of the prion diseases for all three origins (Prusiner 1996). Spontaneous conversion of PrP^{C} into PrP^{Sc} is believed to underlie the sporadic forms of prion diseases (Fig. 5.1a). The low occurrence rate of sporadic prion disease is likely to reflect the extremely low probability of spontaneous conversion of PrP^{C} into PrP^{Sc}. Inherited forms of the disease have been linked to a number of single-point mutations, truncation, or octarepeat expansion mutations in the *PRNP* gene (a gene that encodes prion protein), with more than 30 disease-inducing mutations identified so far (Prusiner and Scott 1997) (Fig. 5.1b). In addition to sporadic and inherited

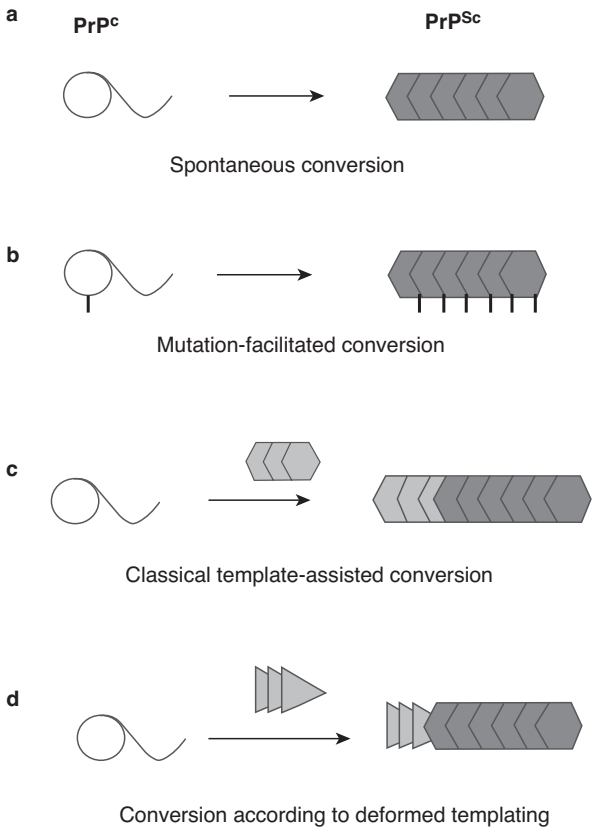

Fig. 5.1 Four mechanisms for PrP^{Sc} formation. (**a**) Spontaneous conversion of PrP^{C} into PrP^{Sc} underlies the sporadic forms of prion diseases. (**b**) Disease-related mutations in prion protein can facilitate the conversion of PrP^{C} into PrP^{Sc}. (**c**) The template-assisted model postulates that PrP^{Sc} replicates its pathogenic structure by recruiting and converting PrP^{C}. According to this model, the folding pattern of a newly recruited polypeptide chain accurately reproduces that of a PrP^{Sc} template. (**d**) The mechanism referred to as deformed templating postulates that the formation of PrP^{Sc} can be seeded by abnormal PrP structures substantially different from that of authentic PrP^{Sc}. A transformation from one cross-β folding pattern to an altered folding pattern occurs during deformed templating

origins, prion diseases can be also acquired via transmission. According to the protein-only hypothesis, the transmissible agent consists of a prion protein in its abnormal, β-sheet-rich, disease-related state (PrP^{Sc}), which propagates its abnormal conformation in an autocatalytic manner by recruiting and converting PrP^{C} into PrP^{Sc} (Prusiner 1982; Griffith 1967). The classical templating mechanism of prion replication postulates that the folding pattern of a newly recruited polypeptide chain accurately replicates that of a PrP^{Sc} template (Fig. 5.1c) (Cohen and Prusiner 1998). As such, the PrP^{Sc}-specific folding pattern replicates endlessly with high fidelity, as far as PrP^{C} molecules are available as a substrate.

This chapter discusses an alternative mechanism of PrP^{Sc} replication designated as deformed templating. Deformed templating involves switching from one cross-β folding pattern present in a template to an altered folding pattern (Fig. 5.1d). Experimental data accumulated in the field over the past decade including the results on synthetic prions provide strong support for this mechanism. The concept of deformed templating offers a new perspective on the genesis, evolution, and adaptation of transmissible prion structures.

5.2 Switching Between Alternative Folding Patterns Within Individual Amyloid Fibrils

According to the prevailing view, multiple amyloid structures could be produced within the same amino acid sequence (Petkova et al. 2005; Makarava and Baskakov 2008). However, the folding pattern within individual amyloid fibrils or PrP^{Sc} particles is believed to be uniform. In amyloid fibrils or PrP^{Sc} particles, β-strands are arranged perpendicularly to the axis of the cross-β spine (Wille et al. 2009; Ostapchenko et al. 2010), and their strain-specific folding pattern provides a template for recruiting and converting a monomeric precursor at the growing edge. Faithful templating of cross-β structures is based on the self-complementation of polypeptide chains involved in cross-β assembly (Eisenberg et al. 2006). Self-complementation can be achieved through several mechanisms including tight complementarity of amino acid side chains in the steric zippers of the cross-β spine; the stacking of side chains in so-called polar zippers, where the side chain hydrogen bonds are formed between β-strands along the fibrillar axis; or domain swapping (Eisenberg et al. 2006).

Our studies that employed single-fibril fluorescence microscopy combined with atomic force microscopy imaging and supplemented with Fourier-transform infrared spectroscopy (FTIR) spectroscopy revealed that the cross-β folding pattern does not always maintain uniform structure upon elongation of individual fibrils (Makarava et al. 2007, 2009) (Fig. 5.2). The cross-seeding reactions, where hamster recombinant PrP (rPrP) fibrils were used to seed fibrillization of mouse rPrP, produced hybrid fibrils consisted of two segments: one composed of hamster and another mouse rPrP (Fig. 5.2b,c). (Makarava et al. 2009). Remarkably, as judged from immunoconformational microscopy assay that probes exposure of PrP epitopes within fibrils (Fig. 5.2a), the folding pattern switched from hamster- to

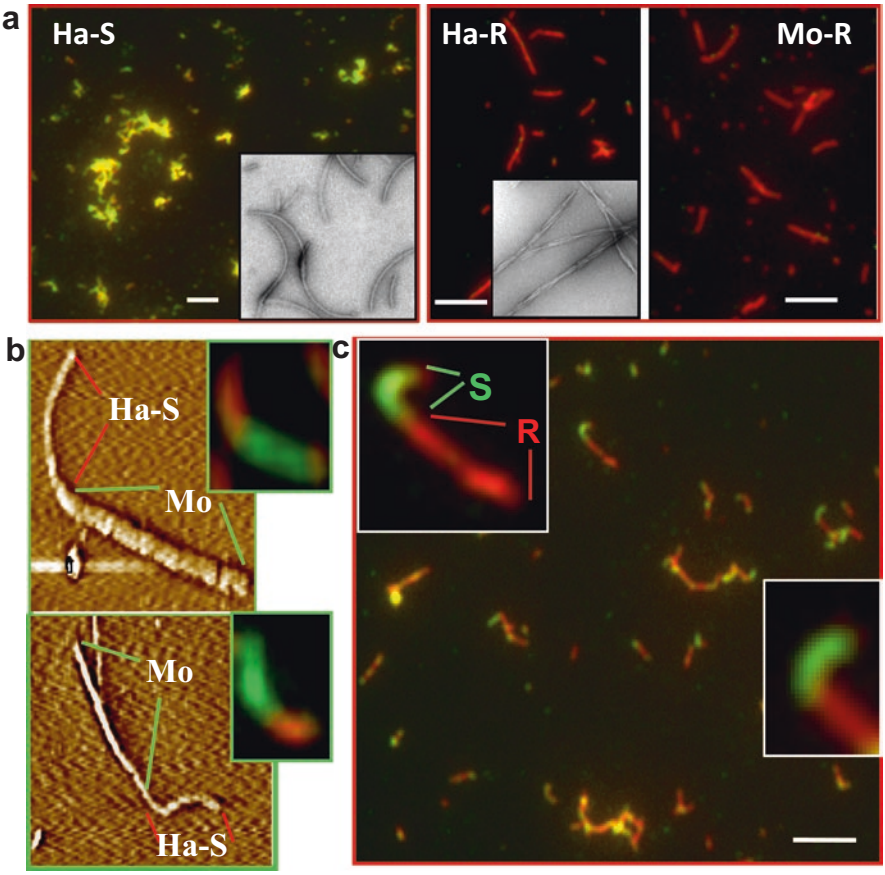

Fig. 5.2 Switching between alternative folding patterns within individual fibril (adapted from Makarava et al. 2009). (**a**) Immunoconformational microscopy imaging assay was designed to probe strain-specific differences in the exposure of epitopes. Examples that immunoconformational assay distinguishes two conformations of rPrP fibrils generated in vitro and designated as S-fibrils (yellow or green) and R-fibrils (red): hamster rPrP S-fibrils (*Ha-S*; *left*), hamster rPrP R-fibrils (*Ha-R*; *center*), and mouse R-fibrils (*Mo-R*; *right*). Insets show electron microscopy images of Ha-S fibrils and Ha-R fibrils. *Scale bars*, 5 μm. (***b, c***). Seeding of mouse rPrP with hamster S-fibrils leads to hybrid mouse-hamster fibrils that show switches in folding patterns from S- to R-specific patterns. (***b***) Atomic Force Fluorescence Microscopy images demonstrate that individual hybrid fibrils consisted of two sections: the sections made of hamster PrP, as detected by hamster-specific anti-PrP antibody (red fluorescence), had a curvy S-like shape, whereas the sections made of mouse PrP, as detected by mouse-specific anti-PrP antibody (green fluorescence), had a straight R-like shape. (***c***) *I*mmunoconformational microscopy imaging assay of hybrid hamster-mouse fibrils showing a switch from S-specific pattern (green) to R-specific pattern (red). Scale bars, 5 μm

mouse-specific within individual hybrid fibrils (Makarava et al. 2009) (Fig. 5.2c). We proposed that for hybrid structures to maintain integrity, alternative folding patterns have to share a common motif (Baskakov 2009).

The observation of a conformational switch within individual fibrils provides a direct illustration of the deformed templating mechanism and highlights the high adaptation potential for amyloid structures. Adaptive conformational switching via deformed templating permits the recruitment of homologous PrP sequences which otherwise are not compatible with the templating structures. Adaptive conformational switching within individual fibrils may provide a mechanistic explanation for strain mutation or modification, phenomena that have been frequently observed upon transmission of prions across species (Peretz et al. 2002; Castilla et al. 2008; Green et al. 2008).

5.3 Generating Transmissible Prion Diseases De Novo

The last two decades witnessed a number of studies, where transmissible prion diseases were generated in animals de novo by inoculating prion material produced in vitro (Legname et al. 2004; Colby et al. 2009, 2010; Makarava et al. 2010, 2011, 2012a, 2015, 2016; Barria et al. 2009; Deleault et al. 2007, 2012a, b; Wang et al. 2010). All studies on generating prion infectivity could be divided into two large groups, where the material for inoculating animals was produced either using (1) serial protein misfolding cyclic amplification (sPMCA) (Barria et al. 2009; Deleault et al. 2007, 2012a, b; Wang et al. 2010) or (2) in vitro fibrillation protocols that utilized rPrP (Legname et al. 2004; Colby et al. 2009, 2010; Makarava et al. 2010, 2011, 2012a, 2015, 2016).

In the studies that employed the first approach, the application of sPMCA accomplished two purposes (1) generating PrP^{Sc} particles de novo and (2) amplification of newly formed PrP^{Sc} to the amounts that can effectively produce clinical disease in wild-type animals with 100% success rate (Barria et al. 2009; Deleault et al. 2007; Wang et al. 2010).

The second approach involved the conversion of rPrP into amyloid fibrils in vitro without the application of sPMCA (Legname et al. 2004; Colby et al. 2009, 2010; Makarava et al. 2010, 2011, 2012a, 2015, 2016). In these studies, transmissible diseases were generated either in transgenic animals with high levels of PrP^{C} expression or in wild-type animals. In transgenic animals, the disease was produced with a 100% success rate in the first passage, although after a relatively long incubation time (Legname et al. 2004; Colby et al. 2009, 2010). In wild-type animals, two or even three serial passages were required for the appearance of clinical prion disease (Makarava et al. 2010, 2011, 2012a, 2015, 2016). Critical concerns that rPrP amyloid fibrils did not induce the disease de novo but only accelerated an ongoing pathogenic process have been raised regarding the studies performed on transgenic mice (Caughey et al. 2009; Caughey and Baron 2006; Soto 2011). Indeed, the transgenic mice that overexpress PrP^{C} were found to develop a neurological disorder that

was accompanied by PrP aggregation, although these disorders were not transmissible in serial passages (Colby et al. 2010). In contrast to the sporadic formation of non-transmissible PrP aggregates, inoculation of rPrP fibrils triggered the formation of authentic PrP^{Sc} that can transmit disease, a process that appears to compete with aggregation of non-transmissible PrP.

Our experiments conducted using Syrian hamsters demonstrated that rPrP fibrils induce transmissible prion disease de novo in wild-type animals (Makarava et al. 2010, 2011, 2012a, 2015, 2016). However, when triggered by rPrP fibrils, only a small fraction of animals showed signs of infection. Furthermore, the clinical disease was observed only at the second or third serial passages (Makarava et al. 2010, 2011, 2012a). Less than a 100% success rate along with a long clinically silent stage raised a number of questions regarding the molecular mechanism underlying the genesis of transmissible prions de novo.

Prior to the discussion of molecular mechanisms for triggering transmissible prion diseases, it is useful to briefly review the data on the structure of rPrP fibrils and PrP^{Sc}. Several studies presented strong evidence that the structures of rPrP amyloid fibrils are different from those of authentic PrP^{Sc} whether isolated from scrapie-infected animals or produced via sPMCA in vitro (Wille et al. 2009; Ostapchenko et al. 2010; Piro et al. 2011; Wang et al. 2020; Kraus et al. 2021). X-ray diffraction experiments revealed substantial differences in equatorial diffraction patterns collected from rPrP fibrils and PrP^{Sc} purified from scrapie brains, suggesting that they have different folding patterns (Wille et al. 2009; Ostapchenko et al. 2010). The results of the X-ray analysis were consistent with the FTIR data, which also pointed to differences between conformations of PrP^{Sc} and rPrP fibrils (Spassov et al. 2006; Makarava and Baskakov 2008). The maxima of the β-sheet absorption collected for PrP^{Sc} isolates varied between 1625 and 1637 cm^{-1} depending on specific PrP^{Sc} strain (Spassov et al. 2006), whereas the maxima of β-sheet absorption for rPrP fibrils was found to be at 1614 and 1626/28 cm^{-1} under the same solvent conditions (Makarava and Baskakov 2008; Ostapchenko et al. 2010). Finally, according to recent cryo-EM studies, both rPrP fibrils and PrP^{Sc} consist of parallel in register β-sheet structure, however, their folding patterns were found to be profoundly different (Wang et al. 2020; Kraus et al. 2021). If rPrP fibrils and PrP^{Sc} have different structures, how can the first template be the last one?

Bearing in mind the results of structural studies, two alternative mechanisms can be considered. According to one mechanism, the preparations of rPrP fibrils contained very small amounts of PrP^{Sc} or particles with a structure similar to authentic PrP^{Sc} (Fig. 5.3a). If this is the case, the low success rate in infecting the animals and the long clinically silent stage should be attributed to the miniscule amounts of PrP^{Sc} in preparation of the fibrils. The second mechanism designated as deformed templating proposes that the formation of PrP^{Sc} and transmissible prion diseases in wild-type animals are triggered by rPrP seeding material that lacks PrP^{Sc} (Fig. 5.3b). According to this mechanism, rPrP fibrils trigger the formation of PrP^{Sc} despite substantial differences in their folding patterns. The low rate of infection in the first passage is due to the stochasticity of the deformed templating process. Moreover, the transformation of rPrP amyloid structure into PrP^{Sc} might involve several steps before authentic PrP^{Sc} emerges (Fig. 5.3b).

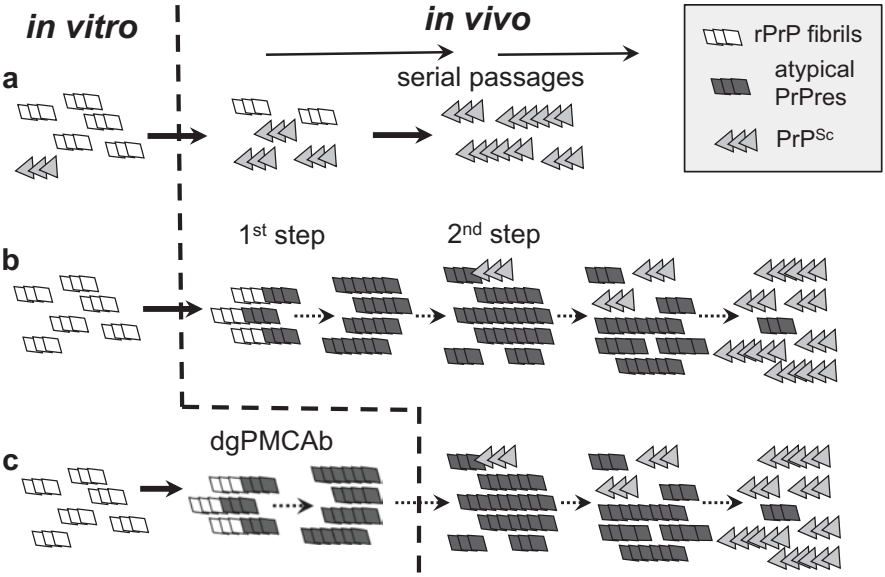

Fig. 5.3 Schematic representation of the mechanisms responsible for generating transmissible prion diseases de novo using rPrP fibrils prepared in vitro. According to the first mechanism, (**a**) the preparations of rPrP amyloid fibrils (schematically shown as *white parallelograms*) contain very small amounts of PrP^{Sc} (shown as *triangles*). The silent stage of the disease is attributed to the long time required for the amplification of this extremely small amount of PrP^{Sc}. (**b**) A second mechanism referred to as deformed templating postulates that there are no PrP^{Sc} particles in the preparations of amyloid fibrils. Instead, when inoculated into animals, amyloid fibrils seed conversion of PrP^{C} into PrP^{Sc}-like structures, although with low efficiency. The process of transformation of rPrP fibrils into PrP^{Sc} involves two steps. In the first step, rPrP fibrils seed atypical PrPres (shown as *dark parallelograms)*, a transmissible form of PrP that replicates silently without causing clinical disease. In the second step, atypical PrPres produces PrP^{Sc} in rare and stochastic seeding events that are described by a deformed templating mechanism. PrP^{Sc} replicates faster than atypical PrPres and eventually replaces it during serial passages. (**c**) An alternative pathway for producing transmissible prion diseases de novo involves the generation of atypical PrPres in dgPMCAb reactions that employ partially deglycosylated PrP^{C} as a substrate upon seeding with rPrP fibrils. Serial transmission of dgPMCAb-derived atypical PrPres in animals leads to the formation of PrP^{Sc} via deformed templating and prion disease (Makarava et al. 2015)

5.4 Experimental Evidence Supporting the Mechanism of Deformed Templating

Several lines of experimental evidence support the idea that synthetic prion strains emerged via the mechanism of deformed templating. First, no PrP^{Sc} could be detected in the preparations of rPrP amyloid fibrils using a highly sensitive sPMCA with beads (sPMCAb) format that detects single PrP^{Sc} particles (Makarava et al. 2011). If one assumes that the first model is correct, the amount of infectivity should be equivalent to approximately 0.5 infectious doses to account for the less than

100% infection rate in the first passage. This amount of infectivity is equivalent to ~10,000–100,000 PrP molecules or to ~100–1000 PrP^{Sc} particles, assuming that an average PrP^{Sc} particle consists of ~100 PrP molecules (Saa et al. 2006; Makarava et al. 2012b). This amount of PrP^{Sc} was well above the detection limits of sPMCAb and should have been easily detected if present in preparations of rPrP fibrils.

Second, the experimental protocol used for producing rPrP amyloid fibrils employs denaturants (a mixture of 1 M GdnHCl and 3 M urea) – the solvent conditions that denature PrP^{Sc}. Using denaturing conditions for fibril formation is possible because rPrP fibrils are much more conformationally stable than PrP^{Sc} (Makarava et al. 2010; Peretz et al. 2001; Sun et al. 2007). Furthermore, the formation of authentic PrP^{Sc} in vitro requires RNA and lipids (Deleault et al. 2007, 2012a, b; Wang et al. 2010), whereas rPrP amyloid fibrils were formed in the absence of these cellular cofactors. Therefore, it is unlikely that authentic PrP^{Sc} could be formed in the preparation of rPrP fibrils conducted in the absence of cofactors essential for authentic PrP^{Sc} structures and under solvent conditions that promote PrP^{Sc} denaturation.

Third, in studies on synthetic prions, a strong correlation between the conformational stability of rPrP amyloid fibrils, the stability of PrP^{Sc} produced in animals upon inoculating rPrP fibrils, and the incubation time to disease were described (Colby et al. 2009). If a minuscule fraction in the preparation of rPrP fibrils is responsible for the disease, the correlation between the stability of rPrP amyloid, which is a bulk property of fibril preparation, and the incubation times would be challenging to explain. Again, these results are consistent with the second model.

Fourth, when transmissible prion disease is triggered by rPrP amyloid fibrils, a decrease in PrP^{Sc} conformational stability was observed during serial passages of synthetic prions (Legname et al. 2005; Makarava et al. 2010; Colby et al. 2009). Similar dynamics in PrP^{Sc} conformational stability were found regardless of whether transgenic mice or Syrian hamsters were inoculated with rPrP fibrils, suggesting that a common pathway in the genesis and evolution of infectious structures might exist (Legname et al. 2005; Makarava et al. 2010; Colby et al. 2009). Observed changes in physical properties illustrate that the PrP^{Sc} structure undergoes a transformation during serial transmission, again providing support to the second model.

Fifth, as judged from the clinical and neuropathological features, the synthetic prion strains generated by rPrP fibrils were remarkably different from prion strains of natural origin or synthetic strains generated via sPMCA (Deleault et al. 2007; Barria et al. 2009; Wang et al. 2010; Makarava et al. 2010, 2011, 2012a, 2020, 2021; Jeffrey et al. 2014). The fact that rPrP fibrils produced a disease phenotype remarkably different from the phenotype expressed by strains generated in sPMCA or strains of natural origin is consistent with the hypothesis that rPrP fibrils gave rise to PrP^{Sc} with a unique structure.

5.5 Deformed Templating In Vivo

How did amyloid fibrils structurally different from authentic PrP^{Sc} give rise to PrP^{Sc} and transmissible disease? An exhaustive search for minuscule amounts of PrP^{Sc} in the preparations of rPrP fibrils yielded negative results (Makarava et al. 2011, 2012a). Instead, a search for intermediate products on a pathway toward PrP^{Sc} revealed that the first product of PrP^{C} misfolding triggered by rPrP fibrils in animals was a new self-replicating PrP state referred to as atypical PrPres (Makarava et al. 2011, 2012a) (Fig. 5.3b). Atypical PrPres displayed an abnormally short, C-terminal proteinase K (PK)-resistant core that was similar to the PK-resistant core of rPrP fibrils with respect to its size and position (Bocharova et al. 2005; Makarava et al. 2011, 2012a). Unlike authentic PrP^{Sc}, atypical PrPres preferentially recruited un- and mono-glycosylated PrP^{C}, while its amplification was RNA-independent arguing that it is structurally different from PrP^{Sc}. Accumulation of atypical PrPres in animal brains did not lead to any notable clinical signs of prion diseases and was associated only with minor lesions (Kovacs et al. 2013). Despite replication and accumulation in the brain, atypical PrPres was a clinically silent state. Over the course of several serial passages, atypical PrPres gave rise to PrP^{Sc} (Makarava et al. 2011, 2012a, 2015, 2016) (Fig. 5.3b). The appearance of PrP^{Sc} was stochastic and always followed the accumulation of atypical PrPres first. The dynamics between the two states suggests that the birth of PrP^{Sc} was a result of a series of deformed templating events and a selection of the most favorable structural variants that were best suited for replication in animal brains (Makarava et al. 2011, 2012a).

Remarkably, atypical PrPres could be generated in vitro via seeding of PMCAb reactions that utilized partially deglycosylated PrP^{C} as a substrate (dgPMCAb) using rPrP fibrils (Makarava et al. 2013, 2015, 2016) (Fig. 5.3c). As far as un- and mono-glycosylated PrP^{C} are available as a substrate, rPrP fibrils give rise to atypical PrPres whether in vivo or in vitro (Makarava et al. 2013, 2015, 2016). However, because di-glycosylated PrP^{C} is not compatible with the structure of atypical PrPres, di-glycosylated PrP^{C} interfered with the replication of atypical PrPres triggering deformed templating events. Upon inoculation into animals, dgPMCAb-generated atypical PrPres gave rise to PrP^{Sc} and prion disease with a phenotype similar to those induced by rPrP fibrils (Makarava et al. 2015) (Fig. 5.3c). These results confirmed that atypical PrPres is an intermediate on the pathway toward PrP^{Sc}, and illustrated that transmissible prion diseases could be produced via two alternative procedures: direct inoculation of rPrP fibrils or in vitro-produced atypical PrPres (Fig. 5.3b,c).

What factors define the rate of deformed templating? In transgenic mice that overexpress hamster PrP^{C}, elevated levels of PrP^{C} expression accelerated the formation of atypical PrPres but did not facilitate the second step, i.e. the transition from atypical PrPres to PrP^{Sc} (Makarava et al. 2016). As deformed templating is believed to be stochastic in nature, the rate of deformed templating does not depend on the concentration of a substrate but is likely to be controlled by the intrinsic rate of conformational errors in templating altered self-propagating states (Makarava et al. 2016).

5.6 Deformed Templating In Vitro

If rPrP fibrils or atypical PrPres can seed authentic PrP^{Sc} via deformed templating, one can assume that the opposite reaction, that is, the seeding of rPrP fibrils by PrP^{Sc}, is possible too. Indeed, while rPrP fibrils and PrP^{Sc} have different structures, they can seed each other upon changes in the replication environment and exposure to an appropriate substrate (Fig. 5.4a,b). In fact, for detecting minuscule amounts of PrP^{Sc}, several assays including quaking and amyloid seeding assays exploited the phenomenon of PrP^{Sc}-seeded conversion of rPrP into amyloid fibrils (Colby et al. 2007; Atarashi et al. 2007). While the amyloid seeding assays are extremely sensitive for detecting minute amounts of PrP^{Sc} seeds (Atarashi et al. 2007), prion infectivity is lost upon PrP^{Sc}-seeded fibrillation of rPrP in vitro arguing that PrP^{Sc}-specific structure is not maintained in seeding assays in vitro (Fig. 5.4a).

5.7 Prion Strain Mutation and Evolution via Deformed Templating

How do prions mutate? What is the origin of strain mutations? The "cloud" hypothesis proposes that pools of PrP^{Sc} particles within individual strains or isolates are intrinsically heterogeneous and that the heterogeneity arises due to spontaneous

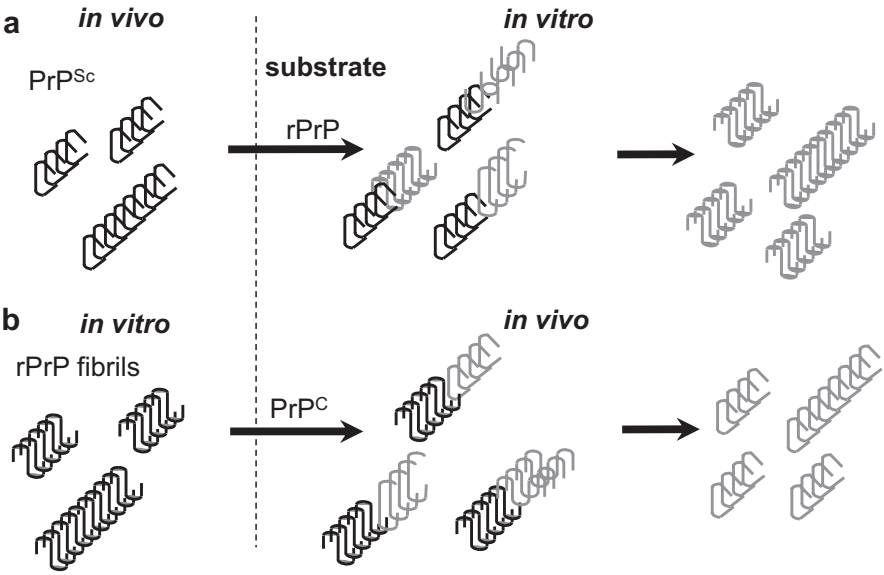

Fig. 5.4 Examples of deformed templating are (**a**) PrP^{Sc}-seeded fibrillation of rPrP in vitro and (**b**) generation of synthetic strains upon serial passaging of rPrP fibrils in animals

mutations in PrP^{Sc} structure (Collinge 2010; Li et al. 2010) (Fig. 5.5a). Upon changes in the replication environment, minor variants that fit best to replicate in the new environment receive selective advantages. Consistent with this view, several studies highlighted the fact that prion strains exhibit high levels of conformational plasticity and are subject to transformation when exposed to new replication environments. For instance, drug-resistant prions emerged in cultured cells following treatment with prion inhibitors swainsonine or quinacrine (Ghaemmaghami et al. 2009; Li et al. 2010). Accumulation of PrP^{Sc} variants in cloned prion material was attributed to ongoing processes of spontaneous 'mutations' of PrP^{Sc} structure (Li et al. 2010). According to the "cloud" hypothesis, changes in the replication environment give a selective advantage to minor PrP^{Sc} variants that are already present in the PrP^{Sc} pool. The "cloud" hypothesis does not explain how minor variants are generated or what is their origin (Fig. 5.5a).

Unlike the "cloud" hypothesis, the deformed templating mechanism proposes that changes in the replication environment play an active role in generating new PrP^{Sc} variants, in addition to its role in imposing a selective pressure (Fig. 5.5b) (Makarava and Baskakov 2013). Under circumstances the PrP^{Sc} template does not fit into a new environment, it can still seed new PrP^{Sc} variants via deformed templating. While the majority of the newly generated variants might not replicate effectively, a variant that fits well to the new environment will eventually emerge through

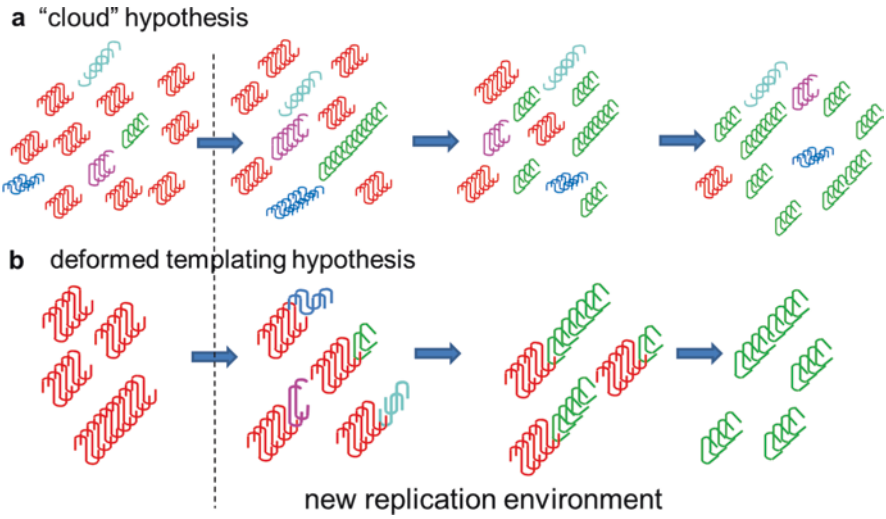

Fig. 5.5 Two hypotheses on the origin of prion strain mutations. (**a**) The "cloud" hypothesis proposes that prion isolates are intrinsically heterogeneous and consist of major (red) and minor (various colors) PrP^{Sc} variants. Changes in the replication environment provide selective advantages for the replication of a minor variant leading to transformations in the composition of PrP^{Sc} variants. (**b**) The deformed templating mechanism postulates that diverse structural variants are generated as a result of changes in the replication environment via numerous trial-and-error deformed templating events. A newly generated variant that fits better than parent PrP^{Sc} to an altered environment replaces the original PrP^{Sc} variant

multiple trial and error seeding events. Therefore, the change in the replication environment boosts the conformational diversity of the PrP^{Sc} pool and selects the variant that is the best fit for that environment.

The two models are not mutually exclusive, and both are likely to be involved in prion evolution. While deformed templating does not argue against structural heterogeneity of a PrP^{Sc} population of natural or synthetic origin, it helps to explain observations that would be difficult to understand solely based on the "cloud" hypothesis. The fundamental difference between the two models is in the origin of altered PrP^{Sc} states. In contrast to the "cloud" hypothesis, the deformed templating proposes that changes in the replication environment play an active role in expanding the pool of altered PrP^{Sc} variants. While new variants emerge with a help of a template they do not faithfully reproduce the parent state.

Experimentally, it is difficult to prove whether upon changing the replication environment, new PrP^{Sc} variants appear via selective amplification of pre-existing minor variants or emerge de novo via deformed templating (Mahal et al. 2012; Cancellotti et al. 2013). Nevertheless, changes in the replication environment were found to generate new PrP^{Sc} states (Gonzalez-Montalban et al. 2013; Katorcha et al. 2018). Adaptation of hamster strains 263K or Hyper to RNA-depleted brain homogenates and then re-adaptation to brain homogenates containing RNA in PMCAb was shown to lead to stable changes in PrP^{Sc} properties including PK-resistance, conformational stability, and amplification rates (Gonzalez-Montalban et al. 2013). Remarkably, upon reversible changes in RNA content, the amplification rate of the newly emerged PrP^{Sc} variants (referred to as $263K^{R+}$ or $Hyper^{R+}$) was 10^4-fold higher than that of brain-derived 263K. Moreover, consistent with the deformed templating mechanism, $263K^{R+}$ was absent in the original 263K brain material and emerged de novo as a result of reversible changes in the replication environment (Gonzalez-Montalban et al. 2013).

5.8 Role of Posttranslational Modifications in Driving Deformed Templating

In classical templating, the folding pattern of a newly recruited polypeptide chain accurately reproduces that of a template. In deformed templating, templates provide seeding too, yet newly recruited polypeptide chains acquire new folding patterns which only partially overlap with the folding pattern of a template. What are the driving forces behind deformed templating? Posttranslational modifications (PTMs) in PrP^{C} and, in particular, two N-linked groups along with Glycosylphosphatidylinositol (GPI) anchor are likely to impose spatial constraints limiting the spectrum of folding patterns available to PrP^{C} upon conversion into PrP^{Sc} (Breydo et al. 2007).

In the absence of PTMs, rPrPs acquire fibrillar structures that are thermodynamically and kinetically preferable (Baskakov et al. 2002; Sun et al. 2007), but do not easily accommodate PTMs. Under the circumstances that PTMs impose spatial or

electrostatic constraints not compatible with the rPrP fibrillar structures, rPrP fibrils select only those PrP^C molecules in vivo that can fit into the fibrillar structure. Indeed, as discussed above, the first product of misfolding triggered by rPrP fibrils in vivo was atypical PrPres, which is predominantly un- and monoglycosylated (Makarava et al. 2011, 2012a) (Fig. 5.3c). For accommodating diglycosylated PrP^C molecules, new structures have to emerge. Not only PTMs drive deformed templating, but N-glycans are also important for maintaining high fidelity of PrP^{Sc} replication. Transmission of prions to hosts expressing PrP^C deficient in N-glycans was found to change strain-specific characteristics of the 79A strain (Cancellotti et al. 2013). Loss of prion infectivity and PrP^{Sc}-specific structure upon PrP^{Sc}-seeded fibrillation of rPrP in vitro also argues that N-glycans are important for maintaining high fidelity of replication. Selective recruitment of PrP^C sialoglycoforms, specified by strain-specific structure, has multiple important implications in prion biology and is discussed elsewhere (Katorcha et al. 2015; Baskakov and Katorcha 2016; Baskakov et al. 2018).

5.9 Deformed Templating as a Mechanism of a Cross-Talk Between Amyloidogenic Proteins

The hypothesis that transmissible prion diseases can be triggered by cross-β PrP structures substantially different from that of authentic PrP^{Sc} has important implications for understanding the etiology of prion and other neurodegenerative diseases. A growing number of studies have documented that amyloid forms of several proteins linked to neurodegenerative diseases were capable of seeding their own aggregation in a prion-like manner in a cell and spreading from cell to cell through the nervous system (reviewed in Miller (2009), Frost and Diamond (2010), and Aguzzi and Rajendran (2009)). It is generally assumed that self-perpetuating aggregation requires identity in amino acid sequence between seeds and substrate. Nevertheless, the possibility of cross-talk between non-related amyloidogenic proteins has been illustrated in several studies (Jean et al. 2007; Yan et al. 2007; Morales et al. 2010; Katorcha et al. 2017). In vivo, amyloidosis of one protein was found to be triggered by fibrils of an unrelated protein in a manner similar to cross-seeded polymerization (Jean et al. 2007; Yan et al. 2007; Morales et al. 2010). Cross-talk between several yeast prion proteins provides another example of how direct interactions between newly forming and preexisting heterologous fibrils might take place in a cell (Derkatch et al. 1997, 2001, 2004). Moreover, protein aggregates produced from two different proteins or peptides, including PrP, Aβ, α-synuclein, immunoglobulin light chain λ, and β_2 microglobulin, often colocalize within the same amyloid plaque in a variety of organs or tissues (Haik et al. 2002; Adjou et al. 2007; Takahashi et al. 1996; Miyazono et al. 1992; Galuske et al. 2004). The promiscuous nature of the propagating activity of amyloid structures can lead to devastating consequences for cellular health. For instance, the cross-talk between non-related amyloidogenic

proteins may offer a possible explanation for the development of age-related conformational disorders that are considered to be sporadic in origin. In an effort to identify the spectrum of structures and sequences capable of triggering the PrP^{C} to PrP^{Sc} conversion, we found that α-synuclein aggregates formed in cultured cells or in vitro, but not non-fibrillar α-synuclein or fibrillar Aβ, triggered misfolding of the PrP^{C} into self-replicating PrP states that induced transmissible prion disease in wild type host (Katorcha et al. 2017).

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Chapter 6
Prion Strain Interference

Ronald A. Shikiya and Jason C. Bartz

Abstract Prions are transmissible agents comprised of a misfolded protein PrP^{Sc} that is post-translationally derived from the normal isoform PrP^{C}. Prion strains are operationally defined by differences in the distribution and intensity of spongiform degeneration and distribution of PrP^{Sc} in the CNS. The mechanism by which prion strains are encoded is not known, however, current evidence suggests that the conformation of PrP^{Sc} encodes prion strain diversity. In natural prion disease, more than one prion strain can exist in an individual. Prion strains, when present in the same host, can interfere with each other, a process that can influence the emergence of a dominant strain from a mixture and can occur during prion adaptation following interspecies transmission. The parameters and mechanisms that influence prion strain interference are beginning to be understood.

Keywords Prion diseases · Adaptation · Strains · Interference

6.1 Introduction

Prions are transmissible agents composed of a misfolded protein PrP^{Sc} that is post-translationally derived from the normal isoform PrP^{C} (McKinley et al. 1983; Prusiner 1982; Bolton et al. 1982; Deleault et al. 2007). PrP^{C} is a cell-surface protein that is attached to the cellular membrane via a glycosylphosphatidylinositol anchor, is expressed in numerous cell types but is most abundant in the central nervous system (CNS) (Prusiner 1991; Basler et al. 1986; Oesch et al. 1985; Kretzschmar et al. 1986). Prion formation is initiated at the cell surface by the binding of PrP^{Sc} to PrP^{C} where these molecules are subsequently endocytosed and the conversion of PrP^{C} to PrP^{Sc} occurs at the cell surface and/or in the endosomal/lysosomal system

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(Caughey and Raymond 1991; Caughey et al. 1990; Caughey et al. 1989). Prion formation is recapitulated in protein misfolding cyclic amplification (PMCA) resulting in PrP molecules with biochemical and infectious properties of PrP^{Sc} (Barria et al. 2009; Saa et al. 2006; Soto et al. 2005; Saborio et al. 2001; Shikiya and Bartz 2011; Vidal et al. 2020; Chianini et al. 2012).

Prion diseases are neurodegenerative diseases of animals including humans. Animal prion diseases include scrapie of sheep and goats, transmissible mink encephalopathy (TME) of ranch-raised mink, bovine spongiform encephalopathy, camel prion disease, and chronic wasting disease (CWD) of captive and free-ranging deer, elk, and moose (Benestad et al. 2016; Williams and Young 1980; Babelhadj et al. 2018; Marsh et al. 1969a; Wells et al. 1987; Cuillé and Chelle 1936). Human prion diseases are comprised of kuru of the Fore people of Papua New Guinea, Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome, and fatal familial insomnia (Prusiner 1998; Brown et al. 1986; Medori et al. 1992). Prion diseases are unique in biology as they have infectious, familial, and sporadic etiologies (Parchi and Gambetti 1995). Infectious prions can be detected in patients from all three disease etiologies suggesting de novo formation of prion infectivity which is consistent with seminal studies where infectious prions were experimentally generated from non-infectious components (Deleault et al. 2007; Legname et al. 2004; Makarava et al. 2011; Colby et al. 2010; Colby et al. 2009). Prion diseases are zoonotic and the emergence of variant CJD (vCJD) is caused by the transmission of bovine spongiform encephalopathy (BSE) to humans by an unknown route of infection (Bruce et al. 1997; Lasmezas et al. 1996).

Prion strains are operationally defined by differences in the distribution and intensity of spongiform degeneration and distribution of PrP^{Sc} in the CNS (Bartz 2016). Prion strains can differ in the incubation period, clinical signs, and distribution of prions within the host and the host range (Ayers et al. 2011; Fraser and Dickinson 1968; Kimberlin et al. 1987, 1989; Beringue et al. 2008). The mechanism by which prion strains are encoded is not known, however, evidence is suggesting that the conformation of PrP^{Sc} may be involved (Bessen et al. 1995; Bessen and Marsh 1992a, 1994; Telling et al. 1996; Caughey et al. 1998; Parchi et al. 2011; Tixador et al. 2010; Laferrière et al. 2013; Safar et al. 1998). The mechanisms of how changes in PrP^{Sc} conformation result in strain-specific differences in the phenotype of disease are largely unknown.

In natural prion disease, more than one prion strain can exist in an individual. Transmission of field isolates of prion disease to rodents can result in the emergence of several distinct prion strains suggesting more than one strain is present in the field isolate (Dickinson 1976; Kimberlin and Walker 1978). Alternatively, the interspecies transmission may result in the generation of new strains that have increased fitness for the new host species (Kimberlin and Walker 1978; Dickinson and Outram 1979; Peretz et al. 2002; Duque Velasquez et al. 2020). Truncated isoforms of PrP^{Sc} with different molecular weights have been identified in individual humans infected with CJD (Rossi et al. 2019; Cassard et al. 2020; Cali et al. 2009, 2020;; Mazza et al. 2010; Notari et al. 2004, 2007; Polymenidou et al. 2005; Puoti et al. 1999;

Schoch et al. 2006; Uro-Coste et al. 2008). This data strongly suggests that more than one prion strain can co-exist in an individual human affected with prion disease.

6.2 Parameters Governing Prion Strain Interference

6.2.1 Overview

Prion strains, when present in the same host, can interfere with each other. Prion strain interference was first described by Alan Dickinson where he determined that inoculation of the 22C agent (the blocking strain) in mice prior to superinfection with the 22A agent (the superinfecting strain) could lead to an extension of the incubation period of 22A (Dickinson et al. 1972). The general parameters that govern prion strain interference are beginning to be understood. The blocking strain must be able to replicate to interfere with the superinfecting strain and increasing the titer of the blocking strain or increasing the interval between blocking strain infection and superinfection correspondingly increases the interference effect. Similarly, the outcome of co-infection with two prion strains is dependent on the ratio of the strains involved. These studies suggest that the onset of prion conversion, and not if prions are co-infected or inoculated at different times, is the parameter that influences which strain will emerge. Prion strain interference occurs between numerous prion strain combinations in two rodent animal models using a variety of routes of infection, including oral infection, suggesting that prion strain interference is a more generalized phenomenon (Table 6.1) (Dickinson et al. 1972, 1975; Bartz et al. 2004, 2007; Kimberlin and Walker 1985; Manuelidis 1998; Nilsson et al. 2010; Eckland et al. 2018; Langenfeld et al. 2016; Shikiya et al. 2010; Schutt and Bartz 2008). Finally, for strain interference to occur in the CNS, the two strains must infect the same population of cells (Shikiya et al. 2010). The evidence supporting these generalizations is outlined below.

6.2.2 The Interval Between Prion Strains Inoculation Influences Interference

Intracerebral (i.c.) inoculation of VM mice (*sinc* genotype *p7p7*) with 22C (a long incubation period mouse-adapted scrapie strain) 9, 5, or 1 week prior to i.c. inoculation of the shorter incubation period strain 22A resulted in all of the mice succumbing to 22A disease (Dickinson et al. 1972). Importantly, the incubation period of the disease in the 9- or 5-week interval group was significantly extended compared to mice inoculated with 22A alone. These results suggested that 22C was able to interfere with the development of 22A pathogenesis. Prions strain interference has also been identified using the two mouse-adapted human strains SY (a long incubation

Table 6.1 Summary of prion interference studies in animals

Blocking strain	Superinfecting strain	Route of inoculation	Host species	Interference effect	References
22C scrapie	22A scrapie	i.c.	VM (*Sinc*$^{p7/p7}$) mice	N,I,B[a]	Dickinson et al. (1972)
22A scrapie	22C scrapie	i.p.	RIII (*Sinc*$^{s7/s7}$) mice	B	Dickinson et al. (1975)
22A scrapie	22C scrapie	i.p.	CW (*Sinc*$^{s7/s7}$) mice	B	Kimberlin and Walker (1985)
22A scrapie	22C scrapie	i.p.	RIII (*Sinc*$^{s7/s7}$) mice	B	Taylor et al. (1986)
SY CJD	FU-1 GSS	i.c.	CD-1 (*Sinc*$^{s7/s7}$) mice	B	Manuelidis (1998), Laura Manuelidis (2003), and Manuelidis and Yun (2000)
SY CJD	FU-1 GSS	i.v.	CD-1 (*Sinc*$^{s7/s7}$) mice	I	Laura Manuelidis (2003)
SY CJD	FU-1 GSS	i.c.	RAG-1$^{-/-}$ (*Sinc*$^{s7/s7}$) mice	N	Laura Manuelidis (2003)
SY CJD	FU-1 GSS	i.c.	C57BlL/6 (Sinc$^{s7/s7}$) mice	I	Laura Manuelidis (2003)
DY TME	HY TME	i.sc.	Syrian hamster	N,I,B	Bartz et al. (2007)
DY TME	263K	i.sc.	Syrian hamster	B	Schutt and Bartz (2008)
DY TME	HaCWD	i.sc.	Syrian hamster	B	Schutt and Bartz (2008)
DY TME	HY TME	i.p.	Syrian hamster	N,I	Bartz et al. (2004)
DY TME	HY TME	Per os	Syrian hamster	N,I	Schutt and Bartz (2008)
DY TME	HY TME	i.c.	Syrian hamster	N,I,B	Bartz et al. (2000)
DY TME	HY TME	i.p.	Syrian hamster	N	Bessen and Marsh (1992b)
DY TME	ME7H	i.c.	Syrian hamster	N	Eckland et al. (2018)
139H	DY TME	i.c.	Syrian hamster	N	Eckland et al. (2018)
139H	Sc237	i.c.	Syrian hamster	N	Eckland et al. (2018)
139H	HY TME	i.sc.	Syrian hamster	N,I,B	Langenfeld et al. (2016)

(continued)

Table 6.1 (continued)

Blocking strain	Superinfecting strain	Route of inoculation	Host species	Interference effect	References
recHaPrP anti-prion	263K	i.c.	Syrian hamster	I	Diaz-Espinoza et al. (2018)
TME	22A scrapie	i.p.	VM (*Sinc*$^{p7/p7}$) mice	N	Taylor et al. (1986)
TME	22C scrapie	i.p.	VL (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
TME	79A scrapie	i.p.	VL (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
TME	79V scrapie	i.p.	VM (*Sinc*$^{p7/p7}$) mice	N	Taylor et al. (1986)
TME	79V scrapie	i.c., i.p.	BRVR (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
TME	87A scrapie	i.c., i.p.	BALB (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
TME	139A scrapie	i.p.	VL (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
TME	ME7H scrapie	i.c., i.p.	BRVR (*Sinc*$^{s7/s7}$) mice	N	Taylor et al. (1986)
Ts-1 scrapie	Ts-2 scrapie	i.c.	CD-1 (*Sinc*$^{s7/s7}$) mice	N	Hirogari et al. (2003)
Ts-1 scrapie	Ts-1 scrapie	i.c.	CD-1 (*Sinc*$^{s7/s7}$) mice	N	Hirogari et al. (2003)

[a]*N* no interference, *I* strain interference, *B* complete blocking

period strain isolated from an sCJD patient) and FU (a short incubation period strain isolated from a Gerstmann–Straussler–Scheinker patient). When SY was i.c. inoculated 80 or 92 days prior to inoculation with FU, PrP^{Sc}, pathology, or clinical signs were not detected, indicating that FU has been blocked (Manuelidis 1998; Manuelidis and Lu 2003). Interference has also been found to occur using i.v. inoculation. When SY was inoculated 80 days prior to FU, there was a significant increase in the incubation time of the superinfected FU (Manuelidis and Lu 2003).

Similar interfering effects have been shown following the sciatic nerve (i.sc.) route of infection in hamsters using strains isolated from transmissible mink encephalopathy (TME). When the DY TME agent was inoculated into the sciatic nerve 30 or 60 days prior to the HY TME agent, there was no evidence of prion strain interference based on clinical signs, Western blot migration, and incubation period of the disease (Bartz et al. 2004, 2007). When the interval was extended to 90 days between i.sc. inoculations, the DY TME agent extended the incubation period of the HY TME agent by 12 days (Bartz et al. 2007). A 120-day interval

between i.sc. inoculations resulted in the DY TME agent completely blocking the HY TME agent (or the 263K and HaCWD agents) from causing disease based on these same three criteria (Bartz et al. 2007; Schutt and Bartz 2008). The ability of DY TME to interfere with or block HY TME corresponded with the accumulation of DY PrP^{Sc} in the lumbar spinal cord, consistent with the hypothesis that replication of the blocking strain is required for the blocking strain to interfere with the conversion of the superinfecting strain (Bartz et al. 2007; Shikiya et al. 2010). Importantly, sciatic nerve inoculation strain interference experiments using the 139H and HY TME strains determined that both the blocking and superinfecting agent conversion can be altered suggesting that the dynamics of mixed strain infections are more complicated than previously appreciated (Langenfeld et al. 2016). The DY TME agent can interfere with HY TME following oral inoculation. Per os infection of hamsters 120 days prior to per os superinfection with the HY TME agent results in an increase in the incubation period of HY TME by 9 days compared to the control group inoculated with the HY TME agent alone (Schutt and Bartz 2008). These experiments illustrate that the interval between inoculations is an important parameter for prion strain interference and that greater intervals between inoculation of the blocking and superinfecting strains allow for higher levels of blocking strain replication increasing the interference effect.

6.2.3 The Relative Titer of the Blocking and Superinfecting Strains Can Influence Interference

Dickinson first indicated that as the titer of the blocking strain was increased, there was a corresponding increase in the interfering or blocking effect, however, details of these experiments were not provided (Dickinson and Outram 1979). Expanding upon this observation, the titer of DY TME can determine if and when the HY TME strain emerges from a mixture (Shikiya et al. 2010; Bartz et al. 2000). Co-Inoculation of hamsters with a mixture of a 10^{-2} dilution of DY TME brain homogenate and a 10^{-6} dilution of HY TME brain homogenate resulted in all of the animals succumbing to HY TME. When a 10-fold higher relative dose of DY TME was used, nearly all of the hamsters succumb to DY TME infection based on clinical signs and the strain-specific electrophoretic migration of PrP^{Sc} on Western blot (Bartz et al. 2000). However, when the brain homogenate from these hamsters was i.c. passaged a second time in hamsters, all the animals succumb to the HY TME agent. When a hundred-fold increase in the relative dose of DY TME agent to HY TME agent was used, the animals succumb to DY TME agent, which was maintained upon the second serial passage in hamsters (Bartz et al. 2000).

The effect of the DY TME agent dose on the emergence of the HY TME agent in vivo has been recapitulated in vitro using PMCA. In these studies, the ratio of the DY and HY TME agents was an important parameter that determined when HY TME would emerge. Similar to in vivo studies, higher ratios of DY TME to HY TME agent resulted in an increase in the ability of DY TME to interfere with or

completely block HY TME from emerging in PMCA (Shikiya et al. 2010). In animal studies where DY TME is able to completely block HY TME from causing disease, small amounts of HY TME can be detected using PMCA suggesting that strain blocking may not be complete (Shikiya et al. 2010). Due to limitations of the life span of the host, exploring if higher ratios of DY TME agent to HY TME agent can result in complete suppression of HY conversion are not feasible. PMCA can overcome this limitation and has been able to examine a wider range of ratios DY to HY TME agent. Using PMCA to study strain interference has identified conditions where complete blockage of HY TME agent replication occurs (Shikiya et al. 2010). Since PMCA replicates HY TME agent with similar efficiency as in animals, these in vitro strain interference studies suggest that complete blockage of agent replication in animals is feasible (Shikiya and Bartz 2011; Shikiya et al. 2010). Overall, the relative onset of replication of the blocking and superinfecting strain is a critical parameter in strain interference, not whether the prion strains are inoculated at the same time or separately.

6.2.4 *Blocking Strain Replication Is Required for Strain Interference*

Transmission of the TME agent to mice does not cause disease (Taylor et al. 1986; Marsh et al. 1969b). Inoculation of mice with TME prior to superinfection with the mouse strains 22A, 22C, 79A, 79V, 87A, 139A, and ME7, indicated that prion strain interference did not occur (Taylor et al. 1986). Similarly, if a prion agent is inactivated by chemical treatment, it is unable to interfere with superinfecting strains. For example, when the 22A scrapie agent was inoculated in mice prior to the 22C scrapie agent, the incubation period for 22C was extended by over 300 days compared to animals inoculated with 22C alone (Kimberlin and Walker 1985). However, when the 22A agent was treated with 12M urea or 5 Mrad of ionizing radiation the interference effect of 22A on 22C was eliminated. Conversely, treatment with 1% β-propionolactone or 1% sodium dodecyl sulfate (SDS) which did not alter 22A infectivity, did not affect the ability of 22A to interfere with 22C (Kimberlin and Walker 1985). Overall, these studies suggest that strain interference requires active replication of the blocking strain.

6.2.5 *Infection of Common Neuroanatomical Pathways Is Required for Interference*

As described above, the DY TME agent was able to extend the incubation period or block the clinical signs of the HY TME agent when both agents are inoculated into the same sciatic nerve (Bartz et al. 2007; Schutt and Bartz 2008). Both HY and DY are transynaptically transported along the same four descending motor pathways

following sciatic nerve inoculation (Ayers et al. 2009). The first cell type that is shared by all four of the descending motor pathways are ventral motor neurons (VMNs) in the lumbar spinal cord, this suggests that these cells are where interference occurs in this system (Shikiya et al. 2010; Ayers et al. 2009). One possible mechanism to explain strain interference is that DY, the blocking strain, at 120 days post infection, has destroyed VMNs and transynaptically spread to other neuronal populations. In this scenario, superinfection with HY TME at 120 days post-DY TME infection will result in the failure of HY TME to establish infection because VMNs have been destroyed and since DY has spread to other locations in the CNS. At this time point, DY can continue to spread eventually leading to the onset of the disease. However, at 120 days post DY TME infection, the only neuropathological change observed in VMNs is the deposition of PrP^{Sc}, indicating that cellular damage to VMNs is not contributing to the interference effect (Shikiya et al. 2010). HY and DY PrP^{Sc} are both deposited on the VMN cell membrane, suggesting this is where strain interference occurs. We hypothesized that strain interference occurs when the blocking and superinfecting strains establish infection in the same population of cells. To directly test this hypothesis, hamsters were superinfected with HY TME on the sciatic nerve contralateral to the sciatic never inoculated with DY TME. Since VMNs on the right and left hemispheres of the lumbar spinal cord are not synaptically connected, prion inoculation of the contralateral sciatic nerve should not be influenced by ipsilateral VMNs. In this scenario, the HY TME agent is directed to VMNs that are not infected with DY TME. Under these conditions, the animals developed HY TME clinical signs with an incubation period similar to animals inoculated with the HY TME agent alone (Bartz et al. 2007). This result indicates that (i) prion strains must infect the same cell for strain interference to occur and (ii) that the interference effect is not due to a paracrine or endocrine effect of a factor that results in resistance to superinfection (e.g., interferons). Overall, these data are consistent with the hypothesis that prion strains must infect the same cell for interference to occur.

6.3 Prion Strain Interference and the Replication Site Hypothesis

6.3.1 The Replication Site Hypothesis

The replication site hypothesis was developed, in part, in an attempt to explain prion strain interference. The ability to block strains to extend the incubation period of superinfecting strains was attributed to the blocking strain occupying replication sites that were common to both the blocking and superinfecting strains (Dickinson and Outram 1979). Occupation of the replication sites by the blocking strain would prevent the superinfecting strain from gaining access to them resulting in a decrease in superinfecting strain replication. The increase in the incubation period or the

complete blockage of the superinfecting strain would be controlled by the degree to which the blocking strain occupied the replication sites. Evidence supporting this hypothesis is the observation that splenectomy, which removes extraneural replication sites, increased the ability of the blocking strain to interfere with the superinfecting strain following intraperitoneal inoculation (Dickinson and Outram 1979). The experiments described above on the parameters that control prion strain interference are consistent with the replication site hypothesis. The replication site, as proposed by Dickinson, is hypothesized to be the gene product of *Sinc*, which is now known to be congruent with PrP^C (Hunter et al. 1987). This would suggest that prion strain interference is due to blocking strain PrP^{Sc} preventing superinfecting strain PrP^{Sc} from interacting with PrP^C either because (i) the PrP^{Sc}/PrP^C interaction prevents superinfecting PrP^{Sc} from gaining access to PrP^C or (ii) that blocking PrP^{Sc} conversion has depleted the available PrP^C for the superinfecting strain to convert.

6.3.2 The Role of PrP^C in Prion Strain Interference

Current evidence suggests that prion strains compete for PrP^C. As described in previous sections, prion strain interference is not mediated by an anatomical deficiency created by the blocking strain or due to a production of a secretory factor that inhibits the conversion of the superinfecting strain. Since strain interference can be recapitulated in PMCA, this indicates that intact cells are not required for strain interference and suggests that prion strains compete for a component required for prion formation. Prion conversion requires PrP^C and is enhanced by cellular cofactors that include RNA, glycosaminoglycans, and lipids (Burke et al. 2020; Miller et al. 2013; Deleault et al. 2003, 2012a, b; Saá et al. 2012). Using recombinant protein as the source of PrP^C, instead of brain homogenate, prion strain interference was observed between two prion isolates in this simplified PMCA reaction (Atarashi et al. 2007). Since this simplified PMCA system does not contain known prion conversion co-factors, it is highly suggestive that the isolates competed for PrP^C.

Using conventional PMCA conditions, PrP^{Sc} accumulates to higher levels in HY TME-seeded PMCA reactions compared with DY TME-seeded reactions (Shikiya et al. 2010). Under these same PMCA conditions, DY TME can interfere with, or completely block, the emergence of HY TME (Shikiya et al. 2010). This data indicates that in the DY TME seeded reactions, DY PrP^{Sc} can convert or sequester PrP^C to inhibit HY PrP^{Sc} formation. To further explore this mechanism, PMCA reactions co-infected with relatively low prion conversion efficiency strains (e.g., 139H and DY TME) resulted in the strains amplifying independently (Eckland et al. 2018). Compared to HY TME-seeded PMCA reactions, the abundance of the remaining PrP^C after PMCA was higher in the PMCA reactions co-infected with the low prion conversion strains (Eckland et al. 2018). Overall, this data indicates that prion strain interference occurs when PrP^C becomes a limiting factor, either via the conversion efficiency of the strain or by the abundance of the convertible PrP^C present in the cell.

6.3.3 Prion Strain Interactions and Interconversion

Direct interaction between the blocking and superinfecting strain PrP^{Sc} may account for strain interference. In this model, blocking strain PrP^{Sc} binds to the PrP^{Sc} from the superinfecting strain. Evidence to support this hypothesis is the detection of hybrid PrP^{Sc} deposits in mice infected with two strains (Nilsson et al. 2010). From this point two main outcomes are possible. First, the blocking strain may be able to convert the superinfecting strain's PrP^{Sc} to the blocking strain's PrP^{Sc} strain-specific conformation. This will allow for a greater accumulation of blocking strain PrP^{Sc} and a reduction in the PrP^{Sc} of the superinfecting strain, leading to strain interference. Prion strain interconversion has been suggested in vitro by strain-induced alterations in the migration of PrP^{Sc}, the sensitivity of PrP^{Sc} to PK digestion, and conformations switching within synthetic PrP fibrils (Makarava et al. 2009; Nishina et al. 2004; Wadsworth et al. 1999). The second possibility is that the direct interaction of PrP^{Sc} from the blocking and superinfecting strain may lead to a hybrid PrP^{Sc} molecule that may have a diminished capacity to convert PrP^{C} to PrP^{Sc} from one or both strains. The observation that combinations of strains with slow conversion activity act independently when co-infected in vivo and in vitro, in spite of high levels of PrP^{Sc}, suggests that PrP^{Sc} interactions between strains do not significantly contribute to prion strain interference (Eckland et al. 2018).

6.4 Prion "Vaccination" and Strain Interference

The concept of a prion "vaccine" was first proposed over 40 years ago (Dickinson and Outram 1979). The envisioned vaccine strain would not cause disease in the vaccinated host but would block subsequent infection with a pathogenic prion strain. This prion vaccine would not protect the host via an immune response to the infectious agent like conventional vaccines but would instead occupy prion replication sites and prevent infection by a pathogenic prion strain. At the time of its proposal, all blocking prion strains eventually would kill the host, and the difficulty, as outlined by Dickinson, was in identifying a prion strain that would not cause disease yet retain the ability to interfere (Dickinson and Outram 1979). While a "vaccine" strain that completely protects the host has not been identified, there are two examples of a prion strain that can interfere with a pathogenic strain yet does not cause disease.

Intraperitoneal or oral inoculation with the DY TME agent does not result in clinical disease within the lifespan of the host (Bartz et al. 2004, 2005). Additionally, in spleen, lymph nodes, peripheral nervous system (PNS), and CNS, DY TME agent replication is not detectable as assessed by animal bioassay or PrP^{Sc} deposition as determined by Western blot or immunohistochemistry (Bartz et al. 2005; Shikiya et al. 2017). Interestingly, inoculation of the DY TME agent can modestly extend the incubation period of the HY TME agent following both i.p. and per os routes of

inoculation (Bartz et al. 2004; Schutt and Bartz 2008). The mechanism underlying this observation is not known, however, several possibilities exist. First, DY TME agent replication is not detected in the spleen; however, DY TME may be occupying replication sites in locations in the host such as lymph nodes that are used by the superinfecting strain for neuroinvasion that was not examined for DY TME agent replication. Second, the DY TME agent is blocking replication sites but the amount of DY TME agent in these tissues is below the sensitivity of animal bioassay. For both possibilities, the large time interval required for DY TME to interfere with superinfecting strains suggests that DY conversion is required for the interference effect. Consistent with this observation, a synthetic hamster prion (anti-prion) that does not cause disease in the lifetime of the host when co-infected with 263K can increase in the incubation period of 263K compared to animals inoculated with 263K alone (Diaz-Espinoza et al. 2018). Consistent with prior superinfection strain interference studies, increasing the interval between inoculation with the anti-prion and superinfection with 263K resulted in an increase in the interference effect (Diaz-Espinoza et al. 2018).

To safely use a non-pathogenic prion strain as a prion vaccine, it must not revert to a pathogenic strain. Much evidence supports the hypothesis that prion strains are comprised of a dynamic mixture of a dominant prion strain and prion substrains (Collinge and Clarke 2007). It is known that altering the ratio of the dominant strain and the substrain can allow for the emergence of a substrain, therefore, the possibility exists that pathogenic substrains could emerge from non-pathogenic vaccine strains. Additionally, while most strains maintain their unique strain properties upon serial passage, some strains are unstable and can randomly change into a new strain (i.e., mutation). Presumably, strain mutation results from the adoption of PrP^{Sc} to a different conformation, however, it is unknown if this is a property of all strains or only a subset of strains. A better understanding of strains is required before this concept can be fully explored.

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Chapter 7
Molecular Mechanisms Encoding Strains of Prions and Prion-Like Misfolded Proteins

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Abstract Yeast, fungal, and mammalian prions determine heritable as well as infectious traits (Shorter J, Lindquist S. Nat Rev Genet, 6:435–450, 2005; Wickner RB, et al. FEMS Yeast Res, 10:980–991, 2010; Prusiner SB, Scott MR, DeArmond SJ, Carlson G. Transmission and replication of prions. In: Prusiner SB (ed). Prion biology and diseases. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 187–242, 2004a). In mammals, prions cause a group of fatal and rapidly progressive neurodegenerative diseases (Prusiner SB, Scott MR, DeArmond SJ, Carlson G. Transmission and replication of prions. In: Prusiner SB (ed). Prion biology and diseases. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 187–242, 2004a), originally described as transmissible spongiform encephalopathies (TSEs) (Gajdusek DC, Gibbs CJ Jr, Alpers M. Nature, 209:794–796, 1966). Variations in prions, which cause different disease phenotypes, are referred to as strains. Mammalian prion strains are differentiated by a number of characteristics, including disease incubation time, clinical symptoms, prion dose–response, proteolytic sensitivity, conformational attributes of pathogenic prion protein (PrP^{Sc}), targeted brain anatomical areas, or by Western blot patterns of glycosylated or deglycosylated PrP^{Sc} (Puoti G, et al. Lancet Neurol, 11:618–628, 2012; Prusiner SB, et al. Some strategies and methods for the study of prions. In: Prusiner SB (ed). Prion biology and diseases. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 857–920, 2004b; Safar J, et al. Nat Med, 4:1157–1165, 1998a). Remarkable progress in the past decade has produced many lines of evidence arguing that extraordinary phenotypic diversity of human prion diseases arises from structurally distinct prion strains that target, at different progression speeds, variable brain structures and cells (Kim C, et al. Nat Commun, 9, 2018; Safar JG, et al. PLoS Pathog,

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11:e1004832, 2015a). This paradigm is supported now with biochemical, genetic, and animal studies, by the recent successful generation of a new synthetic strain of human prions, and by considerable progress in high-resolution structural studies of prions (Kim C, et al. Nat Commun, 9, 2018; Safar JG, et al. PLoS Pathog, 11:e1004832, 2015a). The recent findings of distinct prion-like conformers of amyloid beta (Cohen M, et al. Prion, 9:S76–S77, 2015a (Taylor & Francis Inc., Philadelphia)) and misfolded tau protein expand this concept to Alzheimer's disease (AD) (Kim C, et al. Sci Transl Med, 14:eabg0253, 2022) and monogenic frontotemporal lobar degeneration (FTLD)-MAPT P301L (Daude N, et al. Acta Neuropathol, 139:1045–1070, 2020) and suggest that distinct strains of misfolded proteins drive the phenotypes and progression rates in a number of neurodegenerative diseases (Kang SG, Eskandari-Sedighi G, Hromadkova L, Safar JG, Westaway D. Front Neurol, 1394, 2020a). The emerging concept pointing to structurally distinct prion-like strains of misfolded proteins as the critical differentiating factor in disease development emphasizes the need for personalized structure- and strain-specific therapeutic approaches.

Keywords Prion strains · Conformation of prion protein · Protein misfolding cyclic amplification (PMCA) · Real-Time Quaking-Induced Conversion (RT QuIC) · Conformation-dependent immunoassay (CDI) · Neurodegeneration

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
CDI	conformation-dependent immunoassay
CHO	N-linked complex glycosylation chains
CJD	Creutzfeldt-Jakob disease
CPA	cell panel assay
ER	endoplasmic reticulum
FFI	fatal familial insomnia
FTLD	frontotemporal lobar degeneration
GSS	Gerstmann–Sträussler–Scheinker syndrome
PMCA	protein misfolding cyclic amplification
PrP	prion protein
PrP^{C}	normal or cellular prion protein
PrP^{Sc}	pathogenic prion protein
PRNP	prion protein gene
$rPrP^{Sc}$	protease-resistant conformers of pathogenic prion protein (PrP 27-30)
$sPrP^{Sc}$	protease-sensitive conformers of pathogenic prion protein
sCJD	sporadic Creutzfeldt–Jakob disease
SFI	sporadic fatal insomnia
SSCA	standard scrapie cell assay
TSE	transmissible spongiform encephalopathy
VPSPr	variable protease-sensitive prionopathy

WB Western blot

7.1 Prion Diversity

Unique characteristics of mammalian prion isolates, which cause distinctive disease phenotypes, are referred to as strains. Prion strains were initially isolated based on distinctive clinical symptoms in goats with scrapie (Pattison and Millson 1961). Subsequently, strains were isolated in rodents based on divergent incubation times and neuropathologic profiles (Fraser and Dickinson 1973; Dickinson and Fraser 1977). New strains have been produced upon passage from one species to another (Kimberlin et al. 1987), from non-transgenic (Tg) mice to mice expressing a foreign or artificial PrP transgene (Scott et al. 1997), or most recently in vitro from recombinant prion protein (Legname et al. 2006; Wang et al. 2010).

For several decades, the existence of several prion strains was offered as an argument for the existence of a scrapie-specific nucleic acid (Bruce and Dickinson 1987; Dickinson and Outram 1988). However, despite numerous attempts to find such a nucleic acid using several approaches, and despite mounting evidence against the existence of a strain-coding polynucleotide (Meyer et al. 1991; Kellings et al. 1992, 1994; Safar et al. 2005a), an explanation for prion strains remained a conundrum, and a major challenge to basic principles of molecular biology (Prusiner 1998a; Safar et al. 2005b; Weissmann 2004). Moreover, the discovery that different strains of prions can be propagated indefinitely with high fidelity in inbred mouse lines expressing only a single PrP sequence, and the finding that prion strains were selective with regard to the cells in which they can replicate, raised fundamental questions: (a) How many mammalian prion strains exist? (b) How can cells distinguish different prion strains, as reflected in the cells' ability to propagate them? (c) How are strain-specific characteristics encoded if the prion is composed solely of PrP with the same sequence? A growing body of evidence indicates that the improved understanding of these mechanisms, particularly in human prions, will have major implications for age-related neurodegenerative diseases linked to other misfolded proteins, including an ongoing debate about their potential transmissibility (Daude et al. 2020; Asher et al. 2020; Kang et al. 2020b).

7.2 Distinct Phenotypes of Prion Strains in Bioassay

An important milestone in the history of prion strain research was the experimental transmission of scrapie from sheep to mice ~18 months after intracerebral inoculation of brain extracts (Chandler 1961). On second passage, the incubation periods shortened to 4–5 months and remained constant on subsequent passages. The demonstration that scrapie could be transmitted to a small laboratory rodent made possible many new experimental studies that were previously impracticable in sheep or

goats, and helped to identify and characterize the first prion isolates by distinct clinical symptoms, incubation time, and brain pathology (Fraser and Dickinson 1973; Dickinson et al. 1972). A second milestone occurred with the development of an incubation time bioassay in Syrian hamsters, which reduced the time required to measure prions in samples with high titers by a factor of nearly 6; only 70 days were required instead of the 360 days previously needed. Equally important, four animals could be used instead of the 60 mice that were required for endpoint titrations, and this made possible a large number of parallel experiments (Prusiner et al. 1982, 1999a). However, there were disadvantages to using hamsters instead of mice: (i) the number of inbred hamster strains was small, (ii) only some prion strains were susceptible, and (iii) there were no procedures for transfer and ablation of genes in the hamster. Thus, the third milestone became the production of transgenic (Tg) mice overexpressing prion protein homologous to the original prion host, for example, mouse (Mo), Syrian hamster, or human (Hu) PrP. In contrast to non-transgenic hosts, Tg mouse models of prion diseases produced the original species of prions, and overexpression of the PRNP gene led to significantly shorter incubation times (Carlson et al. 1994a; Scott et al. 1989). Most importantly, the transmission experiments established stable laboratory strains of prions with defined biological characteristics that became standard experimental tools in prion research (Prusiner et al. 1999a, 2004a, b; Scott et al. 2004).

Because of the wealth of data accumulated in animal experiments, the parameters distinguishing distinct mammalian prion isolates fell into qualitative or quantitative categories:

A. Qualitative traits:

1. Clinical symptoms of the host (Pattison and Millson 1961)
2. Anatomical distribution and characteristics of brain lesions (Fraser and Dickinson 1973; Dickinson and Fraser 1977)
3. Anatomical distribution of pathogenic PrP^{Sc} in the brain (Gambetti et al. 2003; Taraboulos et al. 1992)
4. Mass of unglycosylated or deglycosylated $rPrP^{Sc}$ on Western blots (WBs) (Parchi et al. 1996; Bessen and Marsh 1994; Telling et al. 1996)
5. Glycoform pattern of $rPrP^{Sc}$ on WBs (Collinge et al. 1996)
6. Conformational characteristics of PrP^{Sc} in conformation-dependent immunoassay (CDI) (Safar et al. 1998b)
7. Distinct external domains driving their replication (Siddiqi et al. 2021)

B. Quantitative traits:

1. Incubation time (Pattison and Millson 1961)
2. Dose–response curve in endpoint titration (Kimberlin and Walker 1978)
3. Susceptibility of pathogenic PrP^{Sc} to proteases (Safar and Prusiner 1998)
4. Conformational stability of PrP^{Sc} (Safar et al. 1998b, 2011; Peretz et al. 2001)
5. Prion particle size (Kim et al. 2011a, 2012)

7.3 Prion Species

A prion species is defined by the amino acid sequence of the donor's (host's) PrP. Transmission of prions between different animal species frequently results in low transmission rates and long incubation times, which shorten upon repeated transmission to the recipient species (Scott et al. 2004; Safar et al. 2011; Bruce and Dickinson 1979). This so-called "species barrier" is attributed to differences in the PrP sequences between prion donor and new host that hinder the response of host PrP^{C} to the incoming $rPrP^{Sc}$ seed (Scott et al. 2004; Collinge and Clarke 2007). A "species barrier" may also exist within the same animal species; for example, there are two distinct polymorphic PrP alleles in different mouse lines—the Prnpa (108L, 189T) and the Prnpb allele (108F, 189V)—and transfer of prions between mice with divergent PrP alleles is subject to a barrier similar to that observed in the transfer between different animal species (Prusiner et al. 2004a; Carlson et al. 1994b; Tremblay et al. 2004).

In the case of inter-species prion transfer to mice, the barrier may be overcome by replacing the murine PrP genes with their counterpart from the donor (e.g., Syrian hamster (Prusiner et al. 1990), cattle (Scott et al. 1999), human (Telling et al. 1994), or cervids (Browning et al. 2004)). Importantly, in PrP-deficient ($Prnp^{0/0}$) mice, neither prion disease nor prion replication has been found (Büeler et al. 1993). But replacement of the murine PrP gene with its homologs from another species does not recreate the physiology of the donor species, and genes other than PrP may play a role in susceptibility to prions, thereby resulting in different incubation times (Tamguney et al. 2008; Stephenson et al. 2000; Prusiner et al. 1999b). From these experiments and those in vitro, several authors have proposed an auxiliary role for an as yet hypothetical host-derived cofactor in prion replication, which could be a polynucleotide, glycosaminoglycan, lipid, or chaperone-facilitating conversion (Kaneko et al. 1997; Kim et al. 2010; Deleault et al. 2010, 2012; Piro et al. 2011; Geoghegan et al. 2007).

Cumulatively, the expression of foreign, mutant, or chimeric PrP transgenes in mice has created a wealth of knowledge about prions that was previously unattainable. Most importantly, this knowledge helped to separate the phenomena generated by "species barrier" from true strain characteristics encoded in the prion itself (Scott et al. 2004, 2005; Collinge and Clarke 2007). It has also helped to define the central domain (residues 96–167) in the PrP amino acid sequence determining "species barrier" (Scott et al. 2004), demonstrated an inverse relationship between the level of PrP^{C} expression and the incubation time (Scott et al. 1989), and allowed differentiation of the natural prion isolates from de novo prions generated with mutant and recombinant PrP (Legname et al. 2006; Wang et al. 2010; Tremblay et al. 2004; Safar et al. 2000).

7.4 Cell Tropism of Prion Strains

A few traits, such as clinical symptoms, pathology, and central nervous system (CNS) distribution of pathogenic PrP^{Sc} probably indicate distinct susceptibility of different cells to prions (Mahal et al. 2007). Different prion strains are evident in different locations of lesions and PrP^{Sc} deposition in the brain and may exhibit different tropism for cell lines (Mahal et al. 2007). Because the uptake of PrP^{Sc} by cultured cells appears to be a nonspecific process, the distinct susceptibility of various cells to different prion strains probably reflects the capacity of the cell to replicate prions at a rate exceeding natural clearance (Bergstrom et al. 2006; Mishra et al. 2004).

Some authors studying Western blot patterns of PrP 27-30 proposed that the observed differences in glycosylation specify prion strains (Collinge et al. 1996). However, this proposal is difficult to reconcile with the addition of high mannose oligosaccharides to Asn-linked consensus sites on PrP in the ER and subsequent remodeling of the sugar chains in the Golgi (Endo et al. 1989). Modification of the complex CHOs attached to PrP^{C} is clearly completed prior to the PrP^{C} trafficking to the cell surface (Borchelt et al. 1990; Caughey and Raymond 1991), which indicates that the Asn-linked CHOs of PrP^{Sc} do not instruct the addition of such complex-type sugars to PrP^{C}. Mutagenesis of the complex-type sugar attachment sites seemed to increase PrP^{Sc} formation in cultured cells (Taraboulos et al. 1990) but resulted in prolonged incubation times in Tg mice and differences in the patterns of PrP^{C} distribution and PrP^{Sc} deposition in mice expressing mutant PrPs (DeArmond et al. 1997; Tuzi et al. 2008). Finally, the idea that strain recognition is mediated by the nature of the glycans carried by PrP^{Sc} is not supported by the finding that two distinct prion strains could be propagated by PMCA using unglycosylated PrP^{C} (Piro et al. 2009). Cumulatively these studies indicate that Asn-linked glycosylation might alter the stability and susceptibility of PrP^{C} to conversion, thereby resulting in distinctive patterns of PrP^{Sc} deposition and glycosylation on WBs.

An important contribution to the understanding of cellular phenomena related to prion strains came from the cell panel assay (CPA) developed by Charles Weissmann and colleagues. Conventionally, the distinction between mouse-adapted prion strains requires the determination of incubation times in at least two mouse lines extending over 6–10 months. The CPA, which can distinguish between various murine prion strains in less than 2 weeks (Mahal et al. 2007), is based on the standard scrapie cell assay (SSCA)—a method for the rapid and sensitive quantification of prions in vitro. The CPA carried out on a set of four cell lines (PK1, R33, CAD5, and LD9) showed different responses to various prions (Mahal et al. 2007; Karapetyan et al. 2009) and allowed for the reliable distinction of RML, 22L, 301C, and Me7 mouse prion strains. Additionally, when transferred from brain to cultured cells, “cell-adapted” prions outcompeted their “brain-adapted” counterparts, but the opposite occurred when prions were returned from cells to brain. Thus, the authors concluded that prions, although lacking a nucleic acid genome, are subject to mutation and selective amplification (Li et al. 2010).

However, the mechanism underlying specificity for brain areas and for cultured cell lines in vitro are likely to be somewhat different. Persistent infection requires that the rate of PrP^{Sc} synthesis be at least equal to the rate of PrP^{Sc} depletion (Weissmann 2004). In cell culture, depletion of PrP^{Sc} is caused by degradation, secretion, and cell division; whereas in brain—where PrP^{Sc} accumulates predominantly in neurons—depletion does not occur by cell division. Thus, slowing cell division of cultured cells not only increases the accumulation of PrP^{Sc} but may also allow cells to become chronically infected by strains to which they are resistant under normal growth conditions (Ghaemmaghami et al. 2007). The fact that many drugs that "cure" chronically infected cell lines are largely ineffective in abrogating prion disease in vivo, reflects at least in part the fact that in the brain, PrP^{Sc} depletion does not occur by cell division (Ghaemmaghami et al. 2007; Collinge et al. 2009; Trevitt and Collinge 2006).

7.5 Conformational Mechanism of Prion Strain Propagation

Most researchers now accept the model according to which the infectious pathogen responsible for TSEs is solely pathogenic PrP^{Sc} (Prusiner 1982). This protein is a misfolded, β-sheet-rich isoform of the normal cellular prion protein, PrP^{C}, which is predominantly α-helical (Collinge and Clarke 2007; Prusiner 1998b, 2004; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007). The discovery that proteins may be infectious represents a new paradigm of molecular biology and medicine. Although originally deemed heretical, this protein-only model is now supported by a wealth of biochemical, genetic, and animal studies (Collinge and Clarke 2007; Prusiner 1998b, 2004; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007), including recent success in generating infectious prions in vitro (Wang et al. 2010; Kim et al. 2010; Legname et al. 2004; Castilla et al. 2005; Barria et al. 2009; Deleault et al. 2007; Geoghegan et al. 2009). The PrP^{Sc} conformer is believed to self-replicate by a mechanism that remains poorly understood, but which involves binding to PrP^{C}, and causing this protein to convert to the PrP^{Sc} state (Kocisko et al. 1994; Prusiner 1997).

The first suggestion that properties of PrP^{Sc} might be distinct in various strains of prions arose from an analysis of two prion isolates from mink that had been passaged in Syrian hamsters and labeled drowsy (DY) and hyper (HY) according to dominant clinical symptoms (Bessen and Marsh 1992, 1994). The more pronounced resistance of HY PrP^{Sc} to limited proteinase K digestion and distinct sedimentation velocity suggested dissimilar physical properties of PrP^{Sc}, but the results did not correlate with other isolates that produced similar incubation times and indistinguishable patterns of PrP^{Sc} on WBs (Scott et al. 1997). Only when prion strains generated in humans with inherited prion diseases were passaged in Tg(MHu2M) mice could an argument be made for the distinctive conformation or ligands of PrP^{Sc} present in different prion strains (Telling et al. 1996; Prusiner 1997). These studies were fortuitous in the sense that familial CJD (fCJD) (E200K) and fatal familial

insomnia (FFI) produced different sizes of rPrP^{Sc} fragments after limited proteinase K digestion on WBs.

The WB-based studies of PrP^{Sc} were limited to the most protease-resistant fraction of PrP^{Sc}. It has also been difficult to analyze low levels of PrP^{Sc} in the presence of high levels of PrP^{C}. Moreover, the limited digestion by proteinase K resulting in either 19 or 21 kDa bands after deglycosylation of PrP 27–30 could not explain the broad biological diversity observed in more than 30 rodent-adapted prion strains in bioassays. In response to these problems, we developed a rapid, specific, and highly sensitive method for the detection and conformational characterization of PrP^{Sc} designated a conformation-dependent immunoassay (CDI)(Safar et al. 1998b). After assay calibration with recombinant PrP that has refolded into different conformations, we could distinguish α-helical, β-sheet, and random coil conformations of PrP, either alone or in a mixture. Thus, the assay enabled us to directly measure the amount of PrP^{Sc} in brain homogenates without prior digestion with proteinase K to eliminate PrP^{C}. The assay is conformation-sensitive; and with selective precipitation of PrP^{Sc} before differential immunoassay, PrP^{Sc} could be measured in a sandwich format in the presence of ~10,000-fold excess of PrP^{C} with a sensitivity similar to that of bioassays (Safar et al. 2002, 2005b, 2008; Safar and Prusiner 1998; Kim et al. 2011b).

The CDI led to the discovery of a variable fraction of pathogenic prion protein that is actually protease sensitive (sPrP^{Sc}) and allowed us to differentiate all eight strains examined by differently exposed epitopes, response to limited digestion with proteinase K, and stability in achaotropic guanidine hydrochloride (Gdn HCl) (Safar and Prusiner 1998). Thus, our data provided compelling evidence that eight different strains passaged in the same host (Syrian hamsters) possess at least eight distinct conformations. The differences in conformation of PrP^{Sc} detected by CDI in different prion strains in brain homogenates suggested two markedly distinct conformational mechanisms responsible for propagation of different prion characteristics. Under one possibility, each strain would be encoded by the PrP^{Sc} molecules in a definite number of conformations, and a specific mixture (ratio) of the same building blocks would replicate itself in the next passage. The second possibility is that each strain characteristic is encoded in a unique conformer of PrP^{Sc}, which then replicates with a high degree of fidelity and thus reproduces the strain characteristics.

Thus, in addition to a structure for PrP^{C} that is distinct from PrP^{Sc}, our data on prion strains in Syrian hamsters suggested that there may be several PrP^{Sc} conformers with distinct stabilities (energies) (Shirley 1995). This hypothesis represents an obvious departure from earlier work demonstrating that most proteins had a single folded structure that was uniquely encoded in the sequence (Anfinsen 1973). What is the structural basis of these alternative PrP^{Sc} conformers? Work on diphtheria toxin identified distinct crystal forms that displayed different tertiary and quaternary structures for a single polypeptide sequence (Bennett et al. 1995). To describe this observation, the notion of domain swapping was introduced whereby a region of one monomer displaced the corresponding region in another monomer to create an interlocking molecular handshake (Cohen and Prusiner 1998). This phenomenon

has now been observed in a variety of other protein structures with the swapped elements as small as an isolated α-helix or β-strand, and as large as an entire folded domain. We suspect that a similar phenomenon may be responsible for prion strains. The early experimental data obtained with infrared spectroscopy or with mass spectroscopy after hydrogen/deuterium exchange (H/X MS) confirm the conformational plasticity of PrP^{Sc} (Cobb and Surewicz 2009; Jones and Surewicz 2005; Caughey et al. 1998). In fact, conformational polymorphism (i.e. the ability to form different strains) appears to be a general feature of amyloids and was observed, for instance, in fibrils formed by Aβ peptide associated with Alzheimer's disease (Paravastu et al. 2008; Petkova et al. 2002).

The data also argue that PrP^{Sc} must act as a template in the replication of nascent PrP^{Sc} molecules. It seems likely that the binding of PrP^{C} or a metastable intermediate PrP* (Safar et al. 1994a) constitutes the initial step in PrP^{Sc} formation and that this is also the rate-limiting step in prion replication (Safar et al. 1998b; Kaneko et al. 1997; Cohen and Prusiner 1998; Prusiner et al. 1998). The finding that the rate of PrP^{Sc} amplification by PMCA varies considerably for different murine strains supports the view that PrP^{Sc} structure is likely rate-determining also in vivo (Karapetyan et al. 2009). However, the rate of PrP^{Sc} synthesis must also reflect the activation energy required for the conversion process and thus is likely a function of both the conformation of the PrP^{Sc} multimer, which is believed to be strain-dependent and of the conformation of the PrP^{C} serving as substrate. The conformational stability of PrP^{C} may depend on post-translational modifications of PrP such as glycosylation or on association with cellular components, which by favoring certain PrP conformations, could promote preferential propagation of particular strains in different cells. The remarkable affinity of PrP^{C} for nucleic acids (King et al. 2007) and the requirement for polyanions in the PMCA reaction using purified PrP^{C} as substrate (Deleault et al. 2005) together support the view that cell components other than PrP^{C} may play an auxiliary role in prion strain replication (Geoghegan et al. 2007). Thus, the optimal conversion process of different prion strains might require different cofactors, and it is likely that the cofactor content or structure in a particular cell type may contribute to its capacity for propagating a particular strain.

7.6 Molecular Attributes of Human Prion Strains

Although remarkable progress has been made in understanding the pathology, biochemistry, and structure of cloned rodent-adapted prion strains (Prusiner et al. 2004b; Caughey et al. 2009; Cobb and Surewicz 2009; Morales et al. 2007; Watts and Westaway 2007; Telling 2008), understanding of the molecular basis of human prion diseases has lagged behind. The human prion diseases are more complex, and a single pathologic process may present as a sporadic, genetic, or infectious illness (Prusiner et al. 2004a). The most common human prion disease is sporadic Creutzfeldt–Jakob disease (sCJD), accounting for ~85% of cases. Although sCJD was shown to be transmissible to non-human primates more than 50 years ago

(Gibbs Jr et al. 1968; Brown et al. 1994), the origin, pathogenesis, and the number of human prion strains causing the disease are not understood.

Lack of progress in the area of human prions stems from three barriers. First, these diseases present with greater variability on complex genetic background; second, experiments with human material are prohibitive; and finally, relatively few investigators focus on human prion diseases. Nevertheless researchers today generally agree that the genotype at codon 129 of the chromosomal gene PRNP, and to some degree the phenotypes of these diseases, underlie susceptibility to prion diseases (Gambetti et al. 2003; Bishop et al. 2010; Giles et al. 2010). In contrast to the experiments with laboratory rodent prion strains, in which the digestion of brain PrP^{Sc} with proteolytic enzyme proteinase K (PK) consistently results in a single protease-resistant domain with mass ~19 kDa, the outcome in sCJD is more complex. Distinctive glycosylation patterns and up to four PK-resistant fragments of the pathogenic prion protein ($rPrP^{Sc}$) found in sCJD brains are easily distinguishable on Western blot (WB) (Gambetti et al. 2003; Telling et al. 1996; Collinge et al. 1996; Parchi et al. 1997; Wadsworth et al. 1999; Zou et al. 2003).

Although the disease phenotypes of patients with sCJD are remarkably heterogeneous, the WB findings together with human PRNP gene polymorphism led Parchi, Gambetti, and colleagues to posit a clinicopathological classification of sCJD into five or six subtypes. Importantly, it has been shown that the WB characteristics of PrP^{Sc} breed true upon transmission to susceptible transgenic mice and Guinea pigs (*Cavia porcellus*) (Gambetti et al. 2003; Telling et al. 1996; Safar et al. 2011; Parchi et al. 1997). Subsequently, Collinge and collaborators (Collinge et al. 1996; Collinge and Clarke 2007; Wadsworth et al. 1999; Hill et al. 1997) introduced an alternative classification of the PrP^{Sc} types and their pairing with CJD phenotypes that differed from the previous one in two aspects: (a) it recognized three different electrophoretic mobilities of PrP^{Sc}, and (b) differentiated distinct glycoform ratios in PrP^{Sc} (Collinge and Clarke 2007).

Because the disease duration and phenotypes associated with 21 kDa fragments of unglycosylated PrP^{Sc} (type 1) frequently differ from the 19 kDa fragments of PrP^{Sc} (type 2)(Gambetti et al. 2003; Telling et al. 1996; Parchi et al. 1997; Monari et al. 1994), these findings argue that the PrP^{Sc} type may represent another modifier of the phenotype in human prion diseases. Consequently, WB-based clinicopathologic classifications became a useful tool in studies of prion pathogenesis in transgenic mice models of human prion diseases and in human brains (Telling et al. 1996; Collinge and Clarke 2007). Because two distinct PK cleavage sites in PrP^{Sc} types 1 and 2 most likely originate from different conformations, some investigators contend that PrP^{Sc} types 1 and 2 code distinct prion strains (Parchi et al. 1996; Telling et al. 1996; Collinge et al. 1996; Monari et al. 1994). However, the findings of the co-occurrence of PrP^{Sc} types 1 and 2 in 40% or more sCJD cases suggested that the originally observed differences were quantitative rather than qualitative (Puoti et al. 1999; Kovacs et al. 2002; Head et al. 2004; Lewis et al. 2005; Schoch et al. 2006; Cali et al. 2009a, b). Additionally, the extensive phenotypic heterogeneity of sCJD, along with a growing number of studies including bioassays, all suggest that the range of prions causing sCJD exceeds the number of categories

recognized within the original WB-based clinicopathologic schemes (Safar et al. 2005b; Uro-Coste et al. 2008; Polymenidou et al. 2005). Finally, up to 90% of PrP^{Sc} is protease sensitive (s), and the conformation and role of this fraction in the pathogenesis of the disease remains a subject of speculation (Safar et al. 2005b, c; Cronier et al. 2008) because it is destroyed by proteinase K treatment, which is necessary to eliminate PrP^{C} (Safar et al. 2005c). Cumulatively, no direct structural data are available for sCJD brain PrP^{Sc} beyond the evidence that it is variably resistant to proteolytic digestion.

To determine the conformational range and strain-dependent structural characteristics of sCJD PrP^{Sc} in patients who were homozygous for codon 129 of the PRNP gene, and thus advance our understanding of the molecular pathogenesis of human prion diseases, we introduced the conformation-dependent immunoassay (CDI) (Safar et al. 1998b, 2002, 2005b; Safar and Prusiner 1998). The conformational stability of the protein in a denaturant such as Gdn HCl (Shirley 1995) reflects the original conformation of the protein. If the protein has the same amino acid sequence, the difference in stability indicates the difference in conformation. Thus, even relatively minute variations in a protein structure can be determined. Using this concept, we developed a conformational stability assay in which PrP^{Sc} is first exposed to the denaturant Gdn HCl and then to europium-labeled mAb against the epitopes hidden in the native conformation (Safar et al. 1998b). With sequentially increasing concentration of Gdn HCl, PrP^{Sc} dissociates and unfolds from native β-sheet-structured aggregates, and more epitopes become available to antibody binding. Because PrP^{Sc} is an insoluble oligomer and denaturation of this protein is irreversible in vitro, the Gibbs free energy change (ΔG) of PrP^{Sc} cannot be calculated (Safar et al. 1994a). Therefore, we introduced instead the Gdn HCl value found at the half-maximal denaturation ($[GdnHCl]_{1/2}$) as a measure of the relative conformational stability of PrP^{Sc}. The differences in $[GdnHCl]_{1/2}$ reveal evidence of distinct conformations of PrP^{Sc} (Safar et al. 1994a, 1998b; Shirley 1995).

The process of disaggregation and unfolding of PrP^{Sc} in the presence of increasing concentration of Gdn HCl has been described as follows:

$$\left[PrP^{Sc}\right]_n \rightarrow \left[sPrP^{Sc}\right]_n \rightarrow iPrP \rightarrow uPrP$$

where $[PrP^{Sc}]_n$ are native aggregates of PrP^{Sc}, $[sPrP^{Sc}]_n$ are soluble protease-sensitive oligomers of PrP^{Sc}, iPrP is an intermediate, and uPrP is completely unfolded (denatured) PrP (Safar et al. 1993a; b, c, 1994a, b, 1998a, 2011; Kim et al. 2012; Tzaban et al. 2002). Since CDI is not dependent on protease treatment, it allowed us to address fundamental questions concerning the concentration and conformation of different isoforms of sCJD PrP^{Sc}, including protease-sensitive (s) and protease-resistant (r) PrP^{Sc} (Kim et al. 2011b; Safar 2012a). Consequently, the CDI monitors the global transition from native aggregates to fully denatured monomers of PrP^{Sc}. In contrast, the WB-based techniques monitor either the partial solubilization of PrP^{Sc} (Pirisinu et al. 2011) or conversion of $rPrP^{Sc}$ to protease-sensitive conformers (Peretz et al. 2001) after exposure to denaturant. Therefore, stability data on

protease-sensitive oligomers and intermediates of PrP^{Sc} cannot be obtained with WB and may lead to some markedly different values (Choi et al. 2011).

We found with CDI a remarkable heterogeneity of PrP^{Sc} conformations within sCJD patients homozygous for codon 129 plymorphism of the PRNP gene, and a range corresponded to that of stabilities found in ~30 distinct strains of natural and de novo laboratory rodent prions that have been examined so far (Safar et al. 1998b; Peretz et al. 2001; Kim et al. 2011b; Colby et al. 2010). The unexpected differential effect of PK treatment with increasing stability of type 1 and decreasing stability of type 2 PrP^{Sc}(129M) suggests that in contrast to type 1, the protease-resistant core of type 2 is less stable. The increased frequency of exposed epitopes and decreased stability in type 2 PrP^{Sc} after PK treatment (Kim et al. 2011b) are counterintuitive and may indicate one of three possibilities: (i) that the PK sensitivity is not an obligatory measure of protein stability and rPrPSc may be in some prion strains less stable than sPrPSc; (ii) that removal of the N-terminus from PrP^{Sc} resulted in less stable conformation with more exposed 108-112 epitopes; or (iii) that the ligand protecting the 108-112 epitope and stabilizing the PrPSc was removed by PK. Whether the epitopes' hindrance in undigested PrP^{Sc} is the result of lipid, glycosaminoglycan, nucleic acid, or protein binding to the conformers unique to the MM2 sCJD PrP^{Sc} remains to be established. Since sCJD cases with type 2 PrPSc(129M) have generally extended disease durations, the molecular mechanism underlying this effect calls for detailed investigation. Cumulatively, our findings indicate that sCJD PrP^{Sc} exhibits extensive conformational heterogeneity and suggest that a wide spectrum of sCJD prions cause the disease (Kim et al. 2012; Safar 2012a, b). Whether this heterogeneity originates in a stochastic misfolding process that generates many distinct self-replicating conformations (Collinge and Clarke 2007; Prusiner 2001) or in a complex process of evolutionary selection during development of the disease (Li et al. 2010) remains to be established (Kim et al. 2011b; Safar 2012a, b).

7.7 Mechanism of Formation, Replication, and Evolution of Human Prions

Despite the inevitable influence of the potential difficulties in evaluating initial symptoms and variable genetic background, our recent data indicate that the levels as well as biophysical and conformational characteristics of $sPrP^{Sc}$ are a good predictor of the progression rate in sCJD (Kim et al. 2011b, 2012). The disease progression rate and incubation time jointly represent replication rate, propagation, and clearance of prions from the brain (Prusiner et al. 2004a; Safar et al. 2005c). Therefore, the correlations among the levels of $sPrP^{Sc}$, the stability of $sPrP^{Sc}$, and the duration of the disease all indicate that $sPrP^{Sc}$ conformers play an important role in the pathogenesis. When $sPrP^{Sc}$ is less stable than $rPrP^{Sc}$, the difference in stability correlates with less accumulated $sPrP^{Sc}$ and shorter duration of the disease. An

opposite effect is observed when $sPrP^{Sc}$ conformers are more stable than $rPrP^{Sc}$, which results in more accumulation of $sPrP^{Sc}$ and extended disease duration (Kim et al. 2011b; Safar et al. 2005c; Tanaka et al. 2006). A number of studies have now demonstrated that rodent prions can be generated in vitro from bacterially-expressed recombinant mouse or Syrian hamster PrP (Kim et al. 2010; Deleault et al. 2007; Makarava et al. 2010; Zhang et al. 2013; Legname et al. 2005), and these experiments played a fundamentally important role in providing the ultimate proof for the protein-only hypothesis of prion diseases (Zhang et al. 2013; Legname et al. 2005; Theint et al. 2017; Choi et al. 2016; Noble et al. 2015; Klingeborn et al. 2011). However, our early data using hydrogen/deuterium exchange and mass spectrometry indicated that human prions causing sporadic sCJD differ in a major way from both cloned laboratory prions and synthetic prion amyloids, and phenotypically distant MM1 sCJD prions further differ in their structural organization from MM2 sCJD prions (Safar et al. 2015b; Li et al. 2018).

We recently reported the first successful synthesis of new human prion from the recombinant human prion protein expressed in bacteria in reaction seeded with sCJD MM1 prions and cofactor ganglioside GM1. These synthetic human prions were infectious to transgenic mice expressing non-glycosylated human prion protein, causing neurologic dysfunction after 459 and 224 days in the first and second passage, respectively. The neuropathology, replication potency, and biophysical profiling suggest that a novel, particularly neurotoxic human prion strain was created. Distinct biological and structural characteristics of our synthetic human prions suggest that subtle changes in the structural organization of critical domains, some linked to posttranslational modifications of the pathogenic prion protein (PrP^{Sc}), play a crucial role as a determinant of human prion infectivity, host range, and targeting of specific brain structures in mice models. Additionally, by comparing the structural organization of these synthetic human prions synthesized in vitro (Kim et al. 2018) with that of parent sCJD MM1 prions and noninfectious human prion protein amyloid, we have identified critical differences in PrP that are important for the initiation of replication in vivo, that is, their infectivity. Moreover, these data indicated that, in contrast to previous observations on yeast and some murine prion strains, the replication rate of sCJD prions is primarily determined not by conformational stability, but by their unique structural features that control the growth rate of prion protein aggregates (Safar et al. 2015b). Although these structural and functional insights implicate the initial interaction between prion seed and PrP^{C} substrate as a critical step in human prion replication and propagation, the structural elements (domains) driving this process, and the role of different codon 129M or 129V polymorphisms are not known (Daude et al. 2020; Asher et al. 2020; Kang et al. 2020b).

In order to gain insight into the impact of the structural organization of major human brain-derived sCJD MM1, MM2, and VV2 prion strains on their replication and inactivation, we employed recently two different synchrotron hydroxyl radical (•OH) footprinting techniques (Siddiqi et al. 2021; Kiselar et al. 2002, 2011). The first is a novel epitope interference study, where the affinity of a panel of monoclonal antibodies with linear epitopes in PrP monomers and sCJD prions is

progressively altered by modifying key amino acids side chains by hydroxyl radicals produced with increasing doses of synchrotron radiation, and the changes are monitored by quantitative and conformationally dependent immunoassays (CDIs) (Siddiqi et al. 2021). The differences in kinetics of epitope modification evaluated across both PrP monomer forms and sCJD prions indicate differences in accessibility to solvent due to the differences in conformation. Further, the hydroxyl radical-induced modifications are assessed after proteolysis with mass spectrometry (MS) that provide the data on PrP monomers and sCJD MM1, MM2, and VV2 prions at a single residue resolution. Both footprinting techniques indicate that the distribution of critical surface amino acid residues is an important determinant of the structural heterogeneity in human brain-derived sCJD MM1, MM2, and VV2 strains and is responsible for differences in replication rate, resistance to inactivation, and pathological targeting of different brain structures observed in sCJD patients (Siddiqi et al. 2021). Moreover, our data implicate distinct solvent-exposed structural domains in the initial binding of cellular isoform of prion protein (PrPC) as a first critical step in human prion replication and infectivity. Specifically, the major drop in seeding activity in MM1 and MM2 prions correlated with the high rate of modification of residue in domains 129–134, M166, and 206–213, of which the N-terminal residues were implicated in species barrier effect and replication rate of human prions from early bioassays in transgenic mice expressing human and chimeric mouse–human prion protein constructs (Safar et al. 2005b; Korth et al. 2003). Moreover, MM1 sCJD prions exhibit the highest solvent protection in two distant domains (108-112 and 157-167amino acid residues), opposite trends in VV2 sCJD, and intermediate protection in all domains of MM2 sCJD (Siddiqi et al. 2021). Taken together, although the modulating effect of prion clearance in the mammalian brains is likely (Safar et al. 2005c), the surface-exposed residues in human prions are responsible for species barrier effects and initial interaction with normal human PrPC protein, with higher affinity leading lead to faster prion replication and shorter incubation time and faster progression of the disease.

7.8 Expanding the Prion Strain Paradigm to Other Age-Related Neurodegenerative Diseases Caused by Protein Misfolding

Two aspects of late-onset AD that cannot be explained by genetic polymorphisms are (i) discrepancies between amyloid beta and TAU deposit burden and clinical disease severity (Masters and Selkoe 2012), and (ii) the extensive variability of progression rates and phenotypes (Gallardo and Holtzman 2019). We recently described a subgroup of patients with rapidly progressive dementia mimicking prion diseases which, after exhaustive neuropathological investigation and prion protein gene sequencing, was concluded to be rapidly progressive AD (rpAD) (Chitravas et al. 2011). Data from all of the rapidly progressive AD cases collected

independently at prion centers in Germany, Japan, Spain, and France have uniformly confirmed the presence of differentiating clinical characteristics and a low frequency of e4 alleles in the *APOE* gene, while the autosomal dominant history of dementia or comorbidity was absent (Schmidt et al. 2010, 2011, 2012, 2013; Pillai et al. 2018; Cohen et al. 2015b). The high concentrations of distinctly folded conformers of amyloid beta with extended C-termini (Aβ42) that we found in rpAD (Cohen et al. 2015b) were confirmed by nuclear magnetic resonance (NMR) spectroscopy (Qiang et al. 2017). This suggested critical differences in the pathogenesis of rpAD, but the molecular mechanism of rapid progression is not completely understood (Drummond et al. 2017).

To map the conformation of Aβ directly in brain tissue, we recently utilized two chemically distant conformation-sensitive oligothiophenes (LCOs) and investigated different morphotypes of Aβ deposits (diffuse and dense-packed plaques) *in situ* in three anatomical regions of patients with rpAD and spAD (Liu et al. 2021). Upon binding to Aβ deposits, followed by excitations, two LCOs showed different characteristic spectral signatures, indicating the underlying distinct conformations of Aβ. These findings together corroborated the existence of distinct Aβ structural features present in diverse clinical phenotypes with major interindividual variabilities. The structural heterogeneity we observed with LCO has been confirmed recently by cryo-EM which found two major Aβ42 filament types (types I and II) purified from different cases of Alzheimer's disease.

Recent evidence suggest that AD is a dual proteinopathy in which Aβ deposition and the accumulation of aggregated TAU drive AD pathogenesis. However, deposits of abnormal TAU protein seem to be the critical factor in determining cognitive decline (Gallardo and Holtzman 2019). Extensive analysis of aging brain samples indicates that the pathological processes underlying AD begin early with depositing aggregates in anatomical structures of the brain and then spread through neuronal projections (Braak and Del Tredici 2013). An accumulation of data from cell and transgenic mice models suggest that different TAU aggregates generated in vitro or in vivo can replicate in cells, accelerate and propagate the formation of TAU aggregates in transgenics, and thus suggest a prion-like phenomenon (Kaufman et al. 2018; Sanders et al. 2016). By adapting advanced conformation-sensitive biophysical techniques originally developed for strain typing of human prions (Safar et al. 1998b; Kim et al. 2011b, 2012), we investigated the structural organization of different isoforms of TAU protein in the hippocampus of 40 AD cases with variable progression rates. The hippocampus is considered critical in cognitive decline and a crossroad in the spread of pathogenic TAU aggregates. The process begins with early deposits in trans entorhinal cortex (Braak stages I–II) to major projections in the hippocampus, where TAU pathology gradually advances into the CA1 region (Braak II), followed by spread to the limbic structures, inferior temporal neocortex (Braak III), the amygdala and thalamus (Braak IV), and finally spreading into the neocortex (Braak V–VI) (Braak and Del Tredici 2013; Kaufman et al. 2018). Our findings demonstrated major structural diversity of hippocampal tau and established a link between particular conformers (strains) of misfolded tau protein, their seeding potency (replication) in vitro, and the rapid progression of AD (Kim et al. 2022).

The link to disease duration did not arise from the amount of tau aggregates per se, but from their distinct conformations. Further supporting this interpretation, we found that each tau isolate—here defined operationally as deriving from an individual AD case—is rarely a singular conformational entity, but typically a mixture of up to three different conformers that together give rise to distinct rapidly or slowly progressive AD (Kim et al. 2022).

7.9 Outlook

The continuing mystery surrounding high-fidelity replication of human prions and prion-like misfolded proteins poses a fundamental challenge in modern biology, and important questions regarding prion strains remain to be answered. For example, if each strain is composed of an ensemble (spectrum) of conformations—which ones are the most critical? Can they shift their biological properties by selection or by conformational evolution? We recently isolated two distinct populations of human prion particles with different conformational stabilities and aggregate sizes, which co-exist in ~40% of sporadic sCJD[178]. The protein misfolding cyclic amplification (PMCA) replicated each of the PrPSc particle types independently, and in serial propagation with a nonglycosylated mutant PrP^{C} substrate, the dominant PrPSc conformers evolved by natural selection from the subpopulation with the highest replication rate (Haldiman et al. 2013). This mechanism has important implications for species transmissibility as we showed recently on evolving prion strains in chronic wasting disease (CWD) (Duque Velásquez et al. 2020). These and our recent data on AD suggest that human prion and tau strains are not a single conformational entity, but a dynamic collection of distinct populations of particles. Additionally, the conformational concept of prion strain replication raises the question of which conformational domains of prions and prion-like proteins are important for replication, toxicity, and which determine clearance. Although there is now convincing evidence that the PrP^{Sc} conformation of distinct strains is different, it is not known to what extent the conformation or replication rate of different conformers might depend on factors other than conformation of the PrP—for example, the nature of the glycans or additional cell-derived ligands (cofactors). An attractive experiment would be to obtain large quantities of highly purified PrP^{Sc} from a single cell line, infected separately with several different prion strains, determine the glycans carried by each strain-associated PrP^{Sc}, and search for associated molecules such as small RNAs or other cell components. A growing body of evidence indicates that the improved understanding of this mechanism in human CJD prions will have major implications for other age-related neurodegenerative diseases linked to other misfolded proteins, including an ongoing debate about their potential transmissibility (Daude et al. 2020; Asher et al. 2020; Kang et al. 2020b).

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Chapter 8
Cofactor Involvement in Prion Propagation

Surachai Supattapone and Michael B. Miller

Abstract Pure amyloid proteins are responsible for the transmissible properties of yeast prions (Tanaka et al., Nature 428(6980):323–328, 2004; Cell 121(1):49–62, 2005; King and Diaz-Avalos, Nature 428(6980):319–323, 2004). However, it is currently unknown whether the infectious properties of mammalian prions can also be explained by a "protein only" mechanism in which a host-encoded protein, PrP^{C}, undergoes a conformational change into an infectious conformer, PrP^{Sc}. Multiple studies have shown that non-proteinaceous cofactors are necessary for the formation of PrP^{Sc} and mammalian prion infectivity in vitro. Reconstitution studies suggest that different prion variants may preferentially propagate with specific classes of cofactor molecules. The pathogenic roles played by putative prion cofactors remain to be elucidated.

Keywords Prion · Cofactor · RNA · Lipid · Protein-only hypothesis · Strains · Neurotropism · Polyanion

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8.1 The "Protein-Only" Hypothesis

Prions are the infectious agents of fatal neurodegenerative diseases affecting humans and other animals, such as Creutzfeldt–Jakob disease (CJD), kuru, and scrapie (Prusiner 1998); and "prion-like" mechanisms have recently been implicated in the pathogenesis of other disorders, such as Alzheimer's and Parkinson's diseases (Brundin et al. 2010; Cushman et al. 2010; Kim and Holtzman 2010; Lee et al. 2010). Despite decades of investigation, the essential composition of mammalian prions and their mechanism of formation remain unknown (Supattapone 2010).

Experiments showing that scrapie and kuru were transmissible led early investigators to search for a causative pathogen for these diseases (Cuillé and Chelle 1939; Gajdusek et al. 1966). The accepted paradigm for identifying and proving that a pathogen causes an infectious disease, proposed by Robert Koch (1893), required isolation of the diseased organism in pure culture and demonstrating its subsequent ability to cause the disease. As such, efforts were made to characterize the pathogen, in order to facilitate isolation. Aided by the advance of adapting the scrapie agent to mice (Chandler 1961), it was demonstrated that scrapie infectivity could pass through filters with pores as small as 43 nm (Hunter 1969), indicating that the agent was not a bacterium, since the smallest known bacteria measure ~300 nm (Robertson et al. 1975). During this time, all infectious agents smaller than bacteria were thought to be viruses, intracellular parasites with a nucleic acid genome of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) surrounded by a protein capsid and, in some, a lipid envelope. As a result, the infectious agent of scrapie and other spongiform encephalopathies, which display a very long incubation period (Mead et al. 2009; Prusiner 1997), were originally described as slow viruses (Sigurdsson 1954; Gajdusek 1967).

A significant step forward in understanding the nature of the infectious pathogen came from experiments performed by Tikvah Alper in 1967. She found that scrapie infectivity was resistant to high doses of ultraviolet (UV) irradiation (Alper et al. 1967). UV irradiation, known to abolish infectivity of viruses, is thought to inactivate gene-coding nucleic acids by inducing dimerization of pyrimidine nucleotides (Barnhart et al. 1976). These experiments indicated that the scrapie agent lacked a nucleic acid genome, suggesting that a novel class of agent may be responsible.

Griffith proposed three possible molecular mechanisms that could accommodate the experimental observations, including a hypothesis that the scrapie agent may contain only one essential component, a protein (Griffith 1967). Under this "protein-only" hypothesis, this protein would bear a certain conformation and replicate by changing the conformation of a host cell protein. If this hypothesis is correct, then the natural occurrence of multiple prion strains with distinct PrP^{Sc} conformations violates the most fundamental principle of protein folding, originally proposed by Anfinsen, that primary sequence determines tertiary structure (Anfinsen et al. 1961). In 1982, Prusiner and colleagues successfully isolated and characterized infectious prions biochemically (Prusiner 1982). This landmark achievement confirmed that prions are indeed unorthodox infectious agents, identified PrP^{Sc} as a critical

component of infectious prions, and greatly facilitated subsequent efforts to determine the molecular basis of prion infectivity.

8.2 Components of Purified Native Prions

Pulsed-field flow fractionation analysis of purified prion preparations has indicated that the most infectious prion particles are 17–27 nm in size (Silveira et al. 2005). Protease-resistant PrP^{Sc} has been the most consistent and principal substance identified in biochemically purified prion infectivity (Bolton et al. 1982; Prusiner et al. 1984). Still, many efforts have searched for other components in the infectious particles. Studies from the Manuelidis laboratory have identified 25 nm virus-like particles and various nucleic acids in prion-infected brains (Manuelidis et al. 2007; Manuelidis 2011), suggested as the "likely cause" of prion diseases. Various other studies have reported no specific nucleic acids co-purifying with prion infectivity (Hunter et al. 1976; Meyer et al. 1991) or only molecules of variable sequence (Safar et al. 2005). The sum of these findings, put together with UV resistance (Alper et al. 1967) and the successful propagation of prion infectivity in cell-free systems (Castilla et al. 2005), suggests that prions do not contain gene-coding nucleic acids. Thus, neither a virus nor a viroid (RNA lacking protein coat) is likely to be the agent causing prion disease. There has also been a report of co-purifying polysaccharides distinct from the N-linked PrP glycans (Appel et al. 1999). On the basis of disinfection studies with organic solvents and heat, another hypothesis suggests that prions may contain a lipid component in addition to PrP (Gale 2006). Currently, it remains unclear whether native prions contain any essential components other than PrP^{Sc}.

8.3 Prion Replication in Cell-Free Conditions

While purified prion preparations, animals of various *Prnp* sequences, and prion-infected cultured cells have been excellent tools for learning about prion behavior, the development of several in vitro PrP^{Sc} formation techniques has been particularly helpful for studying the composition and propagation mechanism of prions. In a significant advance, Caughey and colleagues carried out the first cell-free conversion of PrP^{C} into PrP^{Sc} (Kocisko et al. 1994). In this method, a stoichiometric excess of infectious PrP^{Sc} is mixed with radiolabeled PrP^{C} molecules, and newly formed, radioactive PrP^{Sc} is detected by its acquisition of protease resistance. Using this technique, it was demonstrated that the distinct PrP^{Sc} biochemical characteristics of prion strains (Bessen and Marsh 1994) were maintained during PrP^{Sc} propagation in vitro (Bessen et al. 1995), providing evidence that another biologic characteristic of prions could be observed under cell-free conditions. However, a large excess of

PrP^{Sc} was required to convert a small amount of PrP^{C}, precluding measurements of the infectivity of in vitro-generated PrP^{Sc} molecules.

Subsequently, Soto and colleagues reported a more efficient method for propagating prions in vitro, protein misfolding cyclic amplification (PMCA) (Saborio et al. 2001). By using alternating steps of incubation and sonication, PMCA facilitated robust PrP^{Sc} amplification in the context of homogenized brain tissue. PMCA was subsequently adapted into a serial format, where the newly generated PrP^{Sc} molecules were used to seed fresh brain homogenate containing unconverted PrP^{C} substrate. Using many serial amplifications in this manner, the input prion infectivity was diluted to undetectable and mathematically negligible levels, and reactions containing newly generated PrP^{Sc} were shown to contain prion infectivity by bioassay (Castilla et al. 2005). Serial PMCA (sPMCA) has also been used to show that specific clinical and neuropathological properties of prion strains may be propagated in a cell-free environment (Castilla et al. 2008; Green et al. 2008), building on the finding of strain-specific PrP^{Sc} pattern propagation in vitro (Bessen et al. 1995).

An alternative method for native PrP^{Sc} formation in vitro employs high-frequency shaking of brain homogenates instead of sonication (Lucassen et al. 2003). Like PMCA, this non-sonication method amplifies PrP^{Sc} levels several folds over the input seed, suggesting that PrP^{Sc} amplification is primarily dependent upon the presence of cofactors in normal brain homogenate rather than sonication. Indeed, subsequent enzyme treatment and reconstitution studies showed that amplification of hamster PrP^{Sc} in this system is dependent upon the endogenous RNA present within the brain homogenate (Deleault et al. 2003).

8.4 Formation of Infectious Prions from Minimal Components: Requirement of Non-PrP Cofactor

The “protein only” hypothesis provides a simple explanation for the infectivity of mammalian prions despite their lack of replicating nucleic acids. One prediction of this hypothesis is that, since PrP^{Sc} molecules in infectious prions are thermodynamically more stable than PrP^{C} molecules, it should be possible to produce infectious PrPSc molecules in vitro by refolding pure recombinant PrP (recPrP) substrate (Cohen 1999). However, attempts to form infectious prions from purified PrP alone have not yielded products that are consistently infectious to wild-type animals. Based on the observation of amyloid fibrils containing PrP in the brains of infected animals (Merz et al. 1987) and potential parallels to self-propagating fungal protein conformations (Wickner et al. 1995; Balbirnie et al. 2001), PrP amyloid fibrils were prepared in vitro from bacterially expressed recombinant PrP (Baskakov et al. 2002). When injected into mice expressing 16-fold greater PrP than endogenous levels, a transmissible neurologic disease resulted after 380–600 days (Legname et al. 2004). However, uninoculated 16x PrP control animals are prone to neurologic dysfunction after ~600 days (Colby et al. 2010), suggesting that the injected

amyloid fibrils may have accelerated a pre-existing disease, similar to the transmission experiments of GSS from mice (Hsiao et al. 1994). Furthermore, this PrP amyloid did not consistently transmit disease to wild-type mice (Colby et al. 2010). A subsequent study also found that PrP amyloid fibrils failed to transmit prion disease to wild-type animals, though fibrils annealed by high-temperature with brain homogenate could trigger infectious PrP^{Sc} formation (Makarava et al. 2010). In another study, PrP fibrillar aggregates formed by PMCA without adding cofactors showed minimal and inconsistent infectivity in animals (Kim et al. 2010).

Preparations formed from purified PrP alone have not reproducibly shown significant levels of prion infectivity in wild-type animals(Supattapone 2014). However, PrP^{Sc} generated from purified PrP substrate mixed with either polyanionic and/or lipid cofactors is infectious to wild-type animals (Deleault et al. 2007, 2012b; Wang et al. 2010; Fernandez-Borges et al. 2018; Burke et al. 2019). Moreover, cofactor removal during serial propagation can produce a non-infectious conformer(Deleault et al. 2012b) and subsequent replenishment with cofactor can restore infectivity(Burke et al. 2019), indicating that non-PrP components may be necessary to form bona fide infectious prions.

8.5 The Protein X Hypothesis

Specific mutant MoPrP molecules can act in a dominant negative manner to prevent the propagation of human prions with HuPrP molecules in transgenic mice (Telling et al. 1995). A potential explanation for this dominant negative effect is that mutant $MoPrP^{C}$ molecules bind and sequester a cofactor that is necessary for prion propagation. Such a cofactor was hypothesized to be a protein, Protein X (Telling et al. 1995). Subsequent investigation identified four C-terminal PrP residues which, when mutated, are capable of exhibiting dominant-negative inhibition of prion propagation in cultured cells (Kaneko et al. 1997; Perrier et al. 2002). It was postulated that these residues form a discontinuous epitope that interacts with Protein X. However, in a polymerization reaction of purified recombinant PrP, one such mutant PrP reduced polymerization of wild-type PrP (Lee et al. 2007). Furthermore, the dominant negative effect can be observed with prions propagating in vitro in purified PrP^{C} substrate and accessory non-protein cofactors (Geoghegan et al. 2009), indicating that Protein X is not responsible for the dominant negative effect. Thus, it is not likely that non-PrP proteins serve as cofactors in prion formation.

8.6 Non-proteinaceous Prion Cofactors

Many different molecules have been proposed to participate in prion propagation. Sulfated glycosaminoglycans (GAGs), such as heparan sulfate proteoglycan (HSPG), can stimulate the formation of protease-resistant PrP^{Sc} (Wong et al. 2001)

and may play a role in PrP^{Sc} formation in cells (Ben-Zaken et al. 2003; Taylor et al. 2009). Copper ions can induce PrP^{C} to form a protease-resistant state (Quaglio et al. 2001; Kuczius et al. 2004), but copper also inhibits PrP^{Sc} propagation in vitro (Orem et al. 2006) and in cultured cells (Hijazi et al. 2003). Plasminogen (Mays and Ryou 2010) and the laminin receptor (Leucht et al. 2003) have also been proposed to participate in prion propagation. PrP also interacts with nucleic acids (Grossman et al. 2003; Cordeiro and Silva 2005; Adler et al. 2003) and lipid membrane vesicles (Morillas et al. 1999; Gabizon et al. 1987).

Specific evidence of a role for RNA in prion propagation came from the observation that transformation of PrP^{C} into PrP^{Sc} in vitro in brain homogenates is reduced after RNase digestion and increased after RNA supplementation (Deleault et al. 2003). Subsequently, the PrP^{C} substrate was purified, and various preparations were tested for their ability to reconstitute PrP^{Sc} amplification (Deleault et al. 2005). PrP-null mouse brain homogenate control and RNA from various sources enabled amplification. Interestingly, various homopolymeric nucleic acids also stimulated PrP^{Sc} amplification, suggesting that the mechanism did not rely on information-coding nucleic acids but instead on polyanionic molecules. Other such polyanions, like HSPG, stimulated conversion to some degree, but less than nucleic acid polyanions (Deleault et al. 2005). Using PMCA, further studies found that polyanions must be at least 40 nucleotides in length to act as PrP^{Sc} propagation cofactors (Geoghegan et al. 2007). Furthermore, during PrP^{Sc} propagation, polyanion cofactors are incorporated into a complex with PrP (Geoghegan et al. 2007). This suggests that the polyanions may act as a structural component of infectious prions.

Not only do polyanion cofactors permit PrP^{Sc} amplification in vitro, but propagation in this minimal component reaction system proceeds indefinitely, and robust in vivo prion infectivity is likewise propagated (Deleault et al. 2007). Thus, infectious prions can be made from a defined mixture of minimal components: prion seed, PrP^{C} substrate, polyanion cofactor, and stoichiometric lipids co-purifying with PrP^{C}. From calculations of the maximal prion seed dilution that could be detected after amplification, these authors estimated that infectious prions could contain as few as 7 PrP^{Sc} monomers. Also, using this recipe but omitting the PrP^{Sc} seed, infectious prions were formed de novo (Deleault et al. 2007), suggesting a potential mechanism for the genesis of sporadic prion diseases such as CJD. Subsequent studies showed that RNA can also serve as a cofactor for the formation of infectious prions from bacterially expressed recombinant (rec)PrP substrate(Wang et al. 2010; Fernandez-Borges et al. 2018).

Prions of different species may display distinct cofactor requirements for propagation. While RNA polyanion cofactors support propagation of hamster PrP^{Sc}, they do not support mouse PrP^{Sc} propagation under the same conditions. Other cofactor molecules, present in PrP-null mouse brain homogenate and resistant to protease and nuclease digestion, appear to be required for mouse PrP^{Sc} propagation (Deleault et al. 2010). One such alternative cofactor was isolated and identified as phosphatidylethanolamine (PE) (Deleault et al. 2012a), a brain-enriched membrane phospholipid. Interestingly, purified conversion reactions reconstituted with PE can be

successfully seeded by a wider variety of prion strains than reactions reconstituted with RNA (Deleault et al. 2012a, b).

8.7 Potential Roles of Cofactors in Prion Formation and Encoding Infectivity

PrP in vitro conversion studies and biological infectivity assays have shown a clear role for non-PrP cofactors in prion propagation (Legname et al. 2004; Makarava et al. 2010; Deleault et al. 2007; Wang et al. 2007, 2010). The function of such cofactors is not known. They could either act as an integral component of the infectious prion or as a catalyst for PrP conformational change (Fig. 8.1). Polyanions may be incorporated into a complex with PrP^{Sc} during propagation in vitro (Geoghegan et al. 2007), possible evidence that they are an integral component. However, photofragmentation of incorporated photolabile nucleic acid polyanion cofactor molecules down to pentanucleotide units did not reduce prion infectivity (Piro et al. 2011), suggesting that cofactor function may be more catalytic in nature. Put another way, while polyanions >40 nucleotides in length are required for such propagation (Geoghegan et al. 2007), fragmentation to five base oligonucleotides permits retention of formed prion infectivity. This finding does not provide definitive proof for the "protein only" hypothesis since co-purified lipids and short oligonucleotides remain present after photodegradation, but it places significant constraints on the possible mechanism by which cofactors facilitate prion formation in vitro.

If cofactors function as an integral component of infectious prions, their contribution could be structural or informational. The resistance of prions to UV

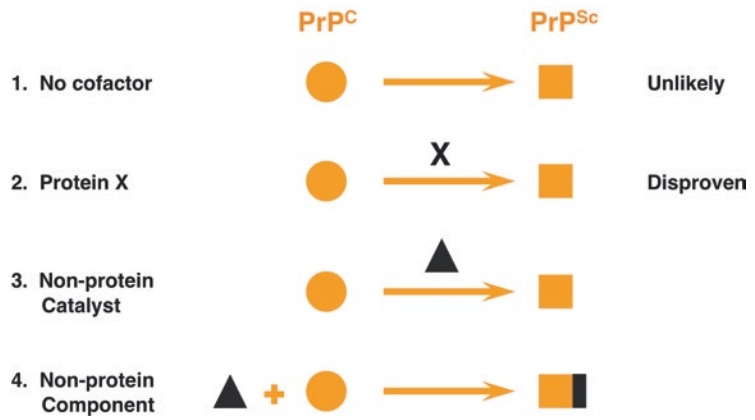

Fig. 8.1 Potential mechanisms of prion formation. A non-proteinaceous cofactor (triangle) is likely to assist the conversion of PrP^{C} (circle) into PrP^{Sc} (rectangle), either as a catalyst or an essential component

irradiation (Alper et al. 1967) and lack of requirement for gene-coding sequence of nucleic acid cofactors (Deleault et al. 2005) argues against such a classical genetic informational role, though such a function could be more subtle. For example, different types of cofactors could support PrP^{Sc} structures in distinct conformations, whereby the cofactor would serve both a structural and informational role. As structure or information, cofactors could also play a role in modulating interactions between PrP^{Sc} and host PrP^{C} molecules, where PrP^{C} polybasic domains appear to provide PrP^{Sc}-binding sites (Miller et al. 2011).

Questions about potential information that cofactors may convey in prions lead to the issue of whether they are universal or specific. The same cofactor molecule could be universally required for the propagation of all prions, or distinct cofactors could participate in propagation of different strains or species of prions (Fig. 8.2). Reconstitution studies suggest that certain PrP^{Sc} molecules propagate best with certain cofactors (Deleault et al. 2010; Burke et al. 2020).

One of the most important challenges to the "protein only" hypothesis is the existence of multiple prion "strains." Strains are defined as natural isolates of infectious prions characterized by distinctive clinical and neuropathological features, which are faithfully recapitulated upon serial passage within the same animal species (Bruce 1993; Carlson 1996). Because prions lack a nucleic acid genome, the mechanism of prion strain variation cannot involve gene mutation (Li et al. 2009).

Studies with yeast models and recombinant mammalian PrP show that pure proteins can adopt multiple, self-propagating conformations (Tanaka et al. 2004, 2005; King and Diaz-Avalos 2004; Jones and Surewicz 2005; Makarava and Baskakov 2008). However, it is difficult to explain the selective neurotropism of native mammalian prion strains on the basis of differential PrP polypeptide folding alone (DeArmond et al. 1997; Mahal et al. 2007).

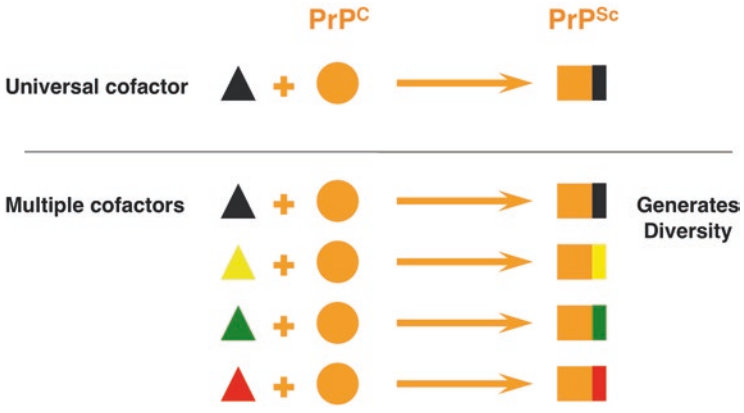

Fig. 8.2 How many cofactors? It is not currently known whether a single, universal cofactor can facilitate the formation of multiple prion species and strains or, alternatively, whether different cofactors are preferentially used by different prions to generate diversity

Some investigators have speculated that strain-dependent differences in PrP^{Sc} glycosylation might encipher the selective neurotropism of prion strains since PrP^{C} glycosylation patterns vary in different regions of the brain (Vorberg and Priola 2002; Beringue et al. 2003; Cancellotti et al. 2005; Khalili-Shirazi et al. 2005; Tuzi et al. 2008). However, this hypothesis was refuted by a study showing that unglycosylated PrP^{Sc} molecules successfully transmit the strain-specific neurotropism of several mouse prion strains (Piro et al. 2009).

Another possible explanation for cellular tropism is that perhaps only a subset of cell types contains the specific cofactor(s) needed to propagate a particular prion strain (Supattapone 2010). In this setting, each prion strain might require a unique set of endogenous cofactors to propagate, that is, a "cofactor variation" hypothesis of strain diversity (Fig. 8.2). The existence of multiple classes of cofactors for prion propagation in vitro is consistent with this hypothesis, which also provides an attractive explanation for the selective neurotropism of prion strains (assuming that different cofactors may be enriched in different brain regions). In support of this hypothesis, it has been shown that purified cofactor molecules dictate both the infectious strain properties (Deleault et al. 2012b) and conformation(Noble et al. 2015) of PrP^{Sc} molecules produced in purified sPMCA reactions regardless of input seed.

8.8 Additional Roles and Applications for Prion Cofactors

Beyond participating in the propagation mechanism of infectious prions, cofactors could also play a role in the mechanism of neurotoxicity. For example, prion infection could deplete or modulate the normal activity of an essential endogenous cofactor molecule. Such a scenario would be compatible with the observation that symptomatic prion disease occurs a long time after maximal infectious titers accumulate in the brains of infected animals, and that the interval period to symptomatic disease is inversely proportional to PrP expression level (Sandberg et al. 2011).

Cofactors may also be required for the pathogenesis of other neurodegenerative diseases involving protein misfolding. For instance, although inoculation brain homogenates containing ABeta plaques can stimulate the spread of similar plaques in the brains of recipient transgenic mice, inoculation of pure synthetic ABeta amyloid into the same recipient mice fails to induce plaque formation (Meyer-Luehmann et al. 2006). One possible explanation for this discrepancy is that additional cofactors are required for amyloid plaques to mature into a form that can propagate in the brain. Structural studies also suggest that tau and synuclein fibrils purified from patient brains may contain cofactor molecules (Falcon et al. 2019; Schweighauser et al. 2020).

It is possible to envision a number of practical applications for prion cofactors. For instance, they could be used to produce large quantities of infectious prions for biophysical studies. Prion cofactors also represent new potential therapeutic targets, and antagonists that block their interaction with PrP might prove to be useful drugs

for treating clinical prion disease. In addition, cofactors could be used in the area of prion diagnostics, either by facilitating the amplification of prions in vitro or by serving as a biomarker of prion disease in histological studies.

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Chapter 9
Prion Protein Conversion and Lipids

Jiyan Ma and Xiangyi Zhang

Abstract The conversion of α-helical rich normal prion protein to a β-sheeted pathogenic isoform is central to prion disease. Decades of studies provided strong evidence supporting the involvement of nonprotein cofactors in prion protein conformational change and in generating prion infectivity. Among all the candidates, lipid appears to be a critical cofactor because of its unique biophysical properties and its ability to induce protein conformational changes. Biophysical and biochemical characterizations of lipid–prion protein interaction demonstrated a huge impact of lipids on prion protein conformation. Studies of prion disease-associated mutations and the in vitro generation of infectious prions with recombinant prion protein in the presence of lipids support the relevance of lipid interaction to prion disease. Lipids could potentially influence multiple steps of prion protein conversion. Further studies are required to elucidate the detailed mechanism of lipid-assisted conformational change of prion protein, which will help us understand the molecular basis of prion infectivity and develop effective strategies against these devastating diseases.

Keywords Prion protein · Prion protein conversion · Lipids · TSEs · Prion infectivity

9.1 Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a large group of infectious neurodegenerative disorders characterized by an unusual infectious agent (Prusiner 1998; Caughey et al. 2009; Aguzzi et al. 2008; Collinge 2001; Collinge and Clarke 2007; Weissmann 2004; Ma and Wang 2014). Prion hypothesis postulates that the infectious agent, PrP^{Sc}, is an altered conformational isoform of host-encoded prion protein (PrP) (Prusiner 1982). PrP is a cell surface localized

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N-linked glycoprotein tethered to lipid membranes through a glycosylphosphatidylinositol (GPI) anchor. The normal form of PrP, PrP^{C}, is an α-helical rich protein containing an unstructured N terminus and a well-structured C terminus with three helices and a short antiparallel β-sheet (Fig. 9.1) (Riek et al. 1996, 1997; Donne et al. 1997). During prion disease, a significant portion of PrP molecules converts to the β-sheeted PrP^{Sc} conformation (Smirnovas et al. 2011; Caughey et al. 1991; Gasset et al. 1993; Spagnolli et al. 2019; Kraus et al. 2021). The two conformational states can be differentiated by biochemical measures such as solubility and protease sensitivity. PrP^{C} is soluble in mild detergents and sensitive to proteinase K (PK) digestion, while PrP^{Sc} is aggregated and the C terminus of PrP^{Sc} is highly resistant to PK digestion (Prusiner 1998; Caughey et al. 2009; Aguzzi et al. 2008; Collinge 2001; Collinge and Clarke 2007; Weissmann 2004).

Prion hypothesis posits that, due to its self-perpetuating property, PrP^{Sc} serves as a template and converts PrP^{C} into the pathogenic PrP^{Sc} conformation (Prusiner 1998). This prediction was supported by both cell-free conversion and protein misfolding cyclic amplification (PMCA) assays. In cell-free conversion assay, highly

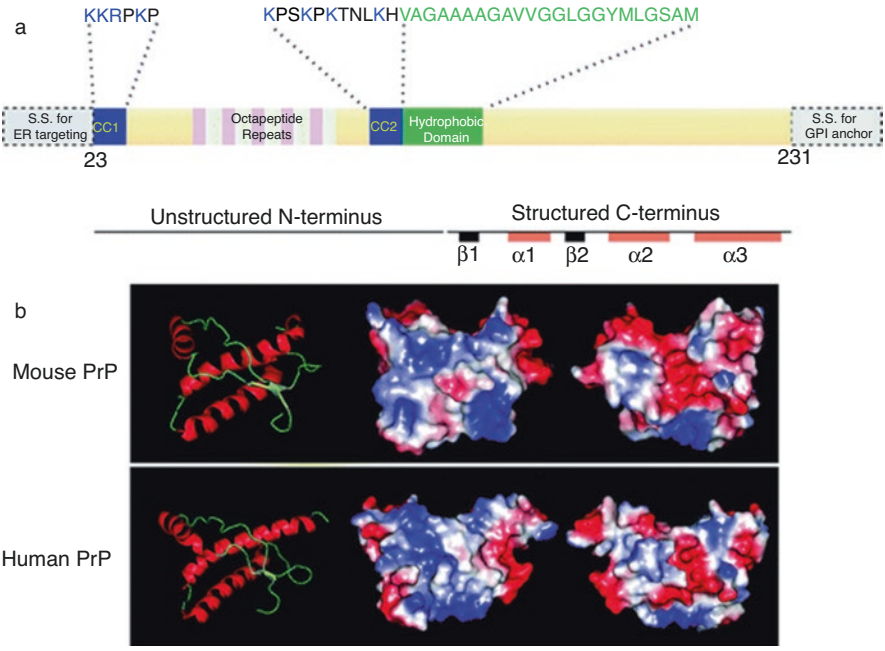

Fig. 9.1 (**a**) PrP contains two positively charged clusters CC1 and CC2 (*blue*) and a hydrophobic domain (*green*). The unstructured and structured regions of PrP are indicated. *S.S.*, signal sequence. (**b**) Surface charges of human and mouse PrPs' structured regions (amino acid 121–231) are colored according to electrostatic potential with *blue* for positive charges and *red* for negative charges. The images in the *middle* show the surface charges of PrP structures on the *left*. The images on the *right* are the surfaces after a 180° rotation around the vertical axis of the images in the *middle*. Images were generated by the PyMOL software

purified PrP^{Sc} seeded purified PrP^{C} into PK-resistant PrP^{Sc} conformation (Kocisko et al. 1994; Bessen et al. 1995), demonstrating the seeding capability of PrP^{Sc}. In PMCA assay, whole brain homogenates are subjected to successive cycles of sonication and incubation, which is much more efficient in propagating PrP^{Sc} conformation (Saborio et al. 2001). High efficiency of PMCA led to the landmark study demonstrating simultaneous propagation of PK-resistant PrP^{Sc} and prion infectivity in a test tube (Castilla et al. 2005). Although it is still not completely understood why the efficiency differs so much between these two assays, it has been shown that in vivo factor(s) in the brain homogenate plays a role in facilitating PrP conversion and/or stabilizing the resulting PrP^{Sc} conformation (Deleault et al. 2003). A variety of polyanions have been found to enhance PrP^{Sc}-templated conversion and RNA appears to be the most potent stimulator (Deleault et al. 2003, 2005).

The requirement of factors other than PrP in PrP^{C}-to-PrP^{Sc} conversion is consistent with the notion that two conformational states of PrP are separated by an energy barrier (Baskakov et al. 2001). In vivo, PrP conversion mainly occurs on cell surface or in endocytic pathway (Caughey and Raymond 1991; Borchelt et al. 1992), indicating that the conversion starts with fully folded α-helical rich PrP^{C} conformation. A chaperone-like activity would help PrP^{C} to overcome the energy barrier and convert to the β-sheeted PrP^{Sc} conformation. Since there is little evidence supporting the involvement of another protein in PrP conversion, other biological molecules such as lipids, oligosaccharides, nucleic acids, or proteoglycans have to be considered for this activity.

9.2 Supporting Evidence for the Involvement of Lipids in PrP Conversion

Lipid appears to be a good candidate because of its proximity to GPI-anchored PrP and the unique impact of lipid interaction on protein structure. PrP^{C}-to-PrP^{Sc} conversion requires both unfolding of α-helical rich PrP^{C} and formation of β-sheeted PrP^{Sc} (Wille and Requena 2018). It is well established that protein–lipid membrane interaction is able to unfold structured proteins (van der Goot et al. 1991; Muga et al. 1993; Pinheiro and Watts 1994; Banuelos and Muga 1995; Fisher and Ryan 1999); this effect would lower energy barrier and remove the first thermodynamic obstacle in PrP conversion. Moreover, the interfacial region of lipid bilayer is known to have a potent capability of inducing secondary protein structures, either α-helices or β-sheets (White et al. 2001; Wimley et al. 1998). Thus, PrP–lipid membrane interaction would facilitate both steps in converting α-helical PrP^{C} to β-sheeted PrP^{Sc}.

The involvement of lipids in PrP conversion is also consistent with previous experimental observations. First, GPI-anchored PrP^{C} can be released from cell surface by phospholipase C (PI-PLC) digestion, whereas the converted PrP^{Sc} resists PI-PLC digestion (Caughey and Raymond 1991; Borchelt et al. 1992). A GPI-anchor-independent lipid membrane interaction by PrP^{Sc} is a plausible explanation for the development of PI-PLC resistance, which is also consistent with the

observation that a GPI-independent lipid interaction is essential for PrP conversion in cell-free conversion assay (Baron and Caughey 2003). Second, cell biological studies reveal that changing lipid contents in prion-infected cells markedly alters PrP^{Sc} production (Taraboulos et al. 1995; Naslavsky et al. 1999), which could be due to the alteration of PrP maturation or trafficking (Sarnataro et al. 2004; Hannaoui et al. 2014). Alternatively, changing lipid membrane composition may alter its interaction with PrP, which could consequently influence the production of PrP^{Sc}. Third, various lipid molecules have been identified in "prion rod," one of the most pure preparations of the infectious particle (Klein et al. 1998). Removing lipids from "prion rod" by treatments of SDS, sonication, and SDS-PAGE results in the loss of prion infectivity (Leffers et al. 2005). This observation could be explained by altering PrP conformation during these treatments. On the other hand, SDS and sonication treatments may disrupt PrP–lipid interaction and destabilize the infectious PrP^{Sc} conformation, which would also lead to a loss of infectivity. Consistent with the latter explanation, it has been reported that reincorporation of purified "prion rod" into lipid vesicles resulted in higher infectivity (Gabizon et al. 1987) and PrP^{Sc}-containing microsomes infected cultured cells with ahigher efficiency than detergent-purified PrP^{Sc} (Baron et al. 2006). Collectively, these observations are consistent with the interpretation that the PrP–lipid interaction is involved in the PrP^{C}-to-PrP^{Sc} conversion.

9.3 Biophysical Studies of PrP–Lipid Interaction

Definitive evidence supporting a GPI-anchor independent PrP–lipid interaction comes from in vitro analyses with purified bacterially expressed recombinant PrP (rPrP) and model lipid membranes. Using spectroscopic approaches, Morillas et al. showed that human rPrP binds to anionic lipid-containing membranes, and rPrP–lipid-binding destabilizes the structured C-terminal domain of PrP (Morillas et al. 1999). The facts that rPrP–lipid interaction is highly pH-dependent and rPrP only binds to anionic lipids indicate a role of electrostatic interaction. Since electrostatic interactions are critical for PrP stability and presence of salts destabilizes rPrP (Apetri and Surewicz 2003), the lipid-binding induced PrP destabilization could be, at least partly, due to the disruption of salt bridges in the folded C terminus by electrostatic rPrP–lipid interaction.

Using similar biophysical approaches, Pinheiro and colleagues confirmed the binding of PrP to anionic lipids using hamster rPrP(90–231) (Sanghera and Pinheiro 2002; Critchley et al. 2004). Interestingly, they also reported that hamster rPrP(90–231) could bind to either zwitterionic DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) or a mixture of DPPC, cholesterol and sphingomyelin (molar ratio at 50:30:20) at pH 7, but not at pH 5 (Sanghera and Pinheiro 2002). The binding of hamster rPrP(90–231) to DPPC or DPPC/cholesterol/sphingomyelin is believed to be driven by hydrophobic lipid–protein interactions, which increases the α-helical content of hamster rPrP(90–231) (Sanghera and Pinheiro 2002). DPPC has a phase

transition temperature of 41 °C and it is in a gel phase at room temperature with fully extended and closely packed acyl chains. In contrast, all other lipids used to study rPrP–lipid interaction are in a liquid crystalline phase in which the acyl chains are randomly oriented and in a more fluid state. Notably, the GPI-anchored PrP is localized in the lipid rafts, which are specialized membrane microdomains of tightly packed lipids. The gel phase DPPC may resemble the rigidity of lipid rafts to certain extent, although DPPC is not a major component of PrP-associated lipid raft (Brugger et al. 2004). Whether this particular physical property of DPPC contributes to the binding of hamster rPrP(90–231) remains unclear.

Steven Collins' group showed that the N-terminal unstructured region of PrP binds to large unilamellar vesicles with negatively charged headgroups at pH 5 (Boland et al. 2010). Using several biophysical approaches, they demonstrated that the N-terminus of PrP inserts into the interstitial space between the phospholipid headgroups, but does not penetrate into the acyl tail region. This interaction leads to an increase in lipid order without phase transition (Le Brun et al. 2014). Interestingly, they also reported that the specificity of N-terminal PrP-lipid binding requires a coordination of various motifs in the N-terminus of PrP, particularly the proline motif in the very N-terminal positively charged amino acid cluster (amino acid 23–28) and the octapeptide repeats (Haigh et al. 2015). Thus, the interaction between N-terminus of PrP and lipids is not merely a result of electrostatic attraction. Instead, it selectively binds to certain types of anionic lipids.

Recently, Overduin et al. analyzed the lipid membrane interacting sites of PrP using the Membrane Optimal Docking Area (MODA) program that predicts protein-lipid interaction based on three-dimensional protein structure (Kufareva et al. 2014). Using the available PrP^C structures, they predicted $W^{99}NK$, $V^{122}GGL$, $Y^{169}SN$, and $Y^{225}YQR$ (for clarity, amino acids are numbered according to human PrP throughout the chapter) are lipid interacting sites of PrP^C (Overduin et al. 2021). Notably, the latter two sites have been indicated in the binding to ganglioside GM1 in a previous study (Sanghera et al. 2011). Using the 4-rung β-solenoid model of mouse PrP^{Sc} (Spagnolli et al. 2019), they reported that the pathogenic conformer has a higher lipid membrane binding propensity with 6 membrane interacting sites and these sites are significantly different from those of PrP^C. This difference indicates that PrP-lipid interaction is not just a pathogenic event that results in the PrP^C-to-PrP^{Sc} conversion. Instead, lipid-PrP^C interaction might be important for its normal folding or normal function. Only when PrP^C aberrantly interacts with certain types of lipid (or combinations of lipid), the deleterious conformational change of PrP occurs.

9.4 Analysis of PrP–Lipid Interaction Using Density Gradient and Protease Digestion

In addition to the biophysical methods mentioned above, density gradient analysis is a straightforward approach to directly measure protein–lipid interaction. With this approach, it has been shown that full-length α-helical rich mouse rPrP binds to

anionic lipids, but not to zwitterionic or cationic lipids (Wang et al. 2007). The interaction between mouse rPrP and anionic lipids initiates with electrostatic contacts, a process that can be blocked by high concentrations of salt. Once electrostatic interaction brings rPrP to the vicinity of lipid bilayer, the hydrophobic domain of rPrP interacts with acyl chains of lipid membrane hydrophobically. The strength of hydrophobic interaction can be analyzed by extraction of the rPrP–lipid complex using a buffer with a high concentration of salt and/or high pH.

The binding of rPrP to anionic POPG (1-palmitoyl-2-oleoylphosphatidylglycerol) increases β-sheet content of rPrP and results in two C-terminal proteinase K (PK)-resistant bands with apparent molecular weights at 15 and 14.5 kDa. Addition of salt at physiological concentration (150 mM NaCl) to the system induces further rPrP conformational change which is reflected by a further change in far-UV circular dichroism (CD) spectrum, a significantly enhanced PK resistance, and the detection of a single C-terminal 15 kDa PK-resistant band by immunoblot analysis (Wang et al. 2007). Interestingly, binding of rPrP to anionic lipid does not always leads to PK resistance. For example, little PK resistance was detected when rPrP binds to anionic POPS (1-palmitoyl-2-oleoylphosphatidylserine). However, when rPrP binds to vesicles consisting of 1:1 molar ratio of POPS and zwitterionic POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), a strong 15 kDa C-terminal PK-resistant band was detected (Wang et al. 2007). This observation clearly demonstrates that the PK resistance is not simply due to the binding of rPrP to anionic lipid-containing vesicles. Instead, it is due to lipid-induced PrP conformational change, which is influenced by the polar headgroup of phospholipids and the distribution of these headgroups on the lipid membranes. The profound influence of POPG-binding on rPrP conformation is confirmed by deuterium exchange mass spectrometry (Miller et al. 2013).

In addition to the C-terminal 15 kDa PK-resistant band, the rPrP binding to anionic lipid-containing membranes also results in a 13.5-kDa N-terminal PK-resistant band (Wang et al. 2007). The simultaneous appearance of both N- and C-terminal PK-resistant fragments and the fact that the sum of these two fragments is greater than the molecular weight of rPrP suggest that rPrP binds to anionic lipid-containing membranes in two different modes. This interpretation is consistent with the finding that, when lipid bilayer is disrupted by a detergent, only the C-terminal 15 kDa PK-resistant fragment can be maintained by protein aggregation (Wang et al. 2007).

The density gradient analyses provide tools to dissect different aspects of rPrP-lipid interaction, including the initial electrostatic interaction that can be inhibited by high concentrations of salt; the ensuing hydrophobic interaction that can be analyzed by extracting rPrP-lipid complex with an alkaline buffer containing high concentrations of salt; and the lipid-induced rPrP conformational changes that can be analyzed by PK digestion. These tools allow the characterization of various PrP domains, mutations, and polymorphism on PrP-lipid interaction.

9.5 The Influence of PrP Mutations on rPrP–Lipid Interaction

After removing the N-terminal signal sequence for endoplasmic reticulum targeting and the C-terminal signal sequence for GPI anchor addition, the primary amino acid sequence of the mature fragment of PrP (Fig. 9.1a) contains two clusters of positively charged amino acid residues at the N terminus (amino acid 23–27, designated as CC1) and in the middle region (amino acid 101–110, designated as CC2). A hydrophobic domain (amino acid 112–134, designated as HD) is located next to the CC2 region. Besides the clusters of positively charged amino acids, the structured C-terminal domain also contains positively or negatively charged surface patches (Fig. 9.1b), which may also contribute to PrP-lipid interaction.

Mutant rPrP without the hydrophobic domain (designated as ΔH) still binds to anionic lipids, but unlike wild-type rPrP, the ΔH mutant can be extracted from rPrP–lipid complex by an alkaline salt buffer and is without either N- or C-terminal PK-resistant fragments (Wang et al. 2010a; Abskharon et al. 2019). These results show that the hydrophobic rPrP-lipid interaction is largely mediated by the HD domain and the development of both N- and C-terminal PK resistance depends on the hydrophobic rPrP-lipid interaction.

For electrostatic PrP-lipid interaction, CC1, CC2, and positively charged surface patches in the structured C-terminal domain all play a role. The electrostatic PrP-lipid interaction mediated by different PrP regions may orient PrP in such a way that it leads to a difference in hydrophobic PrP-lipid interaction and the resulting PrP conformation. Since the N-terminus of PrP is highly flexible, there is little conformational restraint to prevent CC1 and CC2 regions from orienting PrP in a variety of manners on the surface of lipid bilayer, which could potentially lead to a great variety of stable PrP conformations.

The complexity in the electrostatic PrP–lipid interaction is reflected in the analyses of different rPrP mutants (Wang et al. 2010a). Deletion of N-terminal CC1 region reduces electrostatic interaction between rPrP and anionic POPG, leading to a reduced C-terminal PK resistance. This effect can be attributed to the loss of positive charges of rPrP. In contrast, the rPrP mutant, in which four positively charged lysines in the CC2 region are replaced by isoleucine (designated as K/I mutant), does not appear to alter the strength of either electrostatic or hydrophobic PrP-POPG interaction. But, the C-terminal PK resistance of K/I mutant is significantly reduced. Therefore, although the positive charges in the CC2 region minimally affect PrP's initial electrostatic contact with anionic lipids, the interaction between these lysines and the negatively charged phospholipid headgroups appear to play a role in orienting rPrP on lipid membrane and assisting in the formation of PK-resistant PrP.

Two biochemically similar disease-associated mutants, P102L and P105L, are both located in the CC2 regions and flanked by lysines. Since proline is conformationally restrained, replacing proline with leucine would alter the spatial arrangement of positively charged lysines. Interestingly, the P102L mutation does not affect

rPrP–POPG binding but completely eliminates the anionic lipid-induced PK resistance. In contrast, the P105L mutant significantly reduces the electrostatic rPrP–POPG interaction and the anionic lipid-induced PK resistance. When both rPrP mutants are allowed to bind to anionic POPG, neither P102L nor P105L alters the strength of hydrophobic rPrP–POPG interaction.

Considering all three CC2 mutants analyzed, it can be concluded that, despite the cluster of positively charged lysines, the CC2 region minimally affects the electrostatic interaction between rPrP and anionic POPG. The reduction of electrostatic rPrP–POPG binding caused by P105L is likely due to its influence on the global PrP structure, which alters the positively charged surface patches in the C-terminal structured region or the presentation of the N-terminal CC1 region. Since all three mutants reduce anionic lipid-induced PK resistance, it is likely that the CC2 region is important for orienting rPrP on lipid membranes, which leads to PK resistance.

The hydrophobic region localized 129 methionine (129 M) and valine (129 V) polymorphisms significantly affect the susceptibility and pathogenesis of prion disease (Ironside et al. 2005), yet very few biochemical differences between the two PrP variants can be detected. Analysis of these two variants revealed a stronger hydrophobic interaction between the 129 M variant and total mouse brain lipids. This result seems to be counterproductive since valine is more hydrophobic than methionine. However, all amino acids in the hydrophobic domain are capable of interacting with the hydrophobic acyl chains of lipids. Substituting methionine with valine increases the hydrophobicity, which likely results in tighter binding of the hydrophobic acyl chains to residue 129 and alters the interaction between acyl chains and surrounding hydrophobic amino acids. Thus, the total strength of the hydrophobic lipid interaction is lower in 129 V.

It is important to note that rPrP differs from native PrP^{C} in that it lacks *N*-linked oligosaccharides and a GPI anchor. Model lipid vesicles used in the in vitro studies also differ from in vivo lipid membranes in composition, curvature, and local environments. Therefore, one should not simply assume that these in vitro results could be directly extrapolated to the in vivo condition. However, two important observations from these in vitro studies support the relevance of PrP-lipid interaction to the pathogenesis of prion disease. First, disease-associated PrP mutants and the 129 polymorphisms clearly affect PrP-lipid interaction, indicating a role of altered PrP-lipid interaction in the pathogenesis of prion disease. Second, the lipid interaction is sufficient to convert fully folded α-helical rich rPrP into a conformation that is similar to the pathogenic PrP^{Sc} form, with increased β-sheet contents and a highly PK-resistant C terminus. Previous in vitro conversions of α-helical rich rPrP into various aggregated forms all required treatments of denaturant or reducing agent (Legname et al. 2004; Bocharova et al. 2005; Colby et al. 2010; Apetri et al. 2005; Jackson et al. 1999), yet, the lipid-mediated rPrP conformational change does not. This difference indicates that the lipid interaction is capable of overcoming the energy barrier and converting rPrP to a conformation similar to PrP^{Sc}.

9.6 Forming Recombinant Prions with Lipid as a Cofactor

The similarities between lipid-induced rPrP conformation and PrP^{Sc} suggest that lipids might be a necessary cofactor for the conversion of rPrP into an infectious conformation. Other studies also suggested that polyanions, particularly RNA, facilitate PrP conversion in PMCA (Deleault et al. 2003, 2005). When synthetic POPG and total RNA isolated from normal mouse liver were added to rPrP in PMCA reaction, a PK-resistant form of rPrP was generated and could be propagated indefinitely by serial PMCA (Wang et al. 2010b). Because a portion of rPrP gained PK resistance after PMCA, the term "rPrP-res" was used to represent the rPrP conformational state(s) in the PMCA product. The rPrP-res has all the signature characteristics of PrP^{Sc}: aggregated, C-terminal PK-resistance, the capability of converting endogenous PrP^{C} in the brain homogenate to PrP^{Sc} by PMCA, and the capability of infecting cultured cells (Wang et al. 2010b). Most importantly, it causes bona fide prion disease in wild-type mice with an incubation time similar to that of naturally occurring prions (Wang et al. 2010b). Therefore, rPrP-res is not only infectious but also contains high prion infectivity.

To rule out the possible roles of genetic informal RNA in the total RNA isolated from mouse liver, synthetic polyriboadenylic acid was used to replace the total liver RNA in a follow-up study, which showed that the resulting rPrP-res is competent to infect cultured cells and causes prion disease in wild-type mice (Wang et al. 2012). Thus, infectious prion can be generated by PMCA of all synthetically generated materials: bacterially expressed rPrP, POPG, and polyriboadenylic acid. Besides POPG, phosphatidylethanolamine (PE) has also been found to support the conversion of murine rPrP as a solitary cofactor (Deleault et al. 2012a), and ganglioside GM1 has been reported to facilitate the conversion of full-length human rPrP in the presence of polyadenylic acid (Kim et al. 2018). These studies supported the role of lipids as a cofactor for PrP conversion.

Several groups reported conversion of rPrP in the absence of cofactors (Legname et al. 2004; Makarava et al. 2010; Kim et al. 2010; Colby et al. 2010). The rPrP amyloid fibers have been shown to induce prion disease in transgenic mice overexpressing PrP, but not in wild-type mice (Legname et al. 2004; Colby et al. 2010). Full-length hamster rPrP fibers subjected to an "annealing" procedure (5 cycles of incubations at 80 °C and 37 °C in the presence of normal hamster brain homogenate or bovine serum albumin) are able to induce the formation of infectious prions in a subset of asymptomatic wild-type hamsters (Makarava et al. 2010). Using PMCA seeded by PrP^{Sc} partially purified from 263 K scrapie-infected hamster brain, Kim et al. showed that the converted hamster rPrP (designated as $rPrP^{PMCA}$) is able to cause prion disease in wild-type hamsters, but with a large variability in incubation times and attack rates (Kim et al. 2010).

Compared to PMCA-generated rPrP-res (Wang et al. 2010b) or PrP^{Sc}-seeded $rPrP^{PMCA}$ (Kim et al. 2010), rPrP amyloid fibers appear to have a much lower infectivity, which fails to induce prion disease in wild-type animal (Legname et al. 2004; Colby et al. 2010) or only induce infectious prion formation in asymptomatic

wild-type hamsters (Makarava et al. 2010). The low infectivity of rPrP amyloid fibers suggests a possibility that, instead of mature fibers, the infectivity could be associated with some type of oligomeric rPrP structures, which can be on or off the amyloidogenic pathway. In vivo, the rPrP oligomer may be stabilized by binding to a cofactor. The "annealing" step may rearrange the quaternary rPrP structure to increase the formation and/or stabilization of infectious rPrP oligomers. This hypothesis accounts for the discrepancy of a large amount of fibers in the inoculum and extremely low infectivity in bioassay, and explains the differences between fibers with or without "annealing." It is also consistent with the observation that the most infectious prion particles are oligomers (Silveira et al. 2005). Alternatively, the amyloid fiber might be in a conformation that is different from infectious PrP^{Sc}. In vivo, PrP amyloid fiber may seed endogenous PrP^{C} in an erroneous manner and this "deformed templating" may lead to the generation of infectious PrP^{Sc} conformation in a small fraction of inoculated animals (Makarava and Baskakov 2013).

Although growing rPrP amyloid fiber does not require cofactor, it does require chaotropic agents such as guanidinium hydrochloride or urea (Legname et al. 2004; Bocharova et al. 2005; Colby et al. 2010; Apetri et al. 2005). These chaotropic agents may play a role similar to the binding to lipid membranes, that is, unfolding α-helical rich rPrP to allow the formation of β-sheeted amyloid fibers. The condition used for PrP^{Sc}-seeded formation of $rPrP^{PMCA}$ is different, which does not require chaotropic agents or cofactors such as lipids or polyanions (Kim et al. 2010). The following two reasons may contribute to generation of infectious $rPrP^{PMCA}$. First, the sonication step in PMCA may provide activation energy needed for PrP conversion or directly affect rPrP conformation. Second, the conversion buffer for PrP^{Sc}-seeded $rPrP^{PMCA}$ formation contains anionic detergent sodium dodecyl sulfate (SDS) and nonionic detergent Triton X-100. Both detergents contain a hydrophilic group and a hydrophobic moiety, which resembles the structural characteristics of lipid molecules. Moreover, the anionic SDS has been shown to promote the conversion from α-helical rich rPrP to β-sheeted conformations (Leffers et al. 2005). Thus, in PrP^{Sc}-seeded $rPrP^{PMCA}$ formation, SDS and Triton X-100 may partially replace the function of lipid molecules and/or polyanions in promoting rPrP conversion.

Among all in vitro-generated recombinant prions, rPrP-res produced by PMCA in the presence of phospholipid POPG and RNA appears to contain the highest infectivity. Not only does it cause prion disease in wild-type mice with a relatively short incubation time and 100% attack rate after intracerebral injection, infect cultured cells, and propagate the PK-resistant conformation to native PrP^{C} via PMCA (Wang et al. 2017, 2010b; Zhang et al. 2013), it also causes fatal prion disease in wild-type mice when it is delivered via the intraperitoneal or oral route (Wang et al. 2015; Pan et al. 2020). The high infectivity associated with rPrP-res could be attributed to a variety of reasons, but the presence of lipid molecules, a distinct characteristic of this system, likely plays a role in generating the highly infectious rPrP-res.

9.7 Possible Roles of Lipid in Forming an Infectious Prion

Although the involvement of lipid molecules in generating infectious prions is supported by experimental results, many questions remain to be answered, such as: what type of lipid molecules or which combinations of lipids are the "real" in vivo cofactors for the formation of an infectious prion, or whether different lipid molecules can lead to distinct prion strains. The most fundamental question that needs to be addressed is whether or not lipid is an essential part of the infectious agent. Depending on whether lipid is or is not an essential part of the infectious agent, the following roles of lipids can be envisaged.

If the "protein-only" hypothesis is correct in the most strict term, that is, pure PrP^{Sc} without any other biological molecules is sufficient for the infectivity, then the PrP^{Sc} conformer itself should be able to cause prion disease. In this scenario, lipids could act as a chaperone that facilitates PrP conversion by unfolding α-helical rich PrP and/or promoting the formation of the β-sheeted PrP^{Sc} conformer. Alternatively, lipid molecules may simply enhance the in vivo retention time of PrP^{Sc}. In this case, the infectious agent is the PrP^{Sc} conformer, but its association with lipid molecules may prevent its clearance and thereby enhance the infectivity. The third possibility could be that the lipid molecules facilitate the binding of infectious particle to cellular membranes, where the pathogenic PrP^{Sc} conformer will encounter and convert membrane-attached PrP^{C}. The latter two possibilities would account for the increased infectivity when PrP^{Sc} is associated with lipid membranes (Gabizon et al. 1987; Baron et al. 2006).

In case that lipid is an essential part of the infectious agent, lipid molecules may still play all the roles proposed above, and in addition, they will contribute to the stabilization of infectious PrP^{Sc} conformation. Early studies by Alper et al. showed that oxygen greatly sensitizes the infectious agent to ultraviolet irradiation and such an effect is characteristic for the involvement of lipid molecules (Alper et al. 1978). This observation is in agreement with the notion that lipid is an integral part of the infectious agent and plays an essential role in stabilizing the infectious PrP^{Sc} conformation. Supporting this idea, it has been shown that after withdrawing cofactors in rPrP PMCA, a PK-resistant rPrP form can be generated in the absence of any cofactor, which has a PK-resistant core of about 2-kDa smaller than that of rPrP-res. Despite the ability to be serially propagated by PMCA, this protein-only rPrP-res form lacks infectivity and does not cause any disease when inoculated intracerebrally into wild-type C57BL mice (Deleault et al. 2012b). This finding supports lipids as an essential part of the infectious particle and without lipids, the infectious PrP^{Sc} conformation cannot be maintained. Consistent with this idea, when distinct prion strains were propagated to rPrP in PMCA with a single cofactor PE, they were converted into a single prion strain (Deleault et al. 2012b), suggesting that lipid cofactor is able to modulate rPrP conformation and regulate prion strain properties. If this scenario proved to be true, the self-propagating PrP^{Sc} conformation could be stably maintained by forming a PrP^{Sc}-lipid complex, which would allow the

unorthodox prion phenomenon to be simply explained within Christian Anfinsen's protein folding paradigm.

Further studies are required to rigorously test these possibilities. In addition to addressing a long-lasting question with intellectual significance, elucidating the role of lipid or other cofactors in PrP conformational change and in forming an infectious prion may lead to novel prophylactic, diagnostic, and therapeutic strategies against these fatal neurodegenerative disorders.

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Part IV
Environment and Transmission of Prions

Chapter 10
Prions in the Environment

Shannon L. Bartelt-Hunt, Jason C. Bartz, and Qi Yuan

Abstract Scrapie and chronic wasting disease are two prion diseases of particular environmental concern, as they are horizontally transmissible. Prions are shed from diseased hosts in a diverse set of biologic matrices. There is strong experimental evidence that soil and water chemistry as well as other environmental factors can significantly affect prion sorption, resistance to degradation, persistence, replication efficiency when bound to soil, and ultimately prion infectivity. A more thorough understanding of the interaction of prions with the environment in combination with robust detection methods may lead to the means to reduce or eliminate prion disease in free-range and captive animal populations as well as mitigate the risk of zoonotic prion transmission.

Keywords Prion diseases · Environmental prion contamination · Prion shedding · Chronic wasting disease · Scrapie

10.1 Introduction

Scrapie and chronic wasting disease (CWD) are two prion diseases of particular environmental concern, as they are horizontally transmissible and remain infectious after years in the environment (Greig 1940; Hadlow et al. 1982; Miller and Williams 2003; Miller et al. 2004; Dexter et al. 2009). Experimental and epidemiological studies suggest that prion deposition onto soil and subsequent transmission of soil-bound prions may play a role in natural prion transmission (Saunders et al. 2008a,

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2012a, b; Smith et al. 2011), as cervids and ruminants are known to ingest and inhale large amounts of soil (Arthur and Alldredge 1979). Indirect, environmental transmission has been implicated in multiple CWD and scrapie outbreaks (Georgsson et al. 2006; Miller et al. 2006) and environmental transmission has been demonstrated in a number of studies (Greig 1940; Miller et al. 2004; Dexter et al. 2009; Mathiason et al. 2009; Rhyan et al. 2011). A hypothesized model of environmental prion transmission developed in Saunders et al. (2012a) is provided in Fig. 10.1.

One factor influencing environmental transmission of prion diseases is the long-term survival of prions in the environment. Unbound and soil-bound scrapie and BSE PrP^{Sc} were detectable after 18 months of room temperature incubation in the laboratory (Maddison et al. 2010a), and soil-bound hamster prions remained capable of replication after similar year-long incubations in a separate study (Saunders et al. 2011a). In addition, hamster prions mixed with soil and buried in the field remained orally infectious after 2 years (Seidel et al. 2007). Kuznetsova et al. (2020) demonstrated irreversible binding of CWD prions to soil, but no alternation of infectivity in mice inoculated with unbound CWD brain homogenate or CWD brain homogenate equilibrated with soil.

Prions have been demonstrated to persist in other environmental media in addition to soil. Scrapie and BSE were found to survive 8 and 6 years of incubation in wastewater at room temperature with 2 and 3 log reduction in infectivity, respectively (Marín-Moreno et al. 2016). Prion disease transmission has also been demonstrated from other non-soil materials that can be present in the environment including wood, rocks, plastic, glass, stainless steel and aluminum, among others (Konold et al. 2015; Pritzkow et al. 2018).

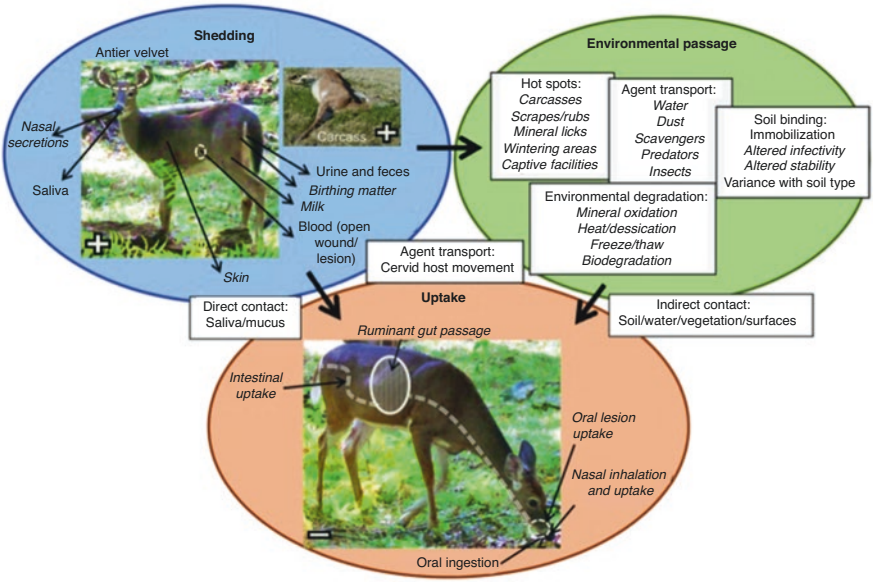

Fig. 10.1 Conceptual model for soil-mediated prion transmission. (From Saunders et al. 2012a)

Epidemiological records indicate numerous instances of scrapie recurrence upon reintroduction of animals on farms previously exposed to scrapie. Scrapie recurrence was documented following fallow periods of 1–19 years (Siguardson 1991; Georgsson et al. 2006) and pastures can retain infectious CWD prions at least 2 years after exposure (Miller et al. 2004). In a natural scrapie-contaminated farm, rigorous decontamination (20,000 ppm free chlorine for 1 h), replacement, and resurfacing of the pen and facilities failed to control scrapie recurrence (Hawkins et al. 2015). In addition, the disposal of mortalities during BSE outbreaks, both in the past and in potential future disposal events, serves as another environmental source of prions with the potential to infect humans. Somerville et al. (2019) determined that BSE infectivity was retained after a 5-year burial period. Therefore, it is clear that understanding environmental factors influencing prion infectivity is critical in prion disease transmission.

Prions are shed from diseased hosts in a diverse set of biologic matrices, including feces, urine, saliva, blood, skin, milk, placenta, and nasal mucus, and a comprehensive review of prion shedding was performed by Gough and Maddison (2010). Prion shedding can occur many months prior to clinical manifestation of the disease (Tennant et al. 2020; Henderson et al. 2015; Gough and Maddison 2010; Tamgüney et al. 2009). Prions also enter the environment after decomposition of diseased animal carcasses (Miller et al. 2004), as prions are present near-ubiquitously throughout a diseased host (Saunders et al. 2012a). Uptake of prions to naïve hosts can occur via ingestion or inhalation of contaminated material (Pritzkow et al. 2015; Hamir et al. 2005, 2008; Kincaid and Bartz 2007; Sigurdson et al. 1999), although the significant routes of natural exposure remain uncertain (Saunders et al. 2012a).

10.2 Prion Sorption to Soil

There is strong experimental evidence that properties of soil and water can significantly affect prion sorption, resistance to degradation, persistence, replication efficiency when bound to soil, and ultimately prion infectivity (Table 10.1). Soil type, which we define broadly here as a soil's distinct texture (particle size distribution), mineralogy, and organic carbon content, is a strong determinant of prion sorption (Table 10.1). PrP^{Sc} has a higher affinity for clays and clay soils compared with sand and sandy soils. For instance, in one study, the sorption capacity of a silty clay loam soil was at least three times higher than a sandy loam soil (or 400 times higher at initial equilibrium) and 2000 times higher than fine quartz sand (Saunders et al. 2009a). In another, sorption of purified PrP^{Sc} to montmorillonite clay was at least 100 times greater than fine quartz sand (Johnson et al. 2006). PrP adsorption kinetics are also significantly different between clay soil and sand or sandy soil. In one study, maximum adsorption for fine quartz sand and a sandy loam soil were observed after 7–30 days, while maximum adsorption for a silty clay loam soil took only 24 h (Saunders et al. 2009a). Thus, prions contacting clay soils could be rapidly immobilized on the soil surface, forming potent reservoirs for efficient transmission. In

Table 10.1 Variance in prion–soil interactions with respect to soil type

Prion–soil property	Soil type/component			References
	Clay/clay soils	Sand/sandy soils	Organic content	
PrP^{Sc} sorption capacity	Higher	Lower	Unknown	Johnson et al. (2006) and Saunders et al. (2009a)
PrP^{Sc} desorption with SDS (% recovery)	Low (<5–50%)	High (20–95%)	Low (5–20%)	Cooke et al. (2007), Jacobson et al. (2009), Maddison et al. (2010a), and Saunders et al. (2010)
PrP^{Sc} sorption kinetics in tissue homogenate	Faster (<1 day)	Slower (>1–30 days)	Unknown	Saunders et al. (2009a)
Role of the PrP^{Sc} N-terminus in sorption	Enhances sorption	Inhibits sorption	Unknown	Cooke et al. (2007), Johnson et al. (2006), Maddison et al. (2010a), and Saunders et al. (2009b, 2010)
Replication efficiency[a]	Reduced	Equal	Reduced	Saunders et al. (2011b)
Intracerebral infectivity[a]	Reduced	Unknown	Unknown	Saunders et al. (2011b)
Oral infectivity[a]	Increased	Unknown	Unknown	Johnson et al. (2007)

Adapted from Saunders et al. (2012b)
[a]Soil-bound prions compared with unbound prions

contrast, prions contacting sandy soils may be more readily transported below the surface and diluted by surface or groundwater. Wyckoff et al. (2016) reported that CWD prions associated with montmorillonite clay increased prion bioavailability *in vivo* in mice.

The role of N-terminal region of PrP^{Sc} in soil adsorption also varies with soil type. Although the N-terminus is not required for prion infectivity (Bessen and Marsh 1994) or for soil sorption (Saunders et al. 2009b), its presence enhances adsorption of PrP^{Sc} to clay, but may hinder adsorption to sand surfaces (Saunders et al. 2009b). In addition, numerous studies have observed cleavage of the N-terminus following PrP desorption from clay surfaces using anionic detergents (Cooke et al. 2007; Johnson et al. 2006; Maddison et al. 2010a; Saunders et al. 2010). Cleavage is not observed following desorption from sand, sandy soils, or organic matter, suggesting the N-terminus is actively involved in PrP sorption to clay particles but not other soil components. Both truncated and full-length forms of PrP^{Sc} will enter the soil environment (Saunders et al. 2008b), and given that the N-terminus is not required for prion infectivity or soil sorption, there may be little effect of interactions between the PrP N-terminus and soil on prion transmission. However, it does strongly suggest mechanistic differences in prion sorption between clay surfaces and other soil surfaces.

Soil–water chemistry can also influence prion adsorption. The chemistry of soil–water–prion mixtures will vary with soil components, soil moisture, and the source

of infectious prions (e.g., excreta, saliva, and tissue). While solution ionic strength and ionic composition may not significantly affect PrP^{Sc} adsorption (Saunders et al. 2011a), the biologic matrix in which prions enter the environment (prion source) can significantly alter soil sorption kinetics and capacity (Saunders et al. 2009a). For example, the magnitude and kinetics of PrP adsorption from tissue homogenate are significantly reduced compared with adsorption of pure or purified PrP (Saunders et al. 2009a), most likely due to competitive sorption (Saunders et al. 2009b). Importantly, adsorption of PrP introduced in biologic matrices besides tissue homogenate has yet to be studied.

Desorption of PrP from soil has not been observed under mild, environmentally relevant conditions or in the presence of harsh chaotropic agents, nonionic detergents, or extreme pH (Cooke et al. 2007; Johnson et al. 2006; Seidel et al. 2007). Thus, desorption of prions once bound to soil may be rare in natural settings. However, it is interesting to note that the ability to desorb PrP with anionic detergents varies with soil type, where extraction from sand and sandy soils is significantly higher than from clays, clay soils, and organic matter (Table 10.1) (Cooke et al. 2007; Maddison et al. 2010a; Saunders et al. 2010; Jacobson et al. 2009).

10.3 Prion Transport in the Environment

Due to their insolubility and high affinity for clays and silts, prions are unlikely to be transported long distances in surface water. Recent studies simulating prion fate in wastewater found that PrP strongly partitioned into the sludge solids (Hinckley et al. 2008; Kirchmayr et al. 2006). Several studies have evaluated the mobility of prions in soil. One found only slight recPrP migration in a soil column over a 9-month incubation (Cooke and Shaw 2007). Jacobson and colleagues observed minimal HY TME PrP^{Sc} migration in columns packed with five different soils (Jacobson et al. 2009, 2010). Purified PrP^{Sc} was more mobile in columns packed with municipal solid waste (Jacobson et al. 2009). The potential for prion transport facilitated by mobile soil colloids has not been investigated. Colloid-facilitated transport has been shown to be a significant transport process for many strongly sorbing contaminants (de Jonge et al. 2004). In addition, infectious prions can form aggregates of colloidal size (Silveira et al. 2005) and might be transported unassociated. Macro-pore colloid-facilitated transport could quickly move prions into groundwater or surface waters and, therefore, warrants further study.

Prion transport in plants has also been evaluated. Rasmussen et al. (2014) determined that recombinant prion protein was found associated with wheat (*Triticum aestivum L.*) roots, but was not translocated to stems. Later studies also found PrP^{Sc} associated with wheat grass roots and leaves and that ingestion of prion contaminated roots and leaves resulted in initiation of prion disease (Pritzkow et al. 2015).

10.4 Degradation and Mitigation of Prions in the Environment

Prions are subject to degradation in the natural environment; however, prions are resistant to degradation and inactivation, especially when compared with bacterial or viral pathogens (Taylor 1999). Bacterial enzymes which effectively degrade prions have been identified, but they are most effective at high pH (10–12) and high temperature (50–60 °C) (McLeod et al. 2004; Yoshioka et al. 2007), conditions which are atypical of most natural environments. Microbiological consortia taken from the rumen and colon of cattle could degrade PrP^{Sc} to undetectable levels within 20 h under anaerobic conditions at 37 °C, although infectivity remained (Scherbel et al. 2006, 2007). Degradation of PrP^{Sc} by select lichen extracts has been shown (Johnson et al. 2011) and treatment with manganese oxide (naturally occurring in certain soils) under acidic conditions also leads to PrP^{Sc} degradation (Russo et al. 2009).

A limited number of studies have investigated degradation of soil-bound prions. Laboratory studies suggest that prions bound to soil with high organic content may degrade more rapidly when compared to prions bound to clay and sand minerals (Maddison et al. 2010a; Saunders et al. 2011a). Soil-bound prions in highly dilute aqueous solutions may also exhibit lower persistence compared to prions in solutions of higher ionic strength (Saunders et al. 2011a). An additional study reported significantly higher survival of clay-bound PrP in the presence of manganese (Davies and Brown 2009). Enzymatic digestion of soil-bound prions under environmentally relevant conditions is effective across all soil types (Saunders et al. 2010), although prions bound to soil organic matter may be more susceptible than prions bound to other surfaces (Saunders et al. 2011c). Among the components of soil organic matter, humic acids (HA) likely play a significant role in prion degradation. Overnight incubation of CWD prions in HA solutions reflecting natural HA concentrations in soils reduced the amount of CWD prions up to 95% and prolonged the incubation periods of transgenic mice intraperitoneally challenged with HA-treated CWD prions (Kuznetsova et al. 2018). Weathering conditions such as wetting and drying and freeze–thaw treatments may serve as natural mitigation methods for prions (Yuan et al. 2015, 2018). Repeated wetting and drying cycles degraded both unbound and soil-bound prions, with greater susceptibility for soil-bound prions. Repeated freeze–thaw treatments were not as effective in degrading prions; however, prion conversion capacity was reduced. This impact of freeze–thaw treatment was not observed in dried prions (pre-incubated at 40 °C for 12 h), suggesting a protective effect of dehydration (Yuan et al. 2018).

In addition to studies evaluating prion persistence in soil, there has been some work to determine the risk of prions in wastewater and biosolids (Epstein and Beecher 2005; Pedersen et al. 2006; Miles et al. 2011). Prions could enter wastewater through effluent from slaughterhouses unknowingly rendering prion mortalities or through contaminated effluent from hospital or research facilities. Hinckley and colleagues determined that most PrP^{Sc} and prion infectivity would associate with the

activated sludge solids, survive mesophilic anaerobic digestion, and be present in the remaining biosolids (Hinckley et al. 2008). Likewise, Kirchmayr et al. found no significant decrease in PrP^{Sc} after 16 days incubation in mesophilic anaerobic sludge and observed PrP^{Sc} solids association (Kirchmayr et al. 2006). PrP^{Sc} degradation was observed in thermophilic anaerobic sludge, although maximum degradation occurred in sterilized samples (Kirchmayr et al. 2006). Others found a large decrease in PrP^{Sc} within 15 days after incubating BSE brain homogenates in municipal sewage at 20 °C (Maluquer de Motes et al. 2008), though the infectivity was retained (Maluquer de Motes et al. 2012; Marín-Moreno et al. 2016). Sheep scrapie brain homogenates were somewhat more resistant to degradation. Based on these studies, it can be assumed that most prion infectivity will be conserved during normal wastewater treatment processes, and prions would thus enter the environment, highly diluted, via landfill disposal or land application of biosolids.

10.5 Do Environmental Factors Influence Prion Incidence?

Prion disease incidence exhibits significant geographic variance, including CJD in humans, CWD, and scrapie (Blanchong et al. 2008; Conner and Miller 2004; Holman et al. 2010; Joly et al. 2006; Stevens et al. 2009; Walter et al. 2011). There are a wide range of potential factors influencing spatial variance in these diseases, including population genetics (Blanchong et al. 2008; Hunter 2007), animal movement patterns and habitat prevalence (Conner and Miller 2004; Joly et al. 2006), predator prevalence (Wild et al. 2011), and human impacts (Krumm et al. 2005; Stevens et al. 2009). Environmental factors such as local climate, the presence of potential vectors, and vegetation, water, and soil characteristics may also influence prion disease incidence for a given area, either by altering the susceptibility of the host to infection or by directly affecting the prion along its transmission pathway.

With respect to the former, a number of groups have investigated trace metal levels in forage, water, and soils of scrapie and CWD endemic areas, given that copper, manganese, or other metals may play key roles in prion pathogenesis (Davies and Brown 2009). No consistent correlations have been observed to-date (Chihota et al. 2004; Imrie et al. 2009; McBride 2007), suggesting that abnormal environmental exposure to trace metals may not be a significant factor in prion incidence. In contrast, a number of studies have observed significant soil factors that may directly affect prion transmission pathways. Although a study of scrapie in Great Britain did not find a significant correlation between soil texture (only roughly delineated as ‘sand’, ‘loam’, ‘peat’, or ‘clay’) and scrapie incidence, a soil drainage factor was significant, where soils classified as ‘naturally wet’ had higher risks of scrapie than ‘freely draining’ soils (Stevens et al. 2009). In addition, Imrie and colleagues found possible correlations between soil pH and organic content and scrapie incidence in Great Britain, but no correlation with soil clay content (Imrie et al. 2009). As the authors acknowledge, these studies must be considered preliminary, as the spatial resolutions were very low and the data sets were limited.

A more robust study of CWD in northern Colorado has suggested a correlation between soil texture and CWD incidence in free-ranging cervids. Along with the previously known risk factors of age and sex, the soil clay content of a deer's home range appeared to be positively correlated with risk of CWD infection (Walter et al. 2011). However, O'Hara Ruiz et al. (2013) and Dorak et al. (2017) found an inverse correlation between CWD incidence and soil clay content.

10.6 Detection of Prions in the Environment

One current limitation in our ability to evaluate environmental prions is that highly sensitive and accurate detection of prion infectivity in the environment is not currently possible. Standard methods such as western blotting fail to detect significant levels of infectivity (Barron et al. 2007; McLeod et al. 2004; Scherbel et al. 2006), and the most reliable method of prion detection, animal bioassay, would be impractical for use on large numbers of environmental samples. Protein misfolding cyclic amplification (PMCA) (Saa et al. 2006), developed by Soto and colleagues for detecting small amounts of PrP^{Sc}, has generated much interest for use as an environmental detection method. PMCA has been used successfully with CWD samples (Kurt et al. 2007) and with hamster PrP^{Sc} exposed to soil (Nagaoka et al. 2010; Seidel et al. 2007). Detection of scrapie PrP^{Sc} on metal and wooden fencing from a scrapie endemic farm using PMCA, but infectivity was not determined (Maddison et al. 2010b). The quake-induced conversion method (Atarashi et al. 2007, 2008), which uses recPrP as a substrate instead of uninfected brain homogenate, is a viable alternative to PMCA as an environmental diagnostic tool. Quantitative tandem mass spectrometric techniques (Onisko et al. 2007) may also be developed as a sensitive environmental detection and quantification method for PrP.

10.7 Conclusion

As prion diseases, and CWD in particular, continue to spread geographically and disease residence times in cervid populations and habitats increase, environmental factors may play an increasingly important role in sustaining or heightening disease prevalence (Almberg et al. 2011). The critical parameters of environmental prion transmission are the mean residence time of prions in environmental reservoirs and the efficiency of transmission via these reservoirs (Sharp and Pastor 2011). We predict that these parameters could vary significantly based on environmental factors, such as soil properties.

Influence of soil factors on disease incidence is certainly not without precedent. Numerous experimental studies have reported variance in the survival, transport, and transmission of enteric pathogens with respect to soil type and soil factors (Cilimburg et al. 2000). Biotic and abiotic soil factors have been linked to the

prevalence of agriculturally relevant soil-borne diseases (Mazzola 2002). Recently, clay soils have been linked to an increased risk of the parasitic nematode *Baylisascaris procyonis* in Texas raccoons (Kresta et al. 2010), organic carbon and clay content was positively correlated with prevalence of ovine Johne's disease, caused by *Mycobacterium avium*, in Australia (Dhand et al. 2009), and poorly drained clay soils with high organic content were associated with the abundance of *Culicoides imicola*, primary vector for the bluetongue virus (Acevedo et al. 2010).

The epidemiological data on prion–soil risk factors are as yet limited. Thus, robust spatial epidemiological studies of well-established CWD-endemic areas should be conducted to build on the work of Walter et al. (2011), O'Hara Ruiz et al. (2013) and Dorak et al. (2017). In addition, reliable methods for detecting and quantifying infectious prions in the soil environment are clearly required.

If soil properties are indeed significant in local prion incidence, a number of important disease management implications arise. In captive settings, herd owners could favor pastures with low-risk soils, perhaps even amending soils to decrease prion transmission. In free-range populations, epidemiological modeling could use soil properties to predict temporal and spatial trends in prion incidence. Soil could be considered to prioritize disease surveillance efforts. High-risk soils, especially those with the potential for human exposure, could be targeted with treatments to reduce transmission (Saunders et al. 2010). These measures offer hope for reducing or eliminating prion disease in free-range and captive animal populations as well as mitigating the risk of zoonotic prion transmission.

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Chapter 11
Environmentally Acquired Transmissible Spongiform Encephalopathy

Paul Brown

Abstract From the ritual cannibalism of kuru to the modern "cannibalism" of iatrogenic and variant forms of Creutzfeldt–Jakob disease (CJD), the history of environmentally acquired spongiform encephalopathy is reviewed. Sources, original recognitions, inter-relationships, and distinctive characteristics of the various forms of disease are discussed, credits (and debits) are acknowledged, and failures and victories recalled as the era of acquired CJD draws to a close.

Keywords Kuru · Iatrogenic Creutzfeldt–Jakob disease · Variant Creutzfeldt–Jakob disease · Bovine spongiform encephalopathy · Human growth hormone · Dura mater grafts · Neurosurgery · Blood-borne infection

11.1 Kuru

The prototype of human transmissible spongiform encephalopathy (TSE), kuru was almost certainly spread through the practice of ritual cannibalism, and was proven to be experimentally transmissible to primates in 1966 (Gajdusek et al. 1966). It is now mainly of historical interest, but certain epidemiological and clinical features are relevant to the later occurrences of iatrogenic and variant forms of Creutzfeldt–Jakob disease (CJD). From oral accounts by elders in the afflicted Foré-speaking peoples in the Eastern Highlands of Papua New Guinea, the disease first appeared early in the twentieth century and rapidly achieved epidemic proportions. The best guess as to its origin is the cannibalistic consumption of a random case of sporadic CJD among the Foré, which then spread via the continued practice of ritual cannibalism through the 1950s, when missionaries and the Australian colonial administration used a "carrot and stick" approach to eliminate the practice (fines or jail versus trade goods). The average incubation period is estimated to have been

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12 years, and the age-specific "dieback" of the disease began with the youngest individuals—i.e., those who had been most recently exposed (Fig. 11.1, Alpers 2008). Since the turn of the century, there have been only eight deaths: three in 2000, two in 2001, one in 2003, one in 2005, and one (the last) in 2009. Four were male and four were female, and all occurred in older adults between 55 and 62 years of age (personal communication, Dr. Michael Alpers).

It is ironic that the high incidence of kuru in children and young women was not, as originally thought, due to hormonal or genetic factors, but a much more prosaic reason: women, surrounded by their infants and young children, prepared the bodies for cooking and were also the principle consumers of brains and viscera. It is also ironic that "morality" rather than medicine brought an end to the disease.

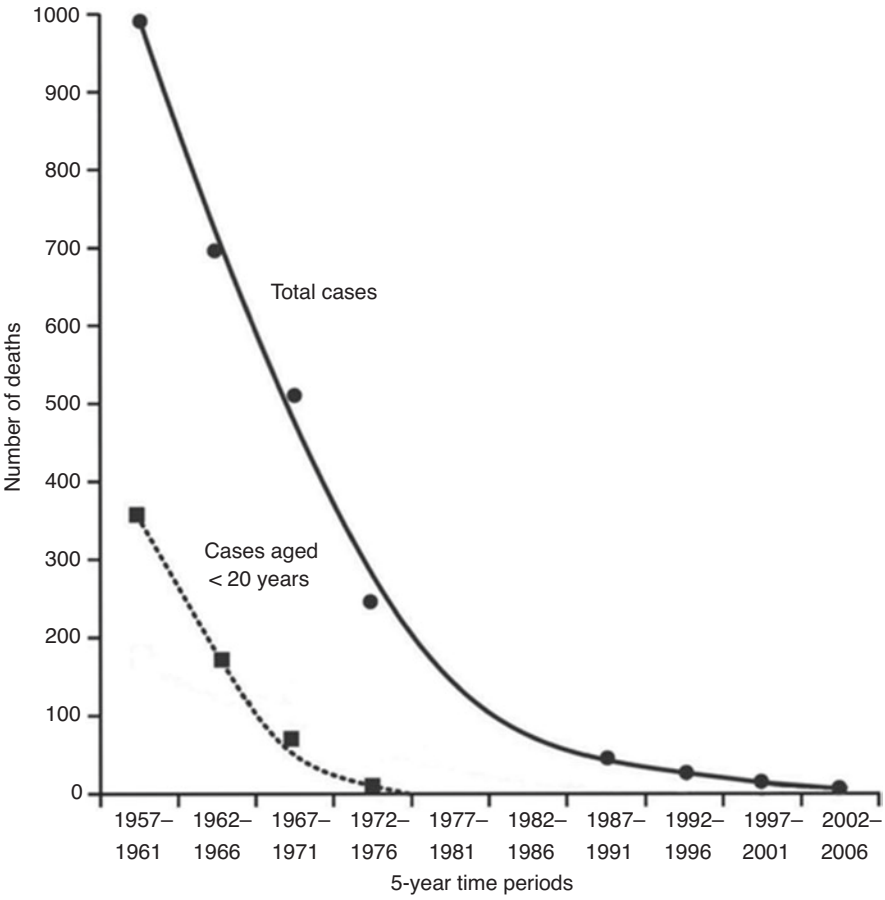

Fig. 11.1 Kuru mortality 1957–2006. Only a single case (in 2009) has occurred in the 15 years since 2006. (Modified with permission from Alpers (2008) The epidemiology of kuru: monitoring the epidemic from its peak to its end. Philos Trans R soc. B 363:3707–3713)

Two further features of kuru are interesting in the context of environmentally acquired CJD. The clinical syndrome was predominantly cerebellar, with little or no dementia, a feature that would also characterize peripheral infection from contaminated cadaveric human growth hormone, but not oral infection from bovine spongiform encephalopathy (BSE). Also, the age at onset of disease (a rough indication of the incubation period) was, on average, considerably shorter in codon 129 homozygotes than heterozygotes, but with a significant overlap between the two, which may yet bear on questions about the future incidence of variant CJD (vCJD) due to infection by the agent of BSE (Cervenáková et al. 1998).

11.2 Creutzfeldt–Jakob Disease

Environmental-acquired forms of CJD occupy a far more important niche in the ensemble of TSE than their numbers would suggest. This importance lies in two facts: they can be prevented (if their cause is recognized), and they stimulate public concern, which translates to public funding of the whole field of TSE, without which research shrinks to the level accorded the category of "orphan diseases." We are seeing this phenomenon today as iatrogenic CJD, BSE, and vCJD recede into the background of public and government consciousness. Their chronology falls conveniently into four successive decades.

11.3 Iatrogenic CJD

The following two tables summarize the national case totals and clinical features of recognized sources of environmentally acquired CJD (Table 11.1).

11.4 The 1970s: Cornea and EEG Depth Electrodes

Somewhat more than a year after publication of the experimental transmission of CJD to a chimpanzee in 1968, a 55-year-old man died of pneumonia following a 2-month history of "incoordination, memory deficit, involuntary movements and myoclonia" (Duffy et al. 1974). At autopsy, a cornea was removed and transplanted into a 55-year-old woman. The autopsy later revealed a diagnosis of CJD in the donor. The recipient became ill 18 months later and had a clinical course typical of CJD, also confirmed at autopsy, and subsequently by transmission of the disease to an intra-cerebrally inoculated primate in D.C. Gajdusek's laboratory at the NIH. The case is interesting for at least three reasons, apart from being the first recognized instance of iatrogenic CJD. First, the interval of 18 months between the operation and onset of disease in the recipient was short enough for the connection to have

Table 11.1 Global distribution of cases of iatrogenic Creutzfeldt–Jakob disease as of December 2021

	Surgical procedures				Medical procedures		
	Dura Mater	Surgical instruments	EEG needles	Corneal transplants[a]	Growth hormone[b]	Gonado-tropin	Packed red cells[c]
Argentina	1						
Austria	4				1		
Australia	5					4	
Brazil					2		
Canada	6						
Croatia	1						
France	14	1			123		
Germany	10			2			
Ireland					1		
Italy	11						
Japan	156			2			
Netherlands	5				2		
New Zealand	3				8		
South Korea	2						
Qatar					1		
South Africa	1						
Spain	8						
Switzerland	3		2				
Thailand	1						
UK	8	3			80		4
USA	4			6	36		
Totals	243	4	2	10	253	4	4

[a]Several of these cases are speculative (donors died of non-CJD or unknown causes)
[b]Brazil and New Zealand hGH was prepared in the USA; Qatar hGH was prepared in France. Additional possible single cases due to hGH (not included in table) in Sweden, Australia, and New Zealand
[c]One of the four patients was asymptomatic and diagnosed at autopsy after death from an unrelated illness. Not included in the table is another asymptomatic autopsy-proven case in a hemophiliac who had been exposed to potentially contaminated Factor VIII

been suspected; had it been many years instead of many months, it might have gone unrecognized and never come to light. Second, it only occurred because of the "lead time" needed for scientific research to disseminate through the general medical community—in this case, the clinical features and transmissibility of CJD. Even a few years later, the diagnosis would certainly have been strongly suspected and cadaveric tissues never used for corneal (or any other) tissue transplant. And third, brain tissue from the recipient that was used in the successful transmission experiment had been stored in formalin for several months prior to inoculation.

Nine further possible or probable instances of corneal transplant transmission have occurred since this case was reported (they cannot be considered definite because none has been proven by experimental transmission studies of the transplanted corneas).

Many of the patients had more than one transplant, and average incubation periods ranged from 8 to 12 years. In one case, CJD developed 16 months after a corneal transplant, but the cause of death in the donor was not established; in another case, both donor and recipient died of neuropathologically verified CJD, but the interval between transplant infection and clinical signs was 30 years. The most recent reported case occurred in 2006 (only the second case in the USA), and the entire group was recently reviewed, together with a statistical estimate that "1 in every 30,000 transplants in the U.S. will be performed with a cornea from a donor between the ages of 31 and 80 with latent CJD" (Martheswaran et al. 2020). The fact that no further cases have been reported could be due to one or more of the following reasons: (1) cases are occurring that are not reported; (2) improving donor exclusion criteria; or (3) the transmissible protein is not present in the corneas of a majority of "latent" donors to cause symptoms before death occurs from other causes.

A second episode of surgical contamination, reported in 1977 (Bernoulli et al. 1977), occurred in 1974 in association with depth electrodes that had been used on a 69-year-old woman with CJD, sterilized with 70% alcohol and formaldehyde vapor (standard practice at that time), and re-used in two patients with intractable epilepsy. The latter two patients developed illnesses consistent with CJD about 2½ years later, and postmortem examinations confirmed the diagnosis in each patient. Two features of this episode merit comment. First, the implicated needles were sent to Gajdusek's laboratory and implanted in the brain of a chimpanzee that subsequently died of CJD, proving the iatrogenic cause of the disease, which to this day remains the only formally proven case of iatrogenic CJD. The second point of interest is that one of the recipients was a 23-year-old woman who became pregnant 14 months after the operative procedure, and who delivered by Caesarian section a normal male infant, who was in good health when last contacted at the age of 12 years.

In two subsequent retrospective studies, neurosurgical cross-contamination of instruments was found to be probably responsible for three cases in the UK and one case in France during the 1950s (Will and Matthews 1982; El Hachimi et al. 1997).

The absence of neurosurgical contamination in recent years is difficult to explain, as operations on patients with undiagnosed CJD continue to occur, and instrument sterilization protocols in many hospitals remain suboptimal. It may be due to a combination of (1) a more widespread awareness of the need to consider CJD among neurological differential diagnoses; (2) more rigorous sterilization protocols and the increasing use of disposable instruments on any suspect or known CJD patient—in the UK, a nation-wide program of optimized sterilization or one-time use of such instruments has been mandated; and (3) a failure to recognize cause and effect without long-term post-operative surveillance.

11.5 The 1980s: Human Growth Hormone (hGH) and Dura Mater Grafts

11.5.1 Human Growth Hormone

The decade began quietly enough, but the possibility of risk from growth hormone was already under study in the Edinborough laboratory of Alan Dickinson, who recognized the potential danger of pituitary–brain proximity. His instincts were correct: in 1985, four young adults dying of CJD within the previous year had all been treated in the 1960s and 1970s with human growth hormone extracted from cadaveric pituitary glands. The first case, in a 21-year-old man whose correct diagnosis was not realized until a post-mortem examination, was the subject of a letter by Dr. Raymond Hintz, a Stanford pediatric endocrinologist, to Dr. Mortimer Lipsett, Director of the NIH institute responsible for the US human growth hormone distribution program (Brown 1988):

> …the patient was treated for 14 years with growth hormone, and I feel that the possibility that this was a factor in his getting Creutzfeldt–Jakob disease should be considered. A careful follow-up of all patients treated with pituitary growth hormone in the past 25 years should be carried out, looking for any other cases of degenerative neurological disease.

Lipsett acted immediately by notifying all prescribing pediatricians at the hormone distribution centers of a possible problem. A few days later, on a flight from Washington to a meeting in Athens, Gajdusek remarked that Lipsett had called him about a possible case of CJD in a growth hormone patient, adding that it looked like there might be an epidemic in the works (his travelling companion, who would subsequently head the NIH investigative panel, did not think it likely). Within a month, two further cases surfaced, prompting Lipsett to shut down the entire program, and the FDA to rush through the approval process for a recombinant product that was then under evaluation.

As more and more cases came to light in the USA, UK, and France, it became clear that contamination was widespread, but its severity could not be predicted—would it become a full-fledged epidemic, or would it remain limited to a comparatively small number of cases? In the event, it lay somewhere between the two extremes, with a total of 245 cases from 1985 through 2021 (Table 11.2). Case numbers for the three principally affected countries were: 29 (USA), 80 (UK), and 123 (France).

Considering the at-risk patient population in each country, these numbers yield frequencies of infection of 1.1% in the USA, where no case has occurred in any patient beginning treatment after 1977 when a chromatography purification step was introduced; 3.6% in the UK, where cases continue to appear in patients infected throughout the entire treatment period, and 10.2% in France, where all cases are thought to have been infected within a 2-year window between 1983 and 1985 from contamination due to both sourcing and processing deficiencies [(Abrams et al. 2011; National Creutzfeldt–Jakob Disease Research Surveillance Unit 2009), and

Table 11.2 Clinical features of environmentally acquired Creutzfeldt–Jakob disease according to the source and route of infection

Source of infection	Agent entry presentation	Mean incubation period (range)	Usual clinical presentation
Corneal transplant	Optic nerve	18 months, 27 years	Dementia/cerebellar
Stereotactic EEG	Intracerebral	16 months, 20 months	Dementia/cerebellar
Neurosurgery	Intracerebral	21 months (18–28 months)	Visual/dementia/ cerebellar
Dura mater graft	Cerebral surface	12 years (16 months–30 years	Cerebellar (visual/dementia)
Growth hormone	Hematogenous (?)	17 years (5–42 years)[a]	Cerebellar
Gonadotrophin	Hematogenous (?)	13.5 years (12–16 years)	Cerebellar
RBC transfusion	Hematogenous	6.5, 7.8, 8.3 years	Psychiatric/cerebellar
BSE-infected tissue	Oral	12–15 years[b]	Psychiatric/sensory

[a]Combined data from France, the UK, and France, based on estimated dates of infection at the mid-point of multi-year therapy: France, 13 years; UK, 20 years; USA, 22 years
[b]Estimate based on epidemiologic data for BSE and vCJD (dates of infection for primary cases of vCJD are unknown)

unpublished data]. The increasingly rare cases of hGH-induced CJD during the past decade have not changed these percentages.

From a clinical standpoint, CJD infection from peripherally administered growth hormone produced a distinctive evolution of symptoms reminiscent of kuru, almost invariably beginning with cerebellar signs, and little or no dementia during the course of the disease (Table 11.2). The incubation period, estimated from the mid-point of what was usually a several-year course of treatment, was approximately 17 years but, like kuru, could extend out to 30 years and beyond. The current record for the longest incubation period in any environmentally acquired disease is 42 years, in a US growth hormone patient.

Susceptibility to infection was to some extent influenced by the polymorphism at codon 129 of the *PRNP* gene: in France and the USA, methionine homozygotes were modestly over-represented (55%) compared to the normal Caucasian population (40%); in the UK, however, valine homozygotes far outnumbered methionine homozygotes, leading to speculation that a different "strain" of CJD was being disseminated in the UK. However, a recent paper in which 11 isolates from each country were phenotyped in transgenic mice found a mix of the same three strains with only minimal differences between countries (Douet et al. 2021a). In all three countries, heterozygotes as a group had somewhat longer incubation periods than homozygotes.

These epidemiological and clinical observations incriminating hGH as the cause of infection were bolstered by the occurrence of virtually identical disease features in four Australian women treated with human pituitary gonadotropin. Formal proof came in 1993 in a report that inoculation of archived samples of 76 US hormone lots into over 200 monkeys and several chimpanzees had produced a transmission of

disease from one lot to one of the two inoculated monkeys, consistent with the occurrence of very low-dose random contamination (Gibbs Jr et al. 1993).

11.5.2 Dura Mater

The original publication discussing the first three cases of CJD in growth hormone recipients concluded with the following paragraph: "We are once again dramatically reminded that human tissues are a source of infectious disease, and that any therapeutic transfer of tissue from one person to another carries an unavoidable risk of transferring the infection. In this context, we must continue to worry about such products as follicle stimulating hormone. Luteinizing hormone, prolactin, and human interferon, as well as skin, bone, bone marrow, dura mater, blood vessel, and nerve grafts and organ transplantation" (Brown et al. 1985). This warning was almost immediately confirmed by the onset of what would be a coincidental outbreak of CJD contamination of dura mater grafts used in neurosurgical operations (Fig. 11.2). As with the growth hormone contamination, recognition of the source of

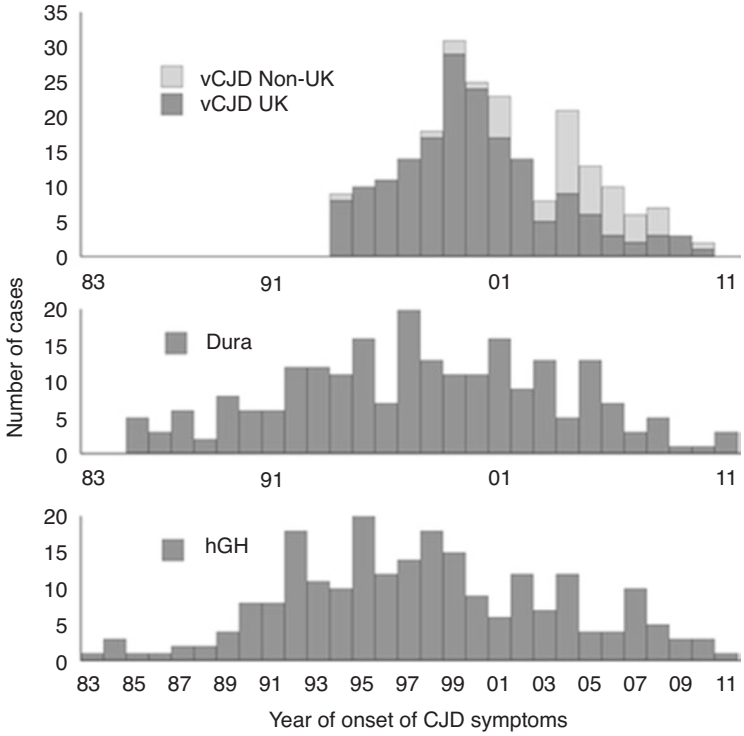

Fig. 11.2 Incidence of iatrogenic CJD due to contaminated cadaveric human growth hormone and dura mater, and of vCJD due to ingestion of BSE-contaminated tissues, 1982–2011

contamination could not help the many victims who were already incubating disease from treatment during the previous two decades, but the resulting substitution of synthetic or non-dural tissues for neurosurgical grafts put an end to iatrogenic disease from this source, although occasional cases with long incubation periods continue to surface.

The first case, reported by neurosurgeons at the Yale University School of Medicine in 1987 (Koch et al. 1985), was in a patient who had received a dural graft following the resection of a cholesteatoma 19 months before the onset of CJD. A second case was reported from New Zealand in 1989, and a third case from Italy, also in 1989. As word spread, further cases came to light in several different countries, especially from Japan, which in time would be the setting for nearly two-thirds of the 226 cases worldwide, almost all of which were the result of graft patches processed in the early 1980s by a single German company. The different national incidences were due to the frequency with which grafts were used, rather than from any particular batch contamination, as the fact that cases occurred in 18 different countries over a span of 25 years suggests that contamination was occurring on a regular basis until manufacturing ceased in 1987.

A predominance of codon 129 methionine homozygotes was heavily influenced by the large number of cases in Japan, where methionine homozygosity occurs in over 90% of the general population. Outside of Japan, heterozygotes as a group had somewhat longer incubation periods than homozygotes (similar to what was seen in growth hormone patients). The overall mean incubation period was 12 years, with a range from 1.5 to 30 years. Clinical presentations were usually cerebellar, although some patients presented with dementia, or more rarely, with visual signs. In the large Japanese case population, analysis of presenting signs according to the site of graft placement showed a significant excess of hemiparesis or hemianopsia in patients with supratentorial grafts, and of brainstem signs in patients with infratentorial grafts. About one-third of the cases had atypical features: slow progression, non-characteristic EEG, plaque deposition (including some patients with "florid" plaques), and an atypical prion molecular signature in Western blots that suggested the possibility of two different strains of infecting agent. One patient also had a pulvinar sign on MRI, a feature that is usually seen only in vCJD.

11.6 The 1990s: BSE and vCJD

In the Report of the Chief Veterinary Officer of the Ministry of Agriculture, Fisheries and Food of 1986, there appeared an anonymous brief report of a scrapie-like disease in a single, 2¾-year-old captive female nyala in an English wildlife park (not published until 1988) (Jeffrey and Wells 1988). There was no evidence of contact with other animals affected by TSE, and at the time, no suggestion that the disease had been transmitted via infected feed. A year later, a scrapie-like disease was reported in the same wildlife park, this time in a captive gemsbok, and similar cases subsequently occurred in an Arabian oryx, a greater kudu, and an eland in other zoos.

All this was surpassed in importance by the discovery in November 1986 of what is now known as BSE in domestic British cattle. Several cattle with an unusual, progressive, and fatal nervous disease had been investigated by staff at Veterinary Investigation Centres in southern England but without any conclusion as to the pathological definition or cause of the disease. "Formalin-fixed brains from two cows in different herds were submitted to the Pathology Department of the Central Veterinary Laboratory and neuropathologically examined by Martin Jeffrey and Gerald Wells who independently concluded that they were affected by a scrapie-like spongiform encephalopathy" (Wells et al. 1987).

During the course of 1987, further cases were identified and there was sufficient evidence available by the early summer to initiate a detailed epidemiological investigation conducted by John Wilesmith, Head of the Epidemiology Department. By the end of the year, he concluded that the cause of the epidemic in cattle (and the similar cases in captive exotic ungulates) was due to the consumption of meat and bone meal (MBM) derived from rendered animal carcasses and waste products that were included in the concentrate rations of weaned calves, especially of dairy cattle, as a protein-rich supplement (Wilesmith et al. 1988).

As is now well-known, the epidemic that followed in the UK, and some years later in other European countries (Fig. 11.3), together with cases in non-European countries—mostly Japan and Canada—became headline news all over the world, seriously affected the beef industry, and led to a global surveillance for BSE. It will never be known if the outbreaks in countries other than the UK were due to infective tissue (dead or alive) imported from the UK, or from simultaneous endogenous mini-epidemics of BSE due to widespread similar changes in rendering practices.

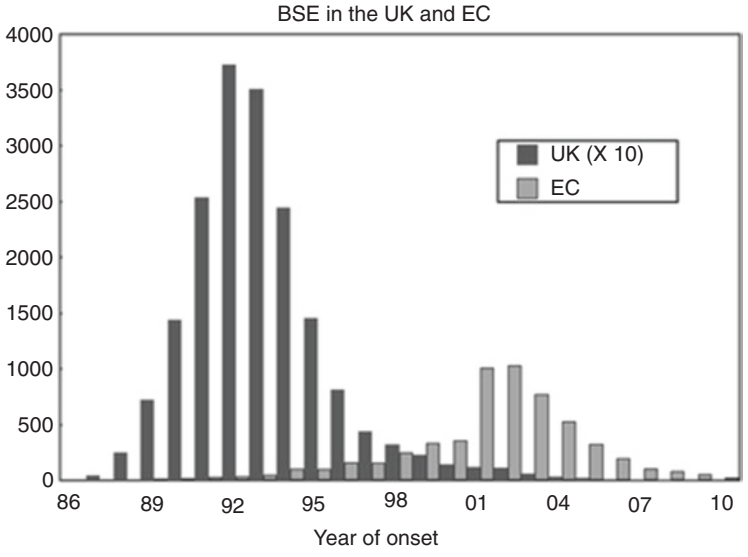

Fig. 11.3 Incidence of BSE in the UK and non-UK European Community, 1986–2010. Note the different scales for the UK and EC case totals

The more important question was whether BSE could spread to humans, and no one had the answer. If, as thought likely, BSE had its origin in the contamination of MBM by scrapie, and scrapie did not cause CJD, how could humans be at risk? The answer lay in a few laboratory experiments that had documented the fact that a given strain of TSE in one species could be unable to transmit disease to a different species unless first passaged through an intermediate species. The analogy of sheep-to-human versus sheep-to-cattle-to-human infection was clear enough, but epidemiology carried the day, and the consensus was that human infection from BSE was highly unlikely. One speaker at a BSE meeting held in Brussels in 1992 went so far as to conclude his presentation by eating a hamburger brought at his request from the UK by a British colleague. To the chagrin of the assembled scientific experts and government authorities, the consensus was wrong: BSE turned out to be infectious for humans, causing a variant form of CJD that was first identified in 1996 in eight cases of disease in young UK adults that had occurred during the previous 2 years (Will et al. 1996). (The speaker, however, is still alive and well 30 years later.)

The author remembers having been contacted by Prof. Robert Will in late 1995 about the neuropathology of young adult cases of sporadic CJD in the NIH collection, and the clandestine disappearance of several members of the Edinburgh CJD Surveillance team at a meeting in Paris in March of 1996 from which, in strict confidentiality, they had been urgently recalled to the UK to make a presentation to the government's TSE advisory committee, as was later described by Richard Rhodes (Rhodes 1997):

> Ironside opened the meeting with slides illustrating the unusual pathology. The SEAC chairman, John Pattison, remembers the moment vividly: "Before he said anything, we could see what it was. It was dramatically different". Another SEAC member, Jeffrey Almond, recalls near-panic. "The atmosphere became genuinely quite tense. Some of us were genuinely afraid of what we were hearing. We were afraid that this really maybe indicated a transmission of BSE to humans.

And with good reason—the number of cases in the UK would rapidly enlarge to attain a peak annual incidence of 29 cases in 1999, and cases also began to appear in other countries in people who had become infected during an earlier period of residence in the UK, or who became infected in their own countries as BSE spread around the world. Indigenous infections were especially prevalent in France, which had been the largest importer of MBM and cattle from the UK. The provisional global total of vCJD through 2021 stands at 240 cases.

The distinctive clinical characteristic of vCJD is its onset in the form of behavioral or sensory abnormalities, rather than the dementia/cerebellar/visual syndrome typical of sporadic CJD (Will and Ward 2004) (Table 11.2). However, as the illness progresses, most of the signs of sporadic CJD supervene, and at an advanced stage vCJD is clinically indistinguishable from sporadic disease. Two pre-mortem tests have enhanced the diagnostic presumption of vCJD: (1) all but one of the symptomatic cases have had a methionine–methionine coding genotype at polymorphic codon 129 of the *PRNP* gene; and (2) in up to 90% of patients the MRI shows a "pulvinar sign"—hyper-intensity of the posterior thalami. The diagnosis can only be established with certainty, however, by post-mortem examination that, as noted

above, reveals the presence of so-called "florid" plaques—globular accumulations of misfolded "prion" protein (PrP^{TSE}) surrounded by a halo of vacuoles. In addition, the plaques stain with an antibody specific for the protein.

The incidence curves of BSE and vCJD in the UK can be used to estimate the average incubation period for vCJD (Fig. 11.4). Observations of naturally infected cattle, and oral dosing experiments using as little as 1 mg of brain (Wells et al. 2007), suggest a reasonable estimate of the incubation period of BSE to be about 5–6 years, with a considerable range upward. Cattle can, therefore, be presumed to have first been infected toward the late 1970s, and maximum human exposure would have occurred in the mid-1980s, after the "silent" epidemic was well underway but before BSE had become a concern for humans. A peak incidence of vCJD applies to vCJD patients infected outside the UK, where a further delay was needed for exported BSE to become established, resulting in a non-UK vCJD peak incidence 5 years later, in 2004.

The outbreak of vCJD was also complicated by the discovery of three secondary cases and an inapparent infection in recipients of packed red blood cells from asymptomatic vCJD donors (Llewelyn et al. 2004; Peden et al. 2004; Wroe et al. 2006; Health Protection Agency 2006), as well as an inapparent infection in a recipient of plasma-derived Factor VIII (Peden et al. 2010).

There are several reasons for thinking that further transmissions of this type will be rare, or not occur at all. The incubation periods of the three symptomatic cases were 6.5, 7.8, and 8.3 years (Fig. 11.5) (Gillies et al. 2009), and 11 of 26 other recipients of red cell transfusions from these same donors remain healthy or have died from non-vCJD illnesses after an interval of at least 20 years (Hewitt et al.

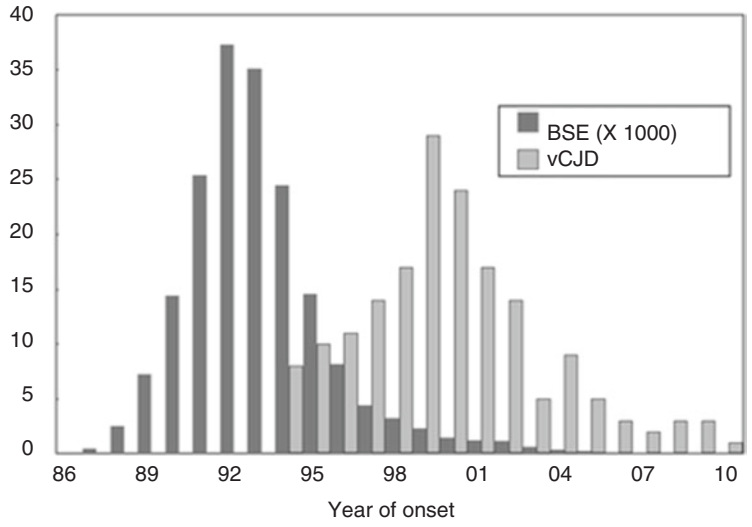

Fig. 11.4 Incidence of BSE and vCJD in the UK, 1986–2010. Note different scales for BSE and vCJD

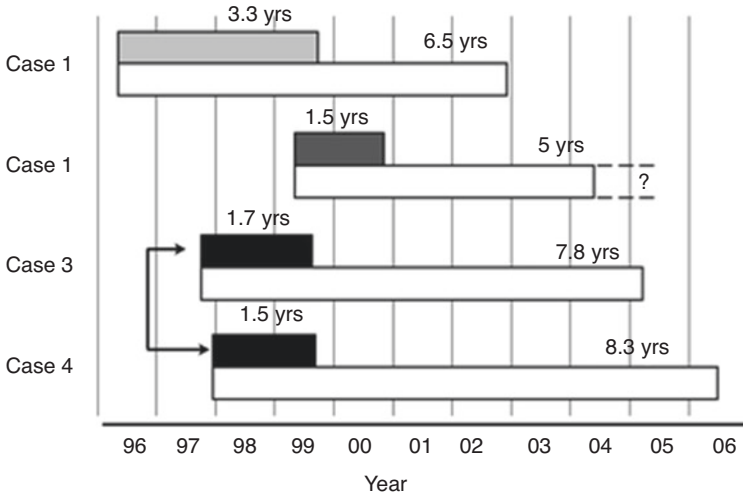

Fig. 11.5 Graph of intervals between transfusions and disease in four instances of secondary vCJD infections transmitted via packed red blood cells from donors who later died of vCJD. *Upper bars* of each pair represent donors and *lower bars* represent recipients. The second recipient died from a non-vCJD illness and was only discovered to have been infected through the use of post-mortem immunohistochemistry. The third and fourth recipients received transfusions from the same donor

2006). Also, leukodepletion of red cells was instituted in the UK in 1999 and no recipient of leukodepleted red cells from these donors has since developed disease. And finally, the near disappearance of primary cases during the past few years signifies a parallel decrease in the risk of individuals incubating vCJD within the blood donor population.

11.7 The Millennium: Denouement

The era of iatrogenic CJD and BSE-induced vCJD is rapidly passing into history, but as with most outbreaks of infectious disease, there are always at least a few cases with unusually long incubation periods that trail out beyond the expected dates of extinction (Table 11.3).

The total of only 41 cases iatrogenic disease during the past decade stands in striking contrast to peak numbers in earlier decades (especially in France and Japan), and it likely that cases will entirely disappear over the next few years.

Nor have we seen the dreaded “second wave” of vCJD cases with long incubation periods due to codon 129 genotypes other than methionine–methionine. This was certainly not an unreasonable concern in view of the tendency toward prolonged incubation periods associated with alternative genotypes in both kuru and hGH-related forms of CJD. In each outbreak, however, the alternative genotypes

Table 11.3 Year-by-year cases during the past decade for the major sources of environmentally acquired infections and countries of incidence

Source of infection	2012	13	14	15	16	17	18	19	20	21	Totals
BSE/vCJD											7
UK		1			1						
France		1	1					1[a]		1[a]	
Italy					1[a]						
hGH											24
UK	5	2	3		1		2	1	1		
France				1		1		1		1	
USA		1	1				2		1		
Dura mater											17
Japan	2	1	2	1	1			1	1		
France	1										
Italy	1		1			1			1		
Spain	1						1				
Germany					1						

[a]Probable laboratory infection

began to appear well before the methionine–methionine cases had been exhausted, and that has not happened with vCJD infections.

We are near the end of the outbreak and only one symptomatic case of vCJD has occurred in a heterozygote or valine homozygote (Mok et al. 2017). The caveat to this observation was the finding of pre-or subclinical infection in the spleens of the heterozygous red cell and Factor VIII recipients mentioned above (Peden et al. 2004, 2010) and in appendices removed from two homozygous valine individuals in a 4-year UK "anonymized" study of over 12,00 specimens (Ironside et al. 2006). As these two individuals were anonymous, there was no possibility of ever knowing their ultimate fate. An analysis of immunohistochemical tests performed on over 32,000 appendix samples removed between 1995 and 1999 yielded 16 positives, and an estimate of latent "carriers" of vCJD infection in the UK yielded a value of approximately 1 per 2000 individuals. In a UK population of around 60 million, of which perhaps 10 million were infants or non-meat eaters, a 1 per 20,000 carrier rate would have totaled around 25,000 infected people, a serious disconnect from the total of 178 known UK cases! Also of interest is the fact that no other animal TSE (scrapie in sheep, Chronic Wasting Disease in cervids, or Mink Encephalopathy) has ever been documented to cause human infection.

One final note about iatrogenic CJD must be mentioned: the possible occurrence of cases in people who work in contact with infectious tissue, i.e., CJD as an occupational hazard. The first patient in whom an iatrogenic cause for the disease was entertained occurred shortly after the discovery in 1968 that CJD was transmissible: a 55-year-old Boston neurosurgeon died of what was considered to be polyarteritis nodosa but at autopsy was found to have typical findings of CJD. Although he had a long career in pediatric neurosurgery, he operated on adults during his early career,

and in the absence of any known penetrating exposure, an iatrogenic origin of his CJD was considered unlikely.

A few years later, a laboratory technician in New York City who worked with CJD tissue samples was diagnosed with CJD and again with no known accidental event. In the past decade, three probable cases have occurred in Europe in laboratory personnel working with CJD or vCJD-infected tissues. All three cases occurred in young/middle aged adult laboratory technicians: one died in 2016 after an estimated incubation period of 7–14 years; another died in 2019 after a 7.5 year incubation period (Brandel et al. 2020); and the third patient is still alive and is the subject of ongoing investigations.

It has been obvious for many years that the most effective means to prevent further environmentally acquired cases of CJD would be a practical laboratory screening test to detect pre- or subclinical infection in people at risk of exposure, as well as blood or organ donors. From many studies in susceptible animal hosts ranging from "humanized" transgenic mice to laboratory primates, both PrP^{TSE} and infectivity have been detected in a wide variety of tissues (including blood) during the incubation period well in advance of illness (Douet et al. 2021b). At the turn of the century, nearly, a dozen of different laboratories and pharmaceutical firms were working to develop a PrP^{TSE} test, but all of them experienced problems in applying their methods to human plasma, and commercial interest flagged as the magnitude of vCJD regressed.

Today, however, there is renewed interest as a result of in-vitro amplification methods to detect PrP^{TSE} that have comparable or even greater sensitivity than infectivity bioassays, the "gold standard" for detecting infectivity thresholds, and are vastly more practical in terms of cost, ease of performance, and time to complete (Wilham et al. 2010). These highly sensitive tests have been used to detect PrP^{TSE} in a wide range of tissues in both experimental and naturally occurring TSE infections, in therapeutic biologicals grown in human tissue culture cell lines (Lyon et al. 2019), and in nasal swabs from patients with sporadic, familial, and variant forms of CJD, as recently reviewed in *Lancet Neurology* (Hermann et al. 2021).

Inadequate sensitivity is, therefore, no longer an issue, but validating a test for the purpose of detecting pre-symptomatic disease begs the question: whom do you test? Random testing of the general population would be impractical, given the rarity of the disease, and even a study of families with heritable forms of disease would not yield very many subjects. One such study is ongoing with as yet unpublished results. Another study took advantage of multiple stored blood samples from two blood donors who subsequently died of vCJD. Their samples tested positive starting 14 and 30 months before the onset of symptoms (Bougard et al. 2016).

We conclude by standing back to view the TSE landscape and see where we have been and where we are going. So far, the only known non-human form of TSE to have infected humans is BSE, which almost certainly represents a species-crossing disease from scrapie-infected sheep to bovines. When coupled with the practice of feeding bovines, MBM made from the carcasses of slaughtered livestock (including sheep and other animals dying of unrecognized infections), the disease spread to epidemic proportions within herds, and finally achieved sufficient frequency to

infect humans. No human infection has ever been documented as coming directly from scrapie-infected sheep tissues, even amongst knife-wielding workers in sheep slaughterhouses.

Chronic wasting disease of cervids (deer, moose, and elk) was probably also contracted from scrapie-infected sheep that may have come in contact with captive deer raised to supply a much smaller population that enjoys eating venison. The disease in wild cervids is gradually spreading around the USA and Canada, and an outbreak of disease recently occurred in herds of wild Norwegian reindeer, and in a moose in Finland, the only other known TSE of animals is transmissible mink encephalopathy, with outbreaks identified in the USA in the 1960s and sporadic outbreaks in Canada, Finland, and Russia as late as 1986. Despite the absence of human infections from any of these animal diseases, many species are susceptible to experimental TSE infections, and we should be wary of ignoring the possibility that these or other animal varieties of TSE may surface in the future.

With respect to iatrogenic disease, infections have resulted from exposure to infected blood, tissues, tissue extracts, and instruments. No infections appear to have followed any organ transplants other than corneas, possibly because other kinds of transplants are less frequently performed, the recipients are older, and their post-transplant survival times are shorter. It is also possible that the eye is more infectious than other transplanted organs. In any case, the donors and/or donations can now be screened using one of the cyclic amplification tests from which results should be available within an acceptable donor-to-recipient transplant interval. Finally, we need to appreciate the fact that the utility of a pre-clinical TSE diagnosis remains moot until some form of effective therapy becomes available. In the meantime, we will need to depend on the other two means of prevention: recognition and screening of individuals in high-risk occupations and special instrument decontamination protocols, all the while maintaining a vigilant attitude toward as yet unidentified future sources of environmental infection.

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Chapter 12
Risk of Transmission of Creutzfeldt–Jakob Disease by Blood Transfusion

Alexander H. Peden and Marcelo A. Barria

Abstract Early epidemiological studies on sporadic Creutzfeldt–Jakob disease (CJD) did not identify blood transfusion as a risk factor for the disease. However, the emergence of variant CJD (vCJD) in 1996 and the identification of PrP^{Sc} in lymphoid tissues in this novel disorder led to concerns that transmission of infectivity by blood transfusion might be a possibility. These concerns were fully realised in 2004, when the first case of vCJD associated with transmission by blood transfusion was identified in a recipient who was a methionine homozygote at codon 129 in the prion protein gene, as in all other vCJD patients at that point in time. Other similar cases have subsequently emerged, along with cases of asymptomatic vCJD infection in a blood transfusion recipient and a plasma product recipient, both of whom were heterozygous at codon 129 of the prion protein gene. This chapter reviews the experimental evidence for the transmission of prion infectivity by blood transfusion in a range of experimental models, discusses the evidence for the transmission of vCJD by blood transfusion and plasma products, and considers the future possibilities for the development and potential uses of blood-based screening tests for human prion diseases.

Keywords Blood transfusion · Creutzfeldt · Jakob disease · Prion disease transmission · Prion protein in blood · vCJD

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12.1 Introduction

Despite several decades of research in many different countries, the cause of the commonest form of human prion disease, sporadic Creutzfeldt–Jakob disease (sCJD), remains unclear. sCJD appears to have been transmitted as an iatrogenic infection following a variety of medical and surgical procedures, but evidence to support infection via blood transfusion, or blood products, appears lacking to date (Collins et al. 1999; Zerr et al. 2000; Urwin et al. 2016; Crowder et al. 2017; Seed et al. 2018; Holmqvist et al. 2020). Although there have been two recent cases of sCJD in UK plasma product recipients, these were likely to have been chance events (Urwin et al. 2017). The emergence of variant Creutzfeldt–Jakob disease (vCJD) in the UK 26 years ago and subsequent evidence for the transmission of vCJD infectivity by the transfusion of non-leucodepleted red cell concentrates from donors who were asymptomatic at the time of donation, but who subsequently died from vCJD, have focused attention on the potential for transmission of other forms of CJD by this route (Puopolo et al. 2011).

In this chapter, we review the evidence for the transmission of prions by blood transfusion in experimental models of prion disease and in sCJD and vCJD in humans, describe recent and developing methods to detect prions in blood, and discuss the prospects of a blood screening test for prions and the issues surrounding the implementation of such a test.

12.2 Experimental Evidence for Prion Disease Transmission by Blood Transfusion

12.2.1 Cellular Prion Protein in Blood

Expression of the cellular prion protein (PrP^C) is thought to be an absolute requirement for the development of prion infection. PrP^C is widely expressed in different tissues and cell types, including neurones in the central nervous system and follicular dendritic cells in lymphoreticular tissues. It is also present in blood, in which the distribution and cellular physiology of PrP^C has been intensively studied. PrP^C is present in plasma and is also found to be cell associated in human blood (MacGregor et al. 1999). Platelets contribute the greatest amount of cell-associated PrP^C to blood with lesser amounts contributed by white blood cells (WBC) and lower levels still by red blood cells (RBC) (MacGregor et al. 1999; Choi et al. 2009). The highest levels of PrP^C (on a per cell basis) in normal human blood are in specific WBC subpopulations (MacGregor et al. 1999; Durig et al. 2000; Choi et al. 2009). Platelets act as a dynamic reservoir for PrP^C in that it is stored in their α-granules, being recruited to the cell surface or released during platelet activation and storage (Perini et al. 1996; MacGregor et al. 1999; Bessos et al. 2001; Holada et al. 2002a). The activation-dependent upregulation of expression in, or release of PrP^C from

leucocytes, dendritic cells, and mast cells, has been interpreted as indicative of the normal cellular functions for PrP^C in blood, suggestive of a role for these cells in prion disease pathogenesis (Durig et al. 2000; Burthem et al. 2001; Lee et al. 2001; Haddon et al. 2009). In so far as PrP^C expression and function in blood might relate to prion disease pathogenesis, it should be noted that clear differences in PrP^C expression between different blood components are evident when human blood is compared with blood of species that are commonly used as models of prion disease, such as rodents and sheep (Barclay et al. 2002).

12.2.2 Animal Models

The study of human prion diseases, such as CJD, continues to be informed by analogous diseases of animals, specifically sheep scrapie and bovine spongiform encephalopathy (BSE) and the establishment of experimental animal models of those animal diseases and of the human diseases themselves. The adaptation of sheep scrapie isolates to rodents has been of fundamental significance to the field, providing a series of well-characterised meta-stable strains in both hamsters and mice, but the modelling of blood transfusion has been particularly well-served by the development of an experimental blood transfusion paradigm using the BSE agent experimentally transmitted to sheep.

12.2.3 Rodent Models

Reports of the existence of a "viraemia" associated with prion disease have a long history and quite naturally these observations raised fears of transfusion-related transmission of CJD. Guinea pig-adapted CJD, serially transmitted by intracerebral (i.c.) inoculation, was reported to have infectivity detectable throughout the incubation period in buffy coat samples, as determined by further i.c. challenge (Manuelidis et al. 1978). This finding was supported by a study using a different human prion disease, a mouse-adapted Gerstmann–Straussler–Scheinker (GSS) disease isolate, termed Fukuoka-1. When challenged with Fukuoka-1 by the i.c route, mice showed detectable infectivity in circulating whole blood from around half way through the incubation period onwards, as determined by intraperitoneal (i.p.) challenge of further susceptible mice (Kuroda et al. 1983). Direct (but limited and poorly documented) testing of blood and buffy coat specimens from CJD patients also indicated the presence of infectivity in human blood during the clinical illness when inoculated into guinea pigs and hamsters (Manuelidis et al. 1985; Deslys et al. 1994).

The further development of high titre, well-characterised rodent scrapie models has provided more consistent and reliable data. A sustained low level of infectivity was found to characterise blood throughout the incubation period in the 263K hamster scrapie model, following i.p. inoculation (Diringer 1984; Casaccia et al. 1989).

At the clinical stage, the infectivity was reported to be associated with the mononuclear leucocyte fraction and not with platelets (Holada et al. 2002b). The hamster model, inoculated with the 263K adapted-scrapie strain, has been used extensively in the development and evaluation of prion reduction filters (Gregori et al. 2004a, 2006a, b; Sowemimo-Coker et al. 2005, 2010; McLeod et al. 2015) and to investigate partitioning during plasma product manufacture (Bett et al. 2017; Lee et al. 2000; Foster et al. 2000; Li et al. 2001; Gregori et al. 2004b; Hartwell et al. 2005; Burdick et al. 2006).

Similar results to those obtained with the 263K model have also been obtained using the Fukuoka-1 mouse model. Following i.c. inoculation, blood was found to contain ~10 infectious units per ml (IU/ml) during the pre-clinical phase, rising to ~100 IU/ml during the clinical phase and largely associated with the buffy coat fraction, as measured by bioassay using the same (i.c.) route (Brown et al. 1998, 1999). Infectivity levels in plasma were found to be low and further reduced by plasma processing (Brown et al. 1998, 1999). When comparisons were made between the blood-borne infectivity levels in the Fukuoka-1 GSS model and RIII mouse-adapted vCJD, the latter was found to contain 20–30 ID/ml at both the pre-clinical and clinical phases, primarily in buffy coat and plasma, with lower levels in platelets and no infectivity detectable in RBC (Cervenakova et al. 2003). More recently, the inoculation of transgenic mouse models overexpressing the human and bovine PrP allowed the direct detection of infectivity in vCJD (and sCJD) patient blood (Douet et al. 2014). The above models all demonstrate clear proof of principle of blood-borne prion infectivity and they also provided information on infectivity levels, on which risk assessments could be based. However, direct extrapolation to blood transfusion and the risk posed by vCJD is difficult due to the possible effects of route and agent/host interaction. Consequently, the use of large animal models offers distinct advantages over rodents, where blood transfusion is concerned.

12.2.4 Primate Models

Early attempts to transmit human spongiform encephalopathy by transfusion of unit quantities of blood to chimpanzees were reported to be negative (Brown et al. 1994). Nevertheless, non-human primates experimentally infected with the BSE/vCJD agent have been used to model vCJD (Lasmezas et al. 2001, 2005; Herzog et al. 2005; Williams et al. 2007). Both brain and buffy coat from a clinically affected lemur (previously exposed by the i.c. route with BSE passaged through macaque) were found to transmit disease when inoculated i.c. into naive lemurs (Bons et al. 2002). Conversely, brain tissue from clinically affected macaques (previously exposed by the i.c. route with BSE) was shown to transmit disease when further macaques were exposed orally or intravenously (Herzog et al. 2004).

The use of a non-human primate model has identified a novel myelopathic syndrome in macaques exposed intravenously (i.v.) to blood components from vCJD-infected macaques and from a vCJD patient (Comoy et al. 2012; Lescoutra-Etchegaray

et al. 2012). More recently, it has been shown that both macaques and wild-type mice exposed to vCJD blood display a new class of neurological syndromes in the absence of detectable abnormal PrP. (Comoy et al. 2017, 2018). However, the prion aetiology of these conditions was demonstrated by the re-transmission to mice, which showed the presence of abnormal PrP. These findings underscore the need for vigilance in surveillance systems for human prion disease, for novel prionopathies or clinicopathological profiles, that may emerge as a result secondary transmission via blood.

12.2.5 Sheep Models

To date, only sheep models have relevant agents (principally BSE) been used to infect animals using the relevant route (orally, to model zoonotic transmission to humans) to produce donors of blood (at clinical and pre-clinical timepoints) that can be used to transfuse recipients using protocols that closely mimic human transfusion practice. The report of one successful transmission by intravenous administration of a unit of whole blood from a pre-clinical BSE orally exposed donor sheep to a naive recipient (Houston et al. 2000, and see an accompanying commentary by Brown 2000) was confirmed and has been fully justified by subsequent publications describing the whole study (Hunter et al. 2002; Siso et al. 2006; Houston et al. 2008; McCutcheon et al. 2011). The overall BSE transfusion transmission rate was 36% and included blood from donors throughout the second half of the (asymptomatic) incubation period, suggesting that either the titre of the infectious agent in blood is higher than anticipated or that transfusion of blood is a very efficient mode of transmission (Houston et al. 2008). Using the same experimental paradigm, components separated from orally exposed pre-clinical BSE sheep blood have shown infectivity to be present in red cell concentrates, plasma and platelet units, even when the blood has first been leucoreduced (McCutcheon et al. 2011). Furthermore, RBC from a BSE-infected donor that had been leucoreduced and treated with a prion reduction filter (P-CAPT) were still shown to harbour infectivity (McCutcheon et al. 2015).

Using the above sheep model, it has also recently been shown that transmission risk is influenced by the *PRNP* genotype and the route of infection. Animals infected with BSE via blood transfusion showed infectivity in their blood sooner after inoculation than animals infected via the oral route (Salamat et al. 2021). Importantly, this study also confirmed that all fractionated blood components were capable of transmitting prion disease, even after leucodepletion. Although many recipient sheep survive for years post-transfusion and were asymptomatic, analysis of their tissues using a highly sensitive amplification method (see sec. 12.5.3) showed low levels of prions in the lymph nodes and brain, but not blood of these animals, indicating that subclinical infection following exposure via this route is a frequent occurrence (Salamat et al. 2022).

Interestingly, efficient transfusion transmission is not a property restricted to the BSE agent. Similar transmission rates (43%) were seen in experiments conducted

using clinical and pre-clinical sheep scrapie (Houston et al. 2008). The neuropathological phenotype of experimental ovine BSE is largely unaffected by route (Siso et al. 2006), whereas that of scrapie appears to differ between natural infection and transfusion transmission (Siso et al. 2009). The efficiency of transfusion mediated transmission has been further explored using the same sheep scrapie model in parallel with a susceptible (ovinised) transgenic mouse model (Andreoletti et al. 2012; Lacroux et al. 2012a, b). In these studies, the transfusion-mediated transmission rates in sheep approach 100%. The results using this model system demonstrate a marked discrepancy between prion titres in sheep blood as defined by i.c. challenge of ovinised transgenic mice and the efficiency of disease transmission following intravenous transfusion of viable cells between sheep. This may not be surprising from a biological perspective, but it does provide an important caveat for calculations previously based on blood infectivity measurements obtained by i.c. inoculation of rodents (Andreoletti et al. 2012).

The above sheep transfusion model has been used to examine the specific role of leucocytes in blood transmission of prion disease. Leucocytes efficiently transmit scrapie via the intravenous route, despite an apparent low infectious titre (Lacroux et al. 2012a, b). Furthermore, mononucleated blood cell populations display different abilities to transmit prion disease via the transfusion route: cell-sorted subpopulations enriched in B and T lymphocytes appeared to be more efficient at transmitting scrapie to recipient animals than fractions enriched for monocytes and macrophages (Douet et al. 2016). This information may enable technologies for blood leucoreduction to be refined to further reduce the risk of prion disease transmission.

Each of the above rodent, primate, and sheep experimental systems is at one or more removes from the events that they seek to model, namely, human–human transmission. Not all of the evidence accumulated to date, such as the kinetics of accumulation or cell types involved, is entirely consistent. Titre is a key case in point. Rodent studies have previously supported an estimate of 10 IU/ml of blood, whereas this was revised downwards to less than 1 IU/unit of blood (~400 ml) based on examination of existing ovine and human data (Gregori et al. 2011). However, when taken together, three conclusions can be drawn: first that low levels of infectivity in blood occur during the pre-clinical phase in these acquired prion diseases. Second, that some of this infectivity is cell associated, and third, that intravenous delivery, especially the transfusion of fresh blood and its components is an efficient mode of prion disease transmission.

12.3 Evidence for vCJD Transmission by Blood Transfusion and Plasma

12.3.1 Secondary Transmission of vCJD by Blood Transfusion

There have been four known cases of vCJD in recipients of blood components from asymptomatic donors who subsequently developed vCJD (Llewelyn et al. 2004; Peden et al. 2004; Wroe et al. 2006; Health Protection Agency 2007) and a fifth case

in which only circumstantial evidence implicates blood transfusion as the cause (Chohan et al. 2010). These individuals were all members of a cohort identified by the transfusion medicine epidemiology review, a collaboration of the National CJD Research & Surveillance Unit (NCJDRSU), and the UK Blood Services (Hewitt et al. 2006; Urwin et al. 2016). Figure 12.1 summarises information on the time of the relevant transfusions and the deaths or onsets of vCJD in both the donors and the recipients. In all four cases, secondary vCJD infection in the recipient appears to have resulted from the transfusion of a single unit of non-leucodepleted red cells from a pre-clinical vCJD donor. These transfusions occurred prior to the phasing in of leucodepletion of all blood for transfusion in the UK during 1998–1999. To date, leucodepletion of blood appears to have been an effective means of preventing the secondary transmission of vCJD in humans, despite the experimental evidence that it does not completely remove infectivity (Douet et al. 2015).

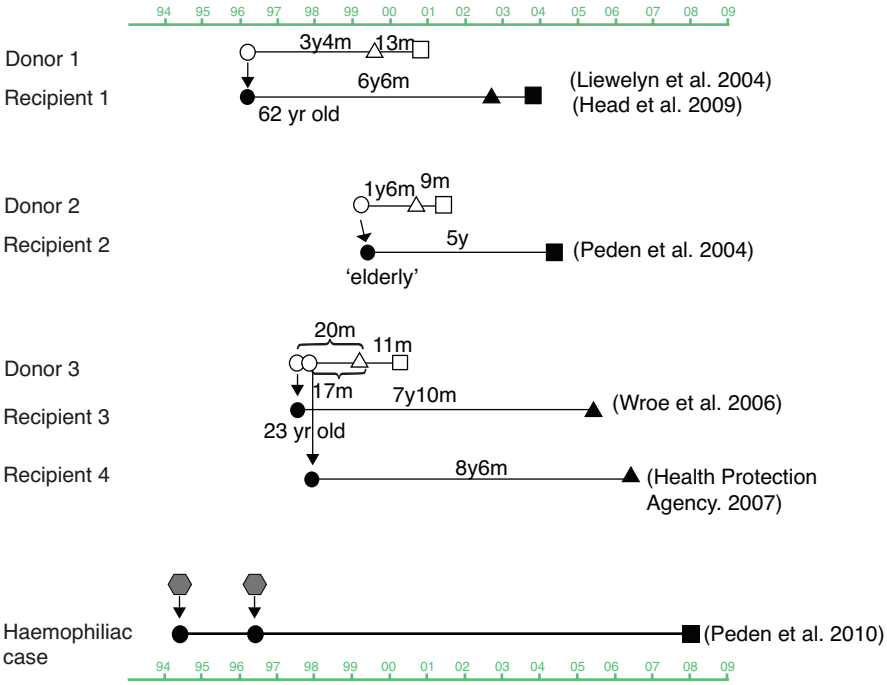

Fig. 12.1 Time lines for the donors and recipients of blood in the four known cases of blood transfusion-associated vCJD infection. The interval between donation/transfusion (*circles*) and death (*squares*) or vCJD disease onset (*triangles*) is represented by *lines* drawn to a scale indicated at the *top* and *bottom* of the figure. The donations/transfusions are indicated by *open symbols* for the blood donors and *filled symbols* for the recipients. The recipients' ages at the time of transfusion (where published) are shown. The asymptomatic haemophiliac patient that showed evidence of vCJD infection in the spleen had been a recipient of two identified vCJD-implicated batches of Factor VIII (*indicated by hexagons*). Key references are shown on the *right*; the data are also reviewed in Hewitt et al. (2006). Recipient 2 and the haemophiliac patient died of non-neurological disorders and recipients 3 and 4 shared a common donor

The clinical reports of recipients 1, 3, and 4 were typical for vCJD and genotype analysis showed that they were all methionine homozygous (M/M) at codon 129 of the prion protein gene *PRNP*. All neuropathologically confirmed clinical cases of vCJD to date have also been homozygous for methionine, apart from one case reported in a methionine/valine (M/V) individual (Mok et al. 2017). The neuropathological findings for recipients 1 and 3 were typical for vCJD (Head et al. 2009; Wroe et al. 2006) (Fig. 12.2a–d). In both of these recipients, western blotting (WB) analysis of brain homogenate following treatment with proteinase K revealed the

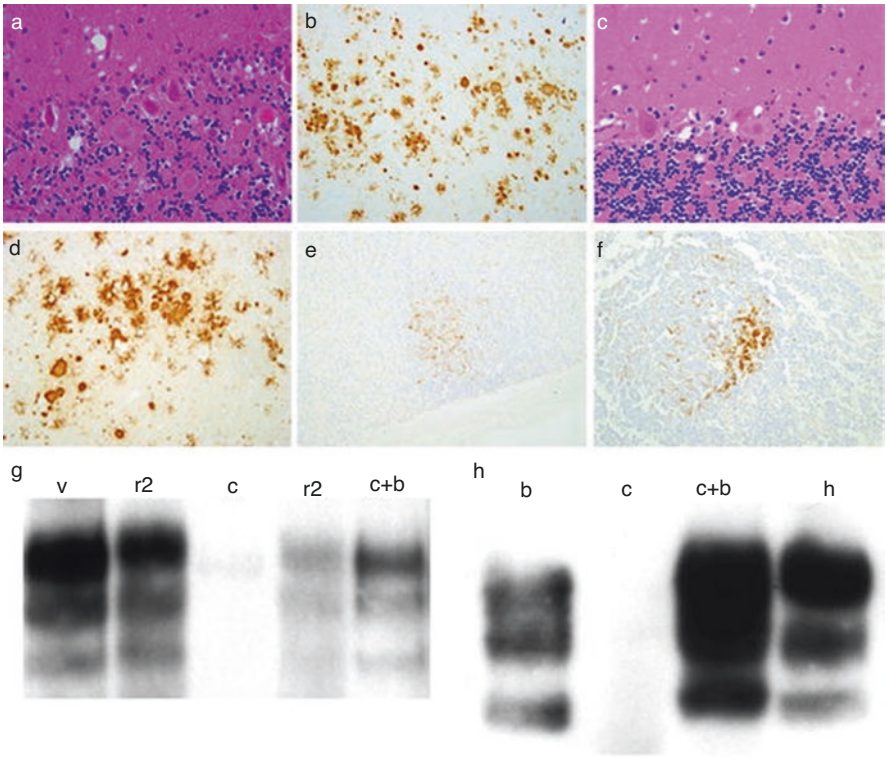

Fig. 12.2 Pathology and biochemistry of autopsy tissues from symptomatic and asymptomatic vCJD-infected individuals following blood transfusion and plasma product administration. Haematoxylin- and eosin-stained sections of the cerebellum from blood donor 1 (**a**) and the corresponding blood transfusion recipient 1 (**c**) show spongiform change and florid plaques. Corresponding immunohistochemistry for PrP in sections of cerebral cortex (**b** for donor 1 and **d** for recipient 1) shows florid plaques, cluster plaques, and other deposits of disease-associated PrP. **e** and **f** show PrP-labelling of germinal centres of the spleen (**e**) and the cervical lymph node (**f**) from the asymptomatic blood transfusion recipient (recipient 2). Panels **g** and **h** show the presence of protease-resistant PrP by NaPTA/WB analysis in spleen from blood transfusion recipient 2 (marked r2) and the case of asymptomatic vCJD infection in a plasma product recipient with haemophilia (marked h). These samples have been run alongside spleen from a clinical case of vCJD ('v'), non-CJD control spleen ('c'), and vCJD brain homogenate alone ('b') or spiked into control spleen ('c + b') for comparison

presence of disease-associated protease-resistant PrP (PrP^{res}) with a banding pattern of type 2B characteristic of vCJD.

Mice inoculated with cerebral frontal cortex samples from recipient 1 became infected with incubation times and brain lesion profiles that were consistent with previous transmissions of vCJD to mice of the same lines, suggesting that there had been no alteration of agent strain (Bishop et al. 2008). Therefore, in *PRNP* codon 129M/M individuals, the strain properties and clinicopathological features of secondary vCJD following blood transfusion are currently indistinguishable from those in patients with vCJD resulting from exposure to BSE.

The second case of blood transfusion-associated vCJD infection differed from the other three in that the transfused recipient (recipient 2) died 5 years after transfusion from a non-neurological disorder and was M/V heterozygous at *PRNP* codon 129 (Fig. 12.1) (Peden et al. 2004). Evidence for vCJD infection in this recipient was obtained when autopsy tissues were examined for the presence of PrP^{Sc} by sodium phosphotungstate precipitation/WB (NaPTA/WB) (Fig. 12.2g), paraffin-embedded tissue (PET) blotting, and immunohistochemistry (IHC). PrP^{res} was found to be restricted to the spleen (NaPTA/WB, PET, and IHC) and a cervical lymph node (IHC). The PrP^{res}-banding pattern in spleen was type 2B. No pathological signs of vCJD were detected in the central nervous system. Recipient 2 thus provided the first evidence that *PRNP* codon 129 M/V individuals might be either susceptible to vCJD or capable of incubating this disease.

Bioassay using wild-type mice confirmed the presence of vCJD infectivity in the spleen of recipient 2 (Bishop et al. 2013). Similar transmission properties were observed for mice challenged with spleen from recipient 2 and corresponding donor, suggesting conservation of strain following propagation in *PRNP* codon 129 M/M and M/V individuals. In agreement with this, conservation of vCJD strain was also observed for the single clinically and pathologically confirmed case of vCJD in a *PRNP* codon 129 M/V individual (Mok et al. 2017; Boyle et al. 2020). Recently, the use of bovine and human transgenic mouse models for bioassay (tgBov-tg110 and tgMet-tg340, respectively) has shown infectivity in additional tissues from recipient 2, including thymus, lung, heart, and pancreas (Douet et al. 2021).

PrP^{res} was not detected in tonsil tissue from recipient 2 (Peden et al. 2004). This finding highlights a potential caveat in the use of resected tonsil for estimating the prevalence of vCJD in the population and the use of tonsil biopsies for the pre-mortem diagnosis of secondary vCJD. Interestingly, PrP^{res} was detected in tonsil tissue taken at autopsy from recipient 3, but a pre-mortem tonsil biopsy had not been performed on this recipient (Wroe et al. 2006).

12.3.2 Evidence for vCJD Transmission by Plasma Products

There has been one case of vCJD infection detected at autopsy in a patient who had been treated with large doses of UK-produced Factor VIII (Peden et al. 2010). The patient was a haemophiliac, who died of a non-neurological disorder in 2008, aged

73. This patient was heterozygous (M/V) at *PRNP* codon 129. PrP^{res} was detected by NaPTA/WB in only one sample of spleen with a banding pattern of type 2B (Fig. 12.2h). All other tissues tested from this patient, including brain and tonsil, were negative.

This case of vCJD infection was identified through a United Kingdom (UK) Department of Health funded study to undertake active surveillance of haemophiliac patients for vCJD infection. All haemophiliacs undergoing surgery on tissues from the central nervous system and lymphoid tissues were invited to participate and give consent for analysis of tissue samples at NCJDRSU for PrP^{res}. In addition, consent was sought for the analysis of samples from autopsy tissues from relatives of patients who died during this study. A variable range of biopsy and autopsy specimens from 17 patients have been analysed by NaPTA/WB, PET, and IHC. All tissues tested negative for PrP^{res} apart from one spleen sample from the patient described above (Peden et al. 2010).

A number of possibilities have been considered to explain how this haemophiliac patient became infected with vCJD. Prior to 1998 in the UK, blood products such as Factor VIII and Factor IX were manufactured from blood plasma sourced in the UK. Units of blood from asymptomatic donors, who went onto develop vCJD, contributed to pooled plasma for the manufacture of batches of clotting factor concentrates (Hewitt et al. 2006). The patient described above had been treated with two of these "vCJD-implicated" batches of Factor VIII, totalling 9025 units, in 1994 and 1996. However, this person's medical history also included treatment with approximately 400,000 units of non-implicated Factor VIII between 1980 and 2001, four blood transfusions, and multiple endoscopic procedures. An assessment of all risk factors, including dietary exposure to BSE, concluded the most likely route of exposure for this patient was non-implicated batches of Factor VIII (Bennett and Ball 2009). This conclusion was based on (1) the large number of units of Factor VIII received by this patient, (2) an estimated prevalence of vCJD in the UK population of 1/10,000 (Spongiform Encephalopathy Advisory Committee 2008), and (3) the routine pooling of around 20,000 units of plasma to make a single batch of clotting factor concentrate (Clarke and Ghani 2005; Clewley et al. 2009; Hilton et al. 2004).

In response to the risk of vCJD transmission presented by pooled plasma from the UK donors who had potential been exposed to BSE, in 1999, UK authorities decide to import plasma for use in the manufacture of plasma products, and from 2004 onwards, imported fresh frozen plasma was used on UK patients born after 1995 (i.e., after the peak period of exposure to BSE). In 2019, a report was published that assessed the implications of resuming the use of UK-source plasma on patients, given the pressures on the supply of imported plasma. The report concluded that the measures should be withdrawn, on the basis of a reduced estimate of the number of future vCJD cases, and the positive effect that lifting the restrictions would have on operational difficulties and costs (SaBTO Advisory Committee on the Safety of Blood, Tissues and Organs 2019; Thomas et al. 2021).

Following on from this, a report by the UK Medicines and Healthcare products Regulatory Agency recommended also lifting the ban on use of UK plasma to generate medicinal products (Medicines and Healthcare products Regulatory Agency

2021; UK Department of Health and Social Care 2021). This report took into account the previous findings of SaBTO and the prion reduction effect of plasma processing methodologies. Indeed, simulations of the manufacturing process for blood products, such as immunoglobulins and Factor VIII, using intermediates spiked with scrapie brain extract, showed that many of the process steps produce a significant reduction in PrP^{Sc} (Roberts et al. 2013).

The level of increased risk associated with relaxation of these measures was considered acceptable when weighed against the benefits in terms of easing operational challenges and reducing considerable costs. The lifting of restrictions on fresh frozen plasma was predicted to result in only one additional clinical case of vCJD per 5.2 million doses administered (SaBTO Advisory Committee on the Safety of Blood, Tissues and Organs 2019; Thomas et al. 2021). The recommendations came with the proviso that all other risk reduction measures, including leucodepletion of blood donations, should remain. A consultation with stakeholders also recommended continuing, or enhancing, surveillance for vCJD in the UK population, and the post-mortem examination of suspected CJD cases (Medicines and Healthcare products Regulatory Agency 2021). It should be noted that a voluntary surveillance study focussed on UK immunodeficiency patients that received immunoglobulin derived from UK human plasma donations is ongoing. So far, this study has shown no evidence of symptomatic or asymptomatic vCJD infection in patients exposed to vCJD-implicated batches of immunoglobulin between 1997 and 2000 (Helbert et al. 2016).

12.4 Evidence for sCJD Transmission by Blood Transfusion and Plasma

Concerns were raised that CJD might be transmissible by blood, and blood products, long before the emergence of vCJD and reports of vCJD being transmitted via blood transfusion. A number of epidemiological studies have been conducted in several countries to address the concern that sCJD might be transmissible via this route. These include case–control studies, where CJD patient cohorts were compared with matched control groups to see if a history of receiving blood transfusions is a risk factor. Alternatively, follow-up studies have been conducted to see if two or more cases are linked via blood transfusion. Nearly, all case–control studies have shown no causal link between sCJD and blood transfusion (van Duijn et al. 1998; Collins et al. 1999; Zerr et al. 2000; Ward et al. 2008). An exception is an Italian study comparing sCJD patients with a control group with alternative diagnoses, showing a significant association between receiving a blood transfusion more that 10 years before clinical onset, and a diagnosis of sCJD (Puopolo et al. 2011). However, this association was not seen in case–control review of UK sCJD patients (Molesworth et al. 2011).

In all retrospective studies conducted to date, no patient with sCJD has been found to have received blood from a donor who went on to develop sCJD. An update

from the UK Transfusion Medicine Epidemiological Review identified no cases of sCJD in 211 recipients of blood from 29 confirmed CJD blood donors, although 5 of recipients had dementia (Urwin et al. 2016). A US study, spanning data collected for 21 years, showed no evidence of sCJD in 826 blood recipients from 65 donors who subsequently developed CJD (Crowder et al. 2017). A Swedish/Danish study, covering more than 50 years, showed no evidence of sCJD in 883 patients receiving blood from 39 donors, who subsequently developed sCJD, and no evidence for clusters of sCJD patients receiving blood from single donors (Holmqvist et al. 2020). Collectively, these studies suggest that the rates of sCJD transmission via blood are extremely low or non-existent.

The theoretical possibility of sCJD transmission via blood and plasma products has also been a longstanding concern. However, the occurrence of sCJD in two recipients of UK plasma products may simply have been chance events, given the large population under surveillance for CJD in the UK and Europe, and no causal link for these cases could be established (Urwin et al. 2017).

12.5 Methods to Detect Prions in Blood and the Prospect of Implementation of a Blood Screening Test for vCJD

12.5.1 The Challenge

The development of a workable blood screening test for vCJD faces a series of formidable obstacles. Some of these are biochemical in nature: if prions are equated with abnormal forms of the prion protein (PrP^{Sc}), then a prospective blood test must be able to detect extremely low levels of PrP^{Sc} in the analyte (whole blood, plasma, or buffy coat), in which the normal precursor protein, PrP^{C}, is more abundant by orders of magnitude. The property of PrP^{Sc} being measured must be unique to the disease-associated or infected state. Whilst brain PrP^{C} and PrP^{Sc} are well-characterised, both PrP^{C} and PrP^{Sc} are now recognised as being biochemically heterogeneous with protease-resistant forms of PrP^{C} being found in normal brain and protease-sensitive forms of PrP^{Sc} being found in CJD brain (Safar et al. 2005; Yuan et al. 2006). Moreover, the exact biochemical form of PrP^{Sc} in blood is unknown. This may result in a practical problem for test development, in that an assay developed with, and optimised for brain PrP^{Sc}, even if spiked into blood or plasma at high dilution, may not be applicable for the detection of endogenous blood PrP^{Sc}. Blood from analogous animal diseases or animal models may, therefore, appear an attractive option, especially since blood from pre-clinical stages can be taken to mimic screening for asymptomatic vCJD infection, but translation may be complicated by differences in the prion strain and host species involved. Given all of these difficulties, a framework for CJD blood test evaluation has been developed by the UK National Institute for Biological Standards and Control (NIBSC).

Implementation presents a further series of challenges: the actual prevalence of vCJD infection in the UK population can only be estimated with very wide confidence intervals (Hilton et al. 2004; Clewley et al. 2009; de Marco et al. 2010; Garske and Ghaini 2010), but the most recent prevalence estimate is of 1:2000 based on retrospective screening of archived appendix specimens in England (Gill et al. 2013; Gill et al. 2020). A routine blood screening test with an exceptionally high specificity, if applied routinely to all blood donations, would still generate significant numbers of false positives (Turner 2006; Ludlam and Turner 2006; Peden et al. 2008). One way to mitigate the effects of these unavoidable false positive screening test results (for donors and for the transfusion services alike) would be to implement a second (confirmatory) assay in parallel with a screening assay. Therefore, two assays are actually being sought. Ideally, the screening assay and confirmatory test would work by different principles, and only one (the screening assay) would need to be high throughput and rapid.

12.5.2 Approaches to Sensitive Detection of PrP^{Sc}

A wide variety of approaches have been taken to the development of blood tests for vCJD (Peden et al. 2008; Knight 2020) and a detailed description is beyond the scope of this chapter. In general, they involve a step that distinguishes PrP^{C} and PrP^{Sc}, followed by a sensitive end detection method. The scientific and commercial interest in developing a test has waned, and an unnamed candidate assay for the diagnosis of vCJD, which progressed to the stage of being evaluated using a protocol set up by the NIBSC failed to meet the criteria necessary for further development (Cooper et al. 2013). Nevertheless, recent technological advancements have enabled the specific detection of PrP^{Sc} (or the potential to seed the amplification of misfolded PrP) in blood from vCJD at clinical and preclinical stages of the disease.

12.5.3 PrP^{Sc} Amplification and Current Blood Test Development

Prion disease pathogenesis is thought to depend on the autocatalytic conversion of PrP^{C} by PrP^{Sc}. Using an in vitro cell-free system model, this process could effectively amplify PrP^{Sc} from sub-detectable levels to levels readily detectable by conventional means. Capitalising on earlier work by Byron Caughey and co-workers (Kocisko et al. 1994; Caughey et al. 1999), Claudio Soto and colleagues developed a method termed protein misfolding cyclic amplification (PMCA) in which a "seed" of PrP^{Sc} promotes the conversion of PrP^{C} "substrate" supplied by an appropriate (usually brain) tissue homogenate. Accelerated by cycles of sonication and incubation, the amplified PrP^{Sc} product is then detected by protease digestion and WB (Saborio et al. 2001). The sensitivity of detection can be further enhanced by using

the product from one PMCA reaction to seed further rounds in a process termed serial PMCA or sPMCA (Bieschke et al. 2004; Castilla et al. 2005). Working with experimental rodent models, serial PMCA has been able to distinguish between bloods from infected and uninfected animals at the clinical phase (Castilla et al. 2005) and during the asymptomatic pre-clinical phase (Tattum et al. 2010; Saa et al. 2006). This general PMCA methodology has been adopted by numerous researchers and has been further developed towards basic science (Deleault et al. 2007; Peden et al. 2021; Moda 2017), medical (Erana et al. 2020; Jones et al. 2007; Saa and Cervenakova 2015), and veterinary (Thorne and Terry 2008; Davenport et al. 2018) applications. Blood or plasma appears to require the introduction of additional preparative steps in part to avoid inhibition of the amplification reaction by plasma constituents (Bougard et al. 2016; Lacroux et al. 2014; Concha-Marambio et al. 2016; Concha-Marambio et al. 2020; Castilla et al. 2005; Saa et al. 2006; Thorne and Terry 2008).

Recently, at least three approaches have enabled the detection of human PrP^{Sc} in vCJD blood by serial PMCA. One relied on the identification of a novel substrate, ovine Q171 PrP, expressed in transgenic mice brains, that was found to provide efficient amplification of human PrP^{Sc}, despite the lack of sequence homology between seed an substrate. This enabled the detection of PrP^{Sc} in blood from 3/4 vCJD patients and from primate and sheep models of the disease at preclinical stages (Lacroux et al. 2014). The other two approaches both use human PrP (M/M at *PRNP* codon 129) expressed in the brains of transgenic mice. One relies on concentration of PrP^{Sc} and the removal of inhibitory components by ultracentrifugation of samples of whole blood and incubating the sample with sarkosyl; this method could detect PrP^{Sc} in 14 cases of vCJD with 100% sensitivity and 100% specificity against a large panel of control samples (Concha-Marambio et al. 2016). The other approach utilises plasmigen-coated magnetic beads to concentrate PrP^{Sc} from blood plasma (Bougard et al. 2016); this technique was able to detect PrP^{Sc} in archived blood collected from patients at the preclinical stage of vCJD, and was also use to assist the diagnosis of a vCJD patient who had probably acquired the disease as a result of a potential occupational exposure (Brandel et al. 2020).

Therefore, the technology now exists for detecting PrP^{Sc} in vCJD blood. However, the serial rounds required make the process lengthy, and the specialised transgenic mouse brain sources of PrP^{C}, and other technical factors, are elements to consider prior to standardising this method for general diagnostic purposes.

Another amplification method termed QuIC has been described in which recombinant PrP replaces natural PrP^{C} substrates, periodic shaking replaces sonication, and in the real-time variant, RT-QuIC, amyloid formation is monitored in real time by thioflavin T fluorescence (Atarashi et al. 2008, 2011, Erana et al. 2020). RT-QuIC is currently being used to assist clinical diagnostic using cerebrospinal fluid (CSF) from suspected cases of sCJD (Green 2019; Orru et al. 2020), but the protocols used are inefficient for the detection prions in vCJD brain and CSF samples (Peden et al. 2012; McGuire et al. 2012).

Problems, both with the relatively inefficient detection of vCJD prions and with inhibitors of RT-QuIC in plasma, were addressed with a further modification of the

methodology (termed e-QuIC) (Orru et al. 2011). e-QuIC was reported to be able to detect a 10^{14}-fold dilution of vCJD brain, making it extremely sensitive as judged by the limit of detection (LoD) of human CJD brain. However, unlike standard RT-QuIC, this method has not yet been successfully transferred to multiple laboratories, or tested on clinical vCJD CSF or blood specimens and relevant controls. Other approaches to overcoming plasma inhibitors of RT-QuIC have since been reported (Elder et al. 2013). However, in its current forms, RT-QuIC is not yet applicable as a test for PrP^{Sc} in blood.

A prospective blood test with a somewhat higher LoD than the one reported for e-QuIC has been tested using whole blood from clinical vCJD patients (n = 21) against 142 blood specimens from donors (n = 100) and neurological controls (n = 42) giving sensitivity and specificities of 71.4% and 100%, respectively (Edgeworth et al. 2011). The novelty and biochemical point of interest of this assay is the use of stainless steel particles to concentrate, modify, or present PrP in advance of a sensitive immunoassay. The 100% specificity of this assay was confirmed on a cohort of US blood donors (n = 5000) presumed to be unexposed to BSE, and 200 healthy UK donors, although two cases of sCJD tested positive from a group of patients for whom prion disease was likely (n = 105) (Jackson et al. 2014a, b). No samples from non-prion disease neurodegenerative disease patients (n = 352) tested positive, indicating no problems with cross-reactivity. Although there was no improvement on the previously reported sensitivity of 70% for vCJD patients, the authors concluded the good sensitivity and optimal specificity of the assay would justify its use as a screening assay for assessing vCJD prevalence in the UK population, and for diagnosing patients (Jackson et al. 2014b). Furthermore, this assay was cable of detecting infectivity in blood at a preclinical stage in a mouse model of prion disease (Sawyer et al. 2015). However, no further progress on this assay has been reported to date.

12.5.4 Future Perspectives

The above assays all have some steps to go before they could be considered validated as vCJD blood screening tests. Moreover, none of these tests currently meet the assay time requirements demanded by blood donation testing. However, PMCA, e-QuIC, and the assay developed by Jackson and Edgeworth (Orru et al. 2011; Jackson et al. 2014a, b) appear to be promising candidates. The high analytical sensitivity achieved using the serial format of PMCA makes this technology a prime candidate for development as a confirmatory blood test (Erana et al. 2020; Peden et al. 2021; Ritchie et al. 2021). Recently, other blood biomarkers, e.g., neurofilament light chain NfL, have been explored for their diagnostic and prognostic value in prion diseases (Zerr et al. 2021), but they lack the essential high specificity for vCJD that would be required for a screening test. However, they may have future use in the diagnostic pathway for patients suspected of having prion disease.

12.6 Conclusion

The emergence of vCJD has had a major impact on blood transfusion in the UK and other affected countries. It is greatly to the credit of the UK transfusion services that several precautionary measures to protect the blood supply were put into place even before the first cases of transfusion-associated vCJD were identified. The measures that were taken to reduce the risks of vCJD transmission by blood and blood products in the UK, and some recent relaxations of these measures, are summarised in Table 12.1. The use of "prion filters" has been investigated as a further precautionary measure, but this has been not been adopted. The most recent data from sheep models indicate that whilst leucodepletion alone does not prevent disease transmission completely, it does have a pronounced effect (Douet et al. 2015; McCutcheon et al. 2011, 2015; Salamat et al. 2021) and that it is the leucoreduction component of combined leucodepletion/prion reduction filters that is responsible the prion removal (Lacroux et al. 2012b). The cases of transfusion-associated vCJD infection all occurred prior to the full introduction of leucodepletion in the UK. However, all blood components should be considered as potential vectors for prion transmission (Salamat et al. 2021).

The lifting of the requirement in the UK to use imported plasma for transfusion and as a substrate for the manufacture of therapeutic products (Table 12.1) was prompted by challenges in the supply of plasma and other operational difficulties. The decision was also based on a reduction in the estimates of the future number of cases of vCJD (SaBTO Advisory Committee on the Safety of Blood, Tissues and

Table 12.1 Measures to reduce the risk of transmission of vCJD taken by UK blood services

Withdrawal and recall of blood components, plasma derivatives, cells or tissues obtained from any individual who later develops variant CJD (1997).
Importation of plasma from countries other than the UK for fractionation to manufacture plasma derivatives (1999). Reversed in 2021 (Medicines and Healthcare products Regulatory Agency 2021; UK Department of Health and Social Care 2021)
Leucodepletion of all blood components (1999).
Importation of clinical fresh frozen plasma for patients born after January 1996 (2004). Reversed in 2019 (SaBTO Advisory Committee on the Safety of Blood, Tissues and Organs 2019; Thomas et al. 2021).
Requirement for patients born on or after 1st January 1996 to receive apheresis (single-donor) platelets whenever possible, Reversed in 2019 (SaBTO Advisory Committee on the Safety of Blood, Tissues and Organs 2019; Thomas et al. 2021).
Exclusion of whole blood and apheresis donors who may have received a blood component transfusion in the UK since 1980, any donors who have been treated with UK plasma derived intravenous immunoglobulin or have undergone plasma exchange (2004). Extended in November 2005 to transfusions anywhere in the world.
Exclusion of blood donors whose blood has been transfused to recipients who later developed vCJD, where blood transfusion cannot be excluded as a source of the vCJD infection and where no infected donor has been identified (2005).
Promotion of appropriate use of blood and tissue products and alternatives throughout the NHS.

Organs 2019; Thomas et al. 2021). Mathematical modelling predicted that lifting the restrictions over fresh frozen plasma might result in only one or two additional deaths from vCJD over the next 50 years, and a comparably low increased risk was determined for reintroducing the use of UK plasma for the manufacture of immunoglobulins (Medicines and Healthcare products Regulatory Agency 2021). The reports cautioned that all other control measures should remain. These changes, combined with uncertainties over the prevalence of asymptomatic vCJD infection in the UK, and the lack of an available blood test, underline the importance of continuing surveillance for vCJD to monitor any future cases in recipients of blood components and blood-derived products. In light of these changes, European countries that might consider importing UK-sourced plasma have been advised to conduct their own risk–benefit analyses (European Centre for Disease Prevention and Control 2021)

The evidence for transmission via blood of forms of CJD other than vCJD is far less clear-cut. A contention that blood transfusion may be a risk factor for sCJD (Puopolo et al. 2011) renewed interest in this field. Data from experimental models indicate that different strains of prions can be transmitted by blood, and that sCJD blood is potentially infectious (Douet et al. 2014). However, the overwhelming epidemiological evidence in humans suggests that blood transfusion, or human-derived blood products, is not risk factors for sCJD. Information continues to be uncovered on the widespread distribution of prion infectivity in peripheral tissues of vCJD patients, (and possibly sCJD patients) (Douet et al. 2021), and the attendant risk of secondary transmission via blood transfusion. These factors, and the very long incubation periods, justify continued surveillance and analysis of risk factors for all forms of human prion disease.

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Chapter 13
Species Barriers in Prion Disease

Suzette A. Priola

Abstract Species barriers in prion diseases are defined by the difficulty that prions from one species have in triggering prion infection in a new species. The fact that bovine spongiform encephalopathy has successfully crossed species barriers to cause disease in human and concerns that chronic wasting disease in deer and elk has the potential to do the same makes understanding the mechanisms underlying species barriers to prion infection critical. The amino acid sequence of the normal host prion protein (PrP^{C}), the conformational diversity of the abnormal and infectious prion protein (PrP^{Sc}), the conformational compatibility between exogenous PrP^{Sc} and the endogenous host PrP^{C}, and the ability to establish a subclinical infection are all important determinants of prion species barriers. However, the potential for host factors and post-translational modifications to PrP^{C} to influence species barriers, and the fact that the critical amino acid residues influencing these barriers differ between species, makes it difficult to predict prion species barriers based upon PrP^{C} sequence alone. Although the recent publication of high-resolution structural information for PrP^{Sc} will be helpful, in vivo or in vitro experiments in relevant models of infection remain the best way to determine species barriers to prion infection.

Keywords Prion · Transmissible spongiform encephalopathy · Species barriers · Prion protein · Scrapie · PrP · TSE · CJD

Abbreviations

PrP	Prion protein
Prnp	Prion protein gene
PrP^{C}	PrP cellular
PrP^{Sc}	PrP scrapie

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TSE	transmissible spongiform encephalopathy
CJD	Creutzfeldt–Jakob disease
sCJD	sporadic Creutzfeldt–Jakob disease
vCJD	variant Creutzfeldt–Jakob disease
TME	transmissible mink encephalopathy
BSE	bovine spongiform encephalopathy
CWD	chronic wasting disease
MBM	meat and bone meal
CNS	central nervous system
Sinc	scrapie incubation time gene
GPI	glycophosphatidyl-inositol
EM	electron microscopy
NMR	nuclear magnetic resonance
PIRIBS	parallel in-register intermolecular β-sheet
NAPA	nonadaptive prion amplification

13.1 Introduction

Prion diseases, also known as transmissible spongiform encephalopathies or TSEs, can be transmitted both within and across species. Intraspecies transmission of prion disease occurs naturally but with variable efficiency. Sheep scrapie, which can be transmitted both vertically and horizontally via placental tissue (Race et al. 1998; Tuo et al. 2001, 2002), can spread to 30–40% of the flock (Hourrigan et al. 1979). Chronic wasting disease (CWD) in deer, where infectivity is present in several tissues (Sigurdson et al. 2001; Spraker et al. 1997, 2002), including saliva, feces, and urine (Haley et al. 2011; Mathiason et al. 2006; Tamguney et al. 2009a), is even more efficient at spreading throughout a herd with up to 100% of the deer becoming infected (Miller and Williams 2003; Sigurdson and Aguzzi 2007). By contrast, bovine spongiform encephalopathy (BSE) in cattle and sporadic Creutzfeldt–Jakob disease (sCJD) in humans, neither of which have detectable infectivity in most tissues outside of the central nervous system (CNS) (Bradley 1996; Brown et al. 1994), do not appear to spread naturally either vertically or horizontally (Brown et al. 1994; Wrathall et al. 2002). Intraspecies transmission of prion infectivity, therefore, correlates with the presence of detectable levels of infectivity in non-CNS tissues.

Regardless of which tissues are positive for infectivity, interspecies transmission of prion infectivity is much more difficult than intraspecies transmission. Species barriers in prion diseases are defined by the difficulty that prion infectivity from one species has in triggering infection in a second species. If low titers are not an issue, a prolonged incubation time upon first passage followed by decreasing disease incubation times in subsequent passages is usually considered indicative of the existence of a prion species barrier. There are no documented instances of naturally occurring prion diseases, such as sheep scrapie, CWD, or sCJD, crossing species barriers

under normal conditions. Thus, natural species barriers to prion infection appear to be very strong.

The only instance in which prion diseases are known to have crossed species barriers outside of a laboratory environment was the result of human intervention. Changes in the rendering of ruminant animal carcasses in the early 1970s allowed material infected either with sheep scrapie (Wilesmith et al. 1988) or a previously unrecognized type of BSE (Beringue et al. 2007) to be processed into meat and bone meal (MBM) which was fed back to cattle. Cattle, which were infected but not clinically ill, were then rendered into MBM and the process repeated until the emergence of clinical BSE was recognized in the late 1980s (Wilesmith et al. 1988; Wells et al. 1987). Although there were concerns at the time that exposure to BSE-contaminated materials could lead to infection of humans, the fact that exposure to sheep scrapie had never been linked to disease suggested that this was unlikely. However, in 1996, a new form of human CJD termed new variant CJD, or more simply variant CJD (vCJD), was identified in young people in the United Kingdom, and it was suggested that this might be the result of exposure to BSE-contaminated materials (Will et al. 1996). Later work confirmed that vCJD was linked both epidemiologically and biologically to exposure to BSE (Bruce et al. 1997; Collinge et al. 1996; Hill et al. 1997). Moreover, it was shown that BSE had crossed species barriers to infect domestic cats, zoo cats, and a variety of exotic ungulates following exposure to BSE-contaminated MBM (Bradley 1996).

Multiple species barriers were, therefore, broken as the result of changes to a common human agricultural process: the possible infection of cattle with sheep scrapie and the infection of humans, felines, and ungulates with BSE. The fact that BSE has successfully and unpredictably crossed species barriers to cause disease in non-ruminant species and concerns that CWD has the potential to do the same makes understanding the mechanisms underlying species barriers to prion infection critical.

13.2 Prion Protein and Prion Species Barriers

Species barriers to prion infection were initially defined based upon the experimental inoculation of different types of TSE agents, as prions were called at the time of these experiments, into multiple mammalian species, including mice, hamsters, ferrets, and mink. For example, transmissible mink encephalopathy (TME) could be transmitted to hamsters but not mice (Marsh et al. 1969). Suffolk sheep scrapie could infect both mice and mink, while Cheviot sheep scrapie could infect mice but not mink (Hanson et al. 1971). Thus, there was a species barrier between mink-derived TME and mice and between some forms of sheep scrapie and mink. Other species, such as rabbits, were found to be resistant to scrapie infection altogether (Gibbs and Gajdusek 1973). Based upon these and multiple other studies, researchers determined that species barriers to TSE infection could be influenced by at least

three different factors: (1) the range of TSE strains in the infectious inoculum; (2) the scrapie incubation time (*Sinc*) gene; and (3) the ability to establish a subclinical infection (Dickinson 1976).

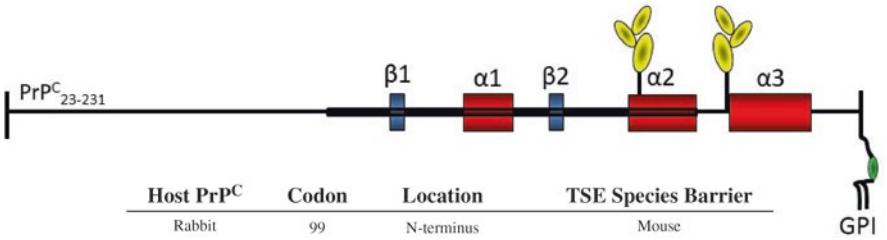
In the early 1980s, it was discovered that an aggregated and protease-resistant mammalian cell-surface glycoprotein-designated prion protein (PrP) was associated with TSE disease (Bolton et al. 1982). As a result, TSE diseases were soon renamed prion diseases and the infectious agent was designated a prion. Soon after its discovery, it was determined that PrP was a normal host protein (Basler et al. 1986; Locht et al. 1986) which was both soluble and protease-sensitive (Bendheim et al. 1988). During prion disease pathogenesis, normal PrP (termed PrP^{C} for PrP cellular) is refolded into an abnormally aggregated, protease-resistant, and infectious form known as PrP^{Sc} (for PrP scrapie) which accumulates, eventually causing disease. Comparison of PrP^{C} molecules from different mammalian species demonstrated that, while the PrP gene *Prnp* is highly conserved, the PrP^{C} amino acid sequence can vary by as much as 20% (Wopfner et al. 1999). This provided a potential molecular basis for prion species barriers: amino acid differences between the incoming infectious PrP^{Sc} and the host PrP^{C} might influence how effectively new PrP^{Sc} could be made and thus determine whether infection and disease could occur.

13.3 Role of PrP Amino Acid Sequence

13.3.1 Region of PrP Involved in Rodent Species Barriers

In order to determine whether or not the sequence of PrP^{C} was a determinant of prion species barriers, researchers took advantage of the strong species barrier to infection that exists between mice and hamsters. In this system, mice are susceptible to infection with mouse scrapie but highly resistant to infection with hamster scrapie. However, when mice were engineered to express hamster PrP^{C}, they became fully susceptible to hamster scrapie, i.e., a prion species barrier had been broken (Scott et al. 1989). Moreover, the incubation time was inversely related to hamster PrP^{C} expression: the higher the expression level of hamster PrP^{C}, the shorter the disease incubation time (Scott et al. 1989). Experiments such as these clearly showed that the amino acid sequence of the host PrP^{C} molecule was a major determinant of species barriers in prion diseases. They also provided an explanation for why earlier studies had implicated the *Sinc* gene in TSE species barriers: the *Prnp* gene and the *Sinc* gene are in fact one and the same (Moore et al. 1998).

Generation of transgenic mice expressing chimeric mouse/hamster PrP^{C} molecules further demonstrated that the major region of PrP^{C} important in the transmission of hamster scrapie to mice resides within the middle portion of the molecule from amino acid residues 108–189 (Fig. 13.1) (Scott et al. 1992, 1993). When this region was derived from hamster PrP^{C}, the mice were susceptible to hamster scrapie. However, when it was derived from mouse PrP^{C}, the mice were resistant to hamster scrapie infection (Scott et al. 1993). Mouse and hamster PrP^{C} are highly



Host PrP^C	Codon	Location	TSE Species Barrier
Rabbit	99	N-terminus	Mouse
Mouse	101	N-terminus	Human (vCJD), Sheep, Hamster
Rabbit	107	N-terminus	Mouse
Rabbit	108	N-terminus	Mouse
Human	129	β1	Bovine (BSE)
Goat	142	β1-α1 loop	Bovine (BSE)
Mouse	138	β1-α1 loop	Hamster
Hamster	155	α1-β2 loop	Mouse
Vole	154	α1-β2 loop	Bovine (BSE)
Dog	163	α1-β2 loop	Ovine (BSE)
Vole	169	β2-α2 loop	Bovine (BSE)
Sheep	171	β2-α2 loop	Bovine (BSE)
Rabbit	173	β2-α2 loop	Mouse
Ferret	179	β2-α2 loop	Mink (TME)
Rabbit	202	α3	Mouse
Rabbit	214	α3	Mouse
Ferret	224	C-terminus	Mink (TME)

Fig. 13.1 PrP amino acid residues involved in prion disease species barriers. The NMR structure of mouse PrP^C following cleavage of the signal peptide and GPI anchor addition sequence is shown (PrP$^C_{23\text{-}231}$). Glycosylation is indicated by the yellow ovals and the location of the GPI anchor at the C-terminus is indicated. The red boxes represent areas of α-helix, while the blue boxes represent areas of β-strand. Areas of disordered/loop/turn structure are represented by the thin black line, while the thicker line indicates the region of PrP^C where most of the amino acid residues important in prion species barriers reside. The table lists some of these amino acid residues using the PrP^C numbering for the corresponding host species. The structural location of each amino acid is given as is the species barrier with which it is associated. References for each residue listed are given in the main text

homologous (Wopfner et al. 1999) and there are only three amino acid differences, residues 138, 154 and 169, between the two species in the region from codon 108–189, suggesting that one or more of these residues might be contributing to the mouse–hamster prion species barrier (Kocisko et al. 1995).

13.3.2 Influence of Single Amino Acid Residues

The influence of the three amino acid residues at 138, 154, and 169 on the species-specific formation of mouse PrP^{Sc} was analyzed in vitro using mouse neuroblastoma (N2a) cells infected with mouse scrapie (Priola and Chesebro 1995). These cells

express mouse PrP^C and generate both mouse PrP^{Sc} and mouse scrapie infectivity. However, when mouse PrP^C expressing an antibody epitope tag is expressed in scrapie-infected N2a cells, its conversion to PrP^{Sc} can be measured against the background of the endogenous, wild-type mouse PrP^{Sc} which does not have the epitope tag. Thus, the influence of mutations in PrP^C on the species-specific formation of PrP^{Sc} can be studied in cell culture. In the case of the mouse–hamster prion species barrier, it was determined that a single hamster specific amino acid at residue 138 in mouse PrP^C prevented the production of mouse PrP^{Sc} in cells (Priola and Chesebro 1995). Substitution of hamster PrP^C amino acid residues at positions 154 and 169 in mouse PrP^C had no effect (Priola and Chesebro 1995). Thus, a single amino acid difference in the host PrP^C molecule was sufficient to prevent the species-specific formation of PrP^{Sc}, suggesting that prion species barriers to infection could be dependent upon relatively minor differences in sequence between the endogenous host PrP^C and exogenous PrP^{Sc} molecules.

Persistent infection of cells in vitro with some types of prions, including BSE and sCJD, can be extremely difficult and is often not successful. This limits the usefulness of cell-based systems in defining and understanding the mechanisms underlying prion species barriers for many species. Fortunately, in addition to both natural and transgenic models of prion disease, there are cell-free systems that are not restricted by prion species (Kocisko et al. 1995; Bossers et al. 1997; Castilla et al. 2005, 2008; Eiden et al. 2011; Kocisko et al. 1994; Raymond et al. 1997) that can be used to analyze the effect of differences in PrP^C sequence on the species-specific formation of PrP^{Sc}. From these studies, it is now clear that the amino acid residues important in the species-specific formation of PrP^{Sc} and transmission of prion infectivity across species barriers differ depending upon the species (Fig. 13.1). For example, species-specific formation of hamster PrP^{Sc} has been mapped to residue 155 in vitro (Priola et al. 2001) and the same residue has been implicated in species barriers in bank voles in vivo (Agrimi et al. 2008). By contrast, this residue in mice has no effect on the species-specific formation of mouse PrP^{Sc} (Priola and Chesebro 1995). Mutation of amino acid residue 101 in mouse PrP^C has been linked to species barriers to infection of mice with human, sheep, and hamster prions (Barron et al. 2001). In ferrets, resistance to TME infection is linked to residues 179 and 224 (Bartz et al. 1994). For rabbits, a species known to be highly resistant to prion infection (Gibbs and Gajdusek 1973), multiple amino acid residues appear to be important for PrP^{Sc} formation (Fig. 13.1) (Vorberg et al. 2003; Eraña et al. 2017).

Depending upon the species, resistance to BSE is associated with different amino acids in PrP^C (Fig. 13.1). In goats, amino acid residue 142, which is analogous to amino acid 138 in mouse PrP, is associated with resistance to BSE (Goldmann et al. 1996). In sheep, it is residue 171 that is associated with susceptibility to BSE (Raymond et al. 1997; Goldmann et al. 1994), while in dogs, which are highly resistant to infection with multiple species of prions, susceptibility to sheep-derived BSE is dependent upon amino acid residue 163 (Vidal et al. 2020). In humans, all clinically positive cases of vCJD have been homozygous for methionine at codon 129 (Mackay et al. 2011), suggesting that susceptibility to BSE correlates with the methionine/valine polymorphism at this residue (Raymond et al. 1997; Wadsworth

et al. 2004). When overlayed onto the structure of PrP^C, it is clear that the amino acid residues important in prion species barriers reside within different regions of PrP^C (Fig. 13.1).

13.3.3 Effect of Prnp Heterozygosity

Heterozygosity at the *Prnp* gene may also influence prion species barriers. In vivo, transgenic mice expressing both mouse and hamster PrP^C are susceptible to infection with mouse and hamster scrapie, but mouse scrapie incubation times are significantly increased when hamster PrP^C is present (Scott et al. 1989). In vitro, expression of hamster PrP^C in mouse scrapie-infected cells can completely abolish PrP^{Sc} formation (Priola et al. 1994). This phenomenon, known either as interference (Priola et al. 1994) or dominant negative inhibition (Zulianello et al. 2000), is seen when heterologous PrP^C and PrP^{Sc} molecules bind, but PrP^C is not subsequently converted to PrP^{Sc}. Interference may explain why all clinical cases of vCJD in humans are homozygous for methionine at codon 129 and why heterozygosity at codon 129 might be protective. A valine at codon 129 would block vCJD PrP^{Sc} formation from the susceptible PrP^C methionine 129 allele in a dominant-negative fashion, slowing down or preventing clinical disease. In this manner, heterozygosity at the PrP^C allele may contribute to the maintenance of prion species barriers.

13.4 Influence of PrP Post-translational Modifications

13.4.1 Glycosylation

Post-translational modifications to PrP^C also appear to impact the species-specific formation of PrP^{Sc}. PrP^C is post-translationally modified by glycosylation at two N-linked glycosylation sites as well as by the addition of a C-terminal glycophosphatidyl-inositol (GPI) membrane anchor (Caughey et al. 1989; Haraguchi et al. 1989; Stahl et al. 1987). In vitro, PrP^C glycosylation can influence PrP^{Sc} formation and the binding between heterologous PrP^C and PrP^{Sc} molecules in a species-specific manner (Priola and Lawson 2001; Burke et al. 2020), and removal of the sialic acids on the ends of the N-linked sugars can lower the barrier to cross species formation of PrP^{Sc} (Katorcha et al. 2014). At a molecular level, less efficient binding of heterologous PrP^C and PrP^{Sc} molecules could result in the production of less PrP^{Sc}, while the negative charge of the sialic acids may affect the stability of PrP^{Sc} aggregates. Finally, abrogation of the second N-linked glycosylation site in mouse PrP^C makes mice more susceptible to infection with some strains of human CJD (Wiseman et al. 2015), suggesting that the second glycosylation site helps to protect against transmission across a species barrier. Thus, PrP^C glycosylation appears to

contribute mechanistically in several ways to the maintenance of prion species barriers in vivo.

13.4.2 GPI Anchor

In vitro, the GPI anchor appeared to have little or no effect on the species-specific formation of abnormal prion protein (Priola and Lawson 2001). However, a recent study has shown that mouse prions without a GPI anchor infect transgenic mice over-expressing human PrP^{C}, which are normally resistant to infection with mouse prions, much more efficiently than mouse prions with a GPI anchor (Race et al. 2015). Anchorless PrP molecules are primarily mono- or un-glycosylated (Kocisko et al. 1994), suggesting again that complex glycosylation may be a protective factor in cross-species transmission of prions, possibly by interfering with the binding of PrP^{C} and PrP^{Sc} (Priola and Lawson 2001). In addition, removal of the GPI anchor would remove negatively charged sialic acids attached to the GPI moiety, which could potentially increase the efficiency of conversion of PrP^{C} to PrP^{Sc} (Baskakov and Katorcha 2016). Indeed, anchorless PrP^{C} is known to be converted into PrP^{Sc} more efficiently in vitro (Kocisko et al. 1994). Thus, the ability of anchorless PrP^{Sc} to cross a species barrier may be related more to its glycosylation state than to the GPI anchor itself.

13.5 Non-PrP Host Factors

There are several examples in transgenic mice where, even though the host PrP^{C} amino acid sequence is identical to the incoming PrP^{Sc} amino acid sequence, species barriers to infection are maintained. For example, transgenic mice expressing human PrP^{C} can be more resistant to infection with vCJD than wild-type mice while simultaneously being more susceptible to infection with sCJD (Hill et al. 1997; Bishop et al. 2006). Substitution of leucine for proline at position 101 in mouse PrP^{C} can modulate the susceptibility to prions from different mouse strains as well as to prions from different species (Barron et al. 2001). While prion strain-dependent differences in PrP^{Sc} conformation may account for some of these observations, these experiments suggest that host factors other than PrP might play a role in species barriers to prion infection. Consistent with this idea, in vitro studies have identified the phospholipid phosphatidylethanolamine as a cofactor in mouse PrP^{Sc} formation (Deleault et al. 2012) and RNA as a cofactor in hamster PrP^{Sc} formation (Deleault et al. 2003). However, whether these cofactors are important in the transmission of prions across species barriers in vivo remains unclear.

13.6 Prion Protein Structure and Prion Species Barriers

13.6.1 Structural Regions of PrP^C Implicated in Species Barriers

The structure of PrP^C has been determined by both NMR (Donne et al. 1997; Liu et al. 1999; Riek et al. 1996, 1997) and X-ray crystallography (Knaus et al. 2001). For all mammalian species, PrP^C has a disordered N-terminal region starting from the signal peptide cleavage site at residue 23 through to approximately reside 121 [see (Wuthrich and Riek 2001) for review]. This is followed by a folded C-terminal domain which spans residues 122–231 and is composed of two β strands that form a short region of β-sheet and three α-helices. The three α-helices and two β strands are connected by generally poorly defined regions of disordered loop/turn structure (Fig. 13.1).

The NMR structure of PrP^C can be used to provide some insight into the structural components of PrP^C which help to control species-specific formation of PrP^{Sc}. The region of PrP which has been most often implicated in controlling prion disease species barriers extends from approximately residue 100 to residue 190 and includes two of the three α-helices, both β strands and multiple regions of disordered loop/turn structure (Fig. 13.1). The N-terminal region of PrP^C encompassing residues 23–90 does not appear to be involved (Davenport et al. 2016). When amino acid residues that have been experimentally shown to have a strong influence on species-specific PrP^{Sc} formation are superimposed onto the structure of PrP^C, the majority of them reside within the disordered loop/turn regions (Fig. 13.1). This suggests that conformational variability within the loop structures of different species of PrP molecules may influence prion species barriers (Moore et al. 2009).

Polymorphisms within the loop/turn structure that connects the second β-strand to the second α-helix (β2–α2 loop) have been associated with reduced PrP^{Sc} formation and/or resistance to prion infection in sheep (Bossers et al. 1997; Eiden et al. 2011; Goldmann et al. 1994), mice (Striebel et al. 2011), and bank voles (Agrimi et al. 2008; Piening et al. 2006). In PrP^C from mice (Riek et al. 1996), sheep (Lysek et al. 2005), cattle (Lopez Garcia et al. 2000), and humans (Zahn et al. 2000), the β2–α2 loop is disordered. However, in other species such as elk (Gossert et al. 2005), hamsters (Donne et al. 1997), and bank voles (Christen et al. 2008), the β2–α2 loop adopts a well-defined structure called the rigid loop. It has been hypothesized that rigidity within the β2–α2 region may determine susceptibility to prion disease (Gossert et al. 2005), and transgenic mice expressing mouse PrP^C genetically engineered to have the rigid loop appear to be more susceptible to scrapie infection (Sigurdson et al. 2010). However, species that are highly resistant to prion infection such as rabbits (Wen et al. 2010), pigs (Lysek et al. 2005), and horses (Perez et al. 2010) also have the β2–α2 rigid loop. Furthermore, there are multiple polymorphisms outside of this region that clearly influence prion species barriers (Fig. 13.1). Thus, it is unlikely that the presence of a rigid loop structure in the β2–α2 region of PrP^C is by itself sufficient to determine species barriers to prion infection in every case.

Nevertheless, it is clear that species-specific polymorphisms which are outside of the more thermodynamically stable α-helical and β-sheet structures of PrP^{C} have a major impact on the species-specific formation of PrP^{Sc} and prion species barriers. Since detailed mechanistic and structural information on how PrP^{C} refolds into PrP^{Sc} is lacking, it is difficult to determine how these loop structures contribute to species-specific PrP^{Sc} formation. One possible explanation is that these regions have a lower free energy barrier for refolding into β-sheet structures (Rezaei et al. 2002). Another is that certain polymorphisms in PrP^{Sc} may favor the formation of β-oligomers (Sweeting et al. 2010), small ordered aggregates that are believed to be important in the conversion of PrP^{C} to PrP^{Sc}. Structural studies using small peptides derived from regions of PrP^{C} associated with species barriers, including residue 138 in mouse PrP^{C} (Priola and Chesebro 1995), have shown that single amino acid differences can lead to very different β-sheet structures (Apostol et al. 2010, 2011). Thus, a third hypothesis is that these short segments of β-sheet structure may help abnormal PrP stack to form different types of parallel or anti-parallel steric zippers, the stability of which may determine prion species barriers (Apostol et al. 2011). Support for this latter hypothesis comes from studies on the β2–α2 loop which suggest that this region may form a tightly packed steric zipper in PrP^{Sc}, the disruption of which may be important in prion species barriers (Zink 2020; Kurt et al. 2015). All of these hypotheses accommodate the idea that even minor differences in conformation between different PrP species can have outsized effects on PrP^{Sc} production and susceptibility to disease.

13.6.2 Effect of Variable PrP^{Sc} Conformation

Differences in PrP^{Sc} conformation may also help to explain the early observation that the range of prion strains in an infectious inoculum is one determinant of whether or not a prion species barrier is crossed. Conformational differences within a pool of PrP^{Sc} molecules are thought to be the basis of prion strains (Caughey et al. 1998; Safar et al. 1998), which are defined as PrP^{Sc} molecules with the same primary sequence but with different biochemical properties in vitro and different biological phenotypes in vivo [for review, see (Bruce 1996)]. If the conformation of a particular strain of PrP^{Sc} was not compatible with the conformation of the host PrP^{C} molecule then, regardless of the PrP primary sequence, a species barrier to infection would exist. This would explain why a single amino acid change in mouse PrP^{C} can control multiple species barriers and restrict infection with different mouse scrapie strains (Barron et al. 2001) and why mink are susceptible to Suffolk, but not Cheviot, sheep scrapie (Hanson et al. 1971). Thus, differences in PrP^{Sc} conformation could also influence prion species barriers, likely by modulating the effect of species-specific differences in the primary sequence of PrP^{C} (Torres et al. 2014).

It would be very informative to have structural information from multiple prion strains and species of PrP^{Sc} to better understand how differences in its structure could impact prion species barriers. While multiple PrP^{Sc} structures have been

proposed over the years (Moore et al. 2009), the two most prominent are the 4-rung β-solenoid model (Vazquez-Fernandez et al. 2016; Spagnolli et al. 2019) and the parallel in-register intermolecular β-sheet (PIRIBS) model (Groveman et al. 2014). Of the two, atomic-level resolution of hamster PrP^{Sc} using high-resolution Cryo-EM analysis supports the PIRIBS model (Kraus et al. 2021). Analysis of the hamster PrP^{Sc} PIRIBS structure suggests that the asparagine at residue 155, which is critical in maintaining the species barrier between hamsters and mice (Priola et al. 2001), resides in an area of PrP^{Sc} where substitution with the corresponding tyrosine from mouse PrP would lead to steric clashes that could negatively impact conversion (Kraus et al. 2021). In vitro PrP^{Sc} formation studies have also suggested that asparagine residues are important in the formation of PrP^{Sc} across species, because they may help to stabilize intermolecular interactions within the PrP^{Sc} aggregate (Kurt et al. 2017). Future high-resolution PrP^{Sc} structures from different species should help to resolve why, depending upon the species, different amino acid residues impact transmission of prions across species barriers.

13.7 Molecular Model of Prion Species Barriers

13.7.1 Initial Prion Infection and Species Barriers

The fact that critical amino acid residues in the species-specific formation of PrP^{Sc} differ between species as well as the observation that PrP^{C} glycosylation can influence species barriers suggests that it is the tertiary structure of PrP, and not its primary structure, that may ultimately be most important in determining whether or not there are species-specific barriers to PrP^{Sc} formation and prion infection. This in turn suggests a molecular mechanism by which species barriers to prion infection are controlled at the level of PrP conformation (Fig. 13.2).

In intraspecies transmission of prions, where the host PrP^{C} and the exogenous infectious PrP^{Sc} are homologous, both the binding of PrP^{C} to PrP^{Sc} and its subsequent conversion to PrP^{Sc} occur as efficiently as possible, because they are conformationally compatible. Thus, there is no barrier to infection (Fig. 13.2a). By contrast, interspecies transmission of prions can occur when the host PrP^{C} and the exogenous infectious PrP^{Sc} are heterologous, but only if the amino acid residue differences are not within critical regions of the PrP molecule. In this instance, either the amino acid differences do not significantly change the conformation of PrP^{C} or the new conformation is still compatible with the incoming PrP^{Sc}. In either case, the binding of PrP^{C} to PrP^{Sc} and its subsequent conversion to PrP^{Sc} occurs efficiently enough that PrP^{Sc} can "replicate" to pathogenic levels (Fig. 13.2b). Thus, the differences in PrP^{C} conformation are insufficient to cause a species barrier to infection.

Interspecies transmission of prions would not occur if the host PrP^{C} and the exogenous infectious PrP^{Sc} are heterologous, but the amino acid differences reside within critical regions of the PrP molecule. In this case, the amino acid differences

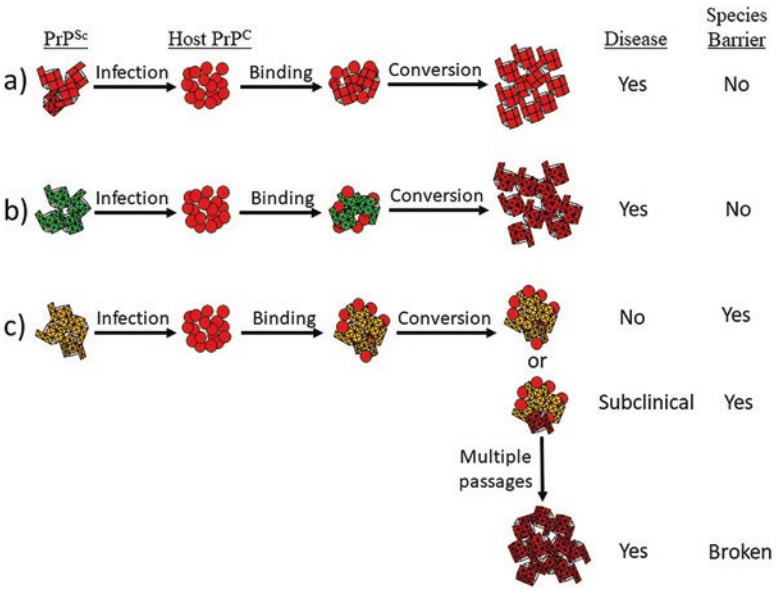


Fig. 13.2 Molecular model of prion disease species barriers. Red indicates PrP molecules derived from the same host species with aggregates of squares representing PrP^{Sc} and circles representing PrP^{C}. The degree of hatching within the squares represents different PrP^{Sc} conformations. Aggregates of green or yellow squares represent PrP^{Sc} molecules from different species. (**a**) Incoming PrP^{Sc} has the same sequence as the host PrP^{C}. Binding of PrP^{Sc} and PrP^{C} occurs, and since there is no conformational incompatibility, new PrP^{Sc} is formed. There is no species barrier and infection leads to disease. (**b**) Incoming PrP^{Sc} has a different primary sequence than the host PrP^{C}. Any resulting conformational differences are still compatible and binding of PrP^{Sc} and PrP^{C} occurs leading to new PrP^{Sc} formation. Despite both amino acid sequence and conformational differences between PrP^{Sc} and PrP^{C}, there is no species barrier and infection leads to disease. (**c**) Incoming PrP^{Sc} has a different primary sequence than the host PrP^{C} and the molecules are conformationally incompatible. Binding of PrP^{Sc} and PrP^{C} still occurs, but no new PrP^{Sc} is formed. Thus, there is a species barrier to infection unless a small, conformationally divergent fraction of PrP^{Sc} is present that can trigger new PrP^{Sc} formation. A subclinical infection would then be established that, given continued passage through the same host species, could eventually lead to clinical disease and a species barrier to prion infection being broken

change the conformation of PrP^{C}, such that it is incompatible with the incoming PrP^{Sc}. As a result, the binding of PrP^{C} to PrP^{Sc} and/or its subsequent conversion to PrP^{Sc} are significantly impaired (Fig. 13.2c). PrP^{Sc} would be unable to "replicate" itself very efficiently and would not accumulate to sufficient levels to trigger disease in the new host, i.e., a prion disease species barrier would exist. Thus, newly formed PrP^{Sc} would not be permanently altered by replication in the new host species, would likely retain its original properties, and would fail to adapt to the new host. This process has recently been termed nonadaptive prion amplification or NAPA (Bian et al. 2017), and suggests that selective pressures on PrP^{Sc} may ultimately dictate its host range (Bian et al. 2017; Duque Velásquez et al. 2020).

13.7.2 Prion Adaptation and Species Barriers

Even if a species barrier is not crossed during primary passage into a new host species, the presence of multiple prion strains in the infectious inoculum coupled with the potential for sub-clinical infection (i.e., prion replication but no disease) might eventually lead to a breach of the barrier to infection. For example, wild-type mice inoculated with hamster scrapie survive 1–2 years with no clinical signs of illness but, after 4–5 serial passages, prions that are mouse tropic, hamster tropic, or dually mouse and hamster tropic can be isolated (Race et al. 2002). In this instance, a species barrier is likely broken, because a minor fraction of the exogenous PrP^{Sc} is conformationally compatible with the endogenous host PrP^{C}, resulting in the generation of low levels of infectious PrP^{Sc} which now have the sequence of the host PrP^{C} molecule. This host compatible PrP^{Sc} begins to accumulate over the lifetime of the infected host but does not reach levels sufficient to cause disease (subclinical disease in Fig. 13.2c). However, when the infectious material is then transferred from the first infected host into a second host, from the second host into a third host, and so on, at each passage, more and more of the incoming PrP^{Sc} is homologous to the host PrP^{C} decreasing the time it takes for PrP^{Sc} to reach pathogenic levels until eventually it causes disease within the lifetime of the host (Fig. 13.2c). In essence, multiple passages in the new host species have allowed time for the prions to adapt and cause disease, a process that may explain the emergence of BSE in cattle. Thus, as long as prions can be transmitted between animals, it is likely that any prion species barrier can be crossed if there are prion strains in the inoculum capable of establishing a subclinical infection in the new host.

13.8 Intermediate Species and Prion Species Barriers

13.8.1 Altered Properties of BSE After Passage into New Species

The fact that hamster prions passaged through mice can acquire a new host range (Race et al. 2002) raises concerns that for both BSE, which has infected humans to cause vCJD (Bruce et al. 1997; Collinge et al. 1996; Hill et al. 1997) and CWD, which is circulating unchecked in wild populations of cervids (Miller and Williams 2003; Sigurdson and Aguzzi 2007), infection of an intermediate species could generate prions that could infect humans. In fact, the species tropism of BSE can be changed by passage through different hosts. While BSE prions derived from cattle cannot infect transgenic mice expressing cervid PrP^{C}, BSE prions passaged through red-tailed deer can (Vickery et al. 2014). Sheep-passaged BSE can infect transgenic mice expressing elk PrP^{C} (Tamguney et al. 2009b) and establish a subclinical infection in transgenic mice expressing human PrP^{C} (Plinston et al. 2011), while BSE from cattle cannot (Tamguney et al. 2009b; Plinston et al. 2011). Similarly, when

different strains of BSE are passaged through transgenic mice expressing different sheep *Prnp* genotypes, some can acquire the ability to infect transgenic mice expressing human PrP^{C} (Marín-Moreno et al. 2020). Mechanistically, in vitro studies suggest that the increased host range of BSE prions following passage through sheep may be because the prions that emerge appear to be more efficient at converting PrP^{C} from other species to PrP^{Sc} (Priem et al. 2014). All these experiments show that crossing species barriers can change the properties of the infectious prion, leading to the unpredictable emergence of prions with distinct species tropisms which can differ from that of the original inoculum.

13.8.2 CWD Host Range and Species Barriers

To date, there are no known cases of human prion disease related to exposure to CWD. Multiple studies have examined whether or not CWD can cross species barriers to cause disease in humans. Non-human primate models of CWD have shown that CWD can be transmitted to squirrel monkeys (Marsh et al. 2005; Race et al. 2014) but not to cynomologous macaques (Race et al. 2018), which are more closely related to humans. Transgenic mice expressing human PrP^{C} are also highly resistant to CWD infection (Sandberg et al. 2010; Wilson et al. 2012), with only one study able to detect potentially low levels of infectivity in approximately 4% of the mice infected (Race et al. 2019). PrP^{Sc} derived from CWD isolates in general does not convert human PrP^{C} very efficiently (Davenport et al. 2015), a resistance that has been mapped to differences in amino acids in the β2–α2 loop of PrP^{C} (Kurt et al. 2015), although using CWD prions stabilized by multiple passages in vivo or in vitro can lead to more efficient conversion (Barria et al. 2011). Indeed, a recent study has shown that elk PrP^{Sc} produced in vitro was able to convert human PrP^{C} into PrP^{Sc} which was then infectious for transgenic mice expressing human PrP^{C} (Wang et al. 2021).

These studies suggest that, while a robust species barrier to infection of humans with CWD prions exists, under the right circumstances, it may be broken. The concern is that, as with BSE, passage of CWD through intermediate species could alter its tropism. CWD can infect several species, including sheep (Cassmann et al. 2021), cats (Mathiason et al. 2013), pigs (Moore et al. 2017), and ferrets (Bartz et al. 1998). Following passage in ferrets, the tropism of mule deer CWD changed, enabling it to infect hamsters (Bartz et al. 1998) that normally have some resistance to initial infection with CWD (Bartz et al. 1998; Raymond et al. 2007). Thus, there is evidence for CWD prions being able to breach a species barrier following passage through a non-cervid species. When combined with the fact that CWD is circulating uncontrolled among wild cervid populations in North America, concerns remain that CWD may one day emerge as a threat to human health.

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Part V
Modelling of Prions

Chapter 14
Modeling the Cell Biology of Prions

Richard Rubenstein, David Doyle, and Robert B. Petersen

Abstract Cell models have been useful for elucidating the function of proteins and/or their role in pathogenesis. Even before the discovery that the prion protein was a normal cellular protein (Oesch et al., Cell 40:735–746, 1985), cell models were developed to investigate prion infection (Rubenstein et al., J Gen Virol 65:2191–2198, 1984). Subsequently, with the discovery of familial forms of human prion diseases (Hsiao et al., Nature 338:342–345, 1989), cell models were developed to investigate the effect of mutations on the metabolism of the prion protein and, in parallel, the normal synthesis and processing of the cellular prion protein. In this chapter, we review the progress made in these two areas to date.

Keywords Cell models · Prion protein · Prions · TSE agent replication · Cellular cultures · Pathogenic mutations

14.1 Cellular Cultures Supporting TSE Agent Replication

Cell cultures represent relevant and useful experimental models to study transmissible spongiform encephalopathies (TSEs) or prion diseases. Our current understanding of the cell biology of both the normal prion protein (PrP^{C}) and the pathogenic isoform (PrP^{Sc}) has utilized infected cell culture models. Cell culture models have also been useful in the development and validation of anti-prion drugs as well as offering an alternative approach to the transmission/infectivity assays historically performed in animal models. Cell culture models have also been used to

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study prion-induced cytopathological changes, which might help to explain the prion disease-associated neuropathogenesis observed in vivo.

Several cell culture models permissive to prion replication are available and some of them allow subpassaging to monitor stable and persistent replication of the infectious agent. The target cell type presumed to be most useful and informative would be cells of neuronal origin from the central nervous system (CNS), since the majority of prion infectivity is found in the CNS and the associated pathology is predominantly neurodegeneration. These include uncloned and cloned mouse neuroblastoma cell lines (N2a, C-1300, N1E-115) (Race et al. 1987; Nishida et al. 2000; Butler et al. 1988; Markovits et al. 1983; Ostlund et al. 2001) and murine GT1 hypothalamic neural cells (Schatzl et al. 1997). The GT1 cells are differentiated gonadotrophin-releasing hormone neurons, and in contrast to some of the neuroblastoma cell lines, they are susceptible to the 139A and 22 L mouse-adapted scrapie strains, as well as prions from familial GSS and sporadic CJD. GT1 cells are particularly useful for studying prion infection-associated cytopathic effects, since they become stably infected in contrast to N2a cells and, therefore, do not require periodic subcloning to maintain an infected culture (Nishida et al. 2000; Schatzl et al. 1997).

In addition, neuronal stem cells isolated from conventional or transgenic mice propagate mouse-adapted prions (Giri et al. 2006; Milhavet et al. 2006). Recently, hippocampal-derived HpL3-4 cells obtained from a PrP^C knockout mouse and transfected with mouse PrP^C were shown to be permissive to the mouse-adapted 22 L scrapie strain (Maas et al. 2007). Finally, cells from the peripheral nervous system, such as MSC80, murine Schwann-like cells, replicate low levels of the Rocky Mountain Laboratories (RML) mouse scrapie strain (Follet et al. 2002). Non-neuronal cell lines can also efficiently propagate prions. Common fibroblast cell lines (Vorberg et al. 2004), a microglial cell line (MG20) established from transgenic mice overexpressing PrP (Iwamaru et al. 2007), and PC12 rat pheochromocytoma cells (Rubenstein et al. 1984, 1991) are susceptible to various murine prion strains. Notably, the mouse-adapted bovine spongiform encephalopathy agent was successfully propagated in MG20 cells. Infection of a skeletal myoblast cell line (C2C12) was recently described (Dlakic et al. 2007), and could be used to investigate the mechanism underlying the prion infection of muscles observed in sheep and cervids.

One well-established feature of the animal bioassay is the species-specificity relationship between the source of the infectious agent and the recipient animal, which dictates both efficiency of infection and latency. In the cell system, both homologous (i.e., species matched) and heterologous (i.e., species mismatched) cell culture model systems have been successfully used. The rationale for using homologous cies-barrier phenomenon observed in animal bioassays in which the efficiency of infection is reduced if there are dissimilar primary amino acid sequences in the PrP of the species from which the prion agent and the host cells were derived. However, cell culture studies have demonstrated that this is not as straightforward as it seems. For example, only a limited number of mouse-adapted scrapie strains

can replicate in murine-derived host cell lines, and rat-derived PC12 cells can only be infected with selected mouse (but not rat)-adapted scrapie strains.

Although the source of the infectious agent is typically homogenized brain tissue originating from infected animals, partially purified preparations of scrapie-associated fibrils or PrP^{Sc} have also been used to achieve a higher-titer inoculum (Race et al. 1987). Cultures are either maintained in a nondividing, neuronal state, or passaged several times, and continually monitored for the disappearance (i.e., dilution) of the initial inoculum and appearance of de novo agent replication. To monitor propagation, cells are harvested at different times after exposure to the source of agent and cell lysates are used in animal bioassays. Alternatively, once it had been demonstrated that there is a close association between PrP^{Sc} and agent replication, the appearance and increase of the proteinase K (PK)-resistant PrP^{Sc} isoforms can be monitored by immunodetection and used as a biomarker of prion agent replication. Cell blotting techniques have been successfully used to detect PrP^{Sc} when only 1% of the cells are infected (Bosque and Prusiner 2000) and a filter retention assay for PrP^{Sc} (Winklhofer et al. 2001), which measures both PK resistance and presence of a detergent-insoluble aggregated state, has also been used. In addition, Vilette et al. have used a post-embedding method able to detect single infected cells (Vilette et al. 2001). This method has the advantage of evaluating the percentage of infected cells present in a particular culture. This is important when one considers the reports that for N2a cells only 1% of the cells were actually infected (Race 1991), although more efficient cell-culture models (Bosque and Prusiner 2000; Nishida et al. 2000) seem to have up to 30% of cells actually accumulating PrP^{Sc}.

The amount of infectivity present in the culture is also an important issue. Recent data on permissible cell lines revealed that cultures have the potential to accumulate as many infectious units per milligram of protein as brain from affected animals (Vilette et al. 2001).

Studying prion propagation in cell culture originally used animal-derived infected cells in which infected cultures were obtained from infected animals. The SMB cell line was established from the brain of a mouse clinically affected by the Chandler scrapie strain (Clarke and Haig 1970a, b). The majority of the initial studies on infecting cells in vitro used murine neuroblastoma cell lines (Race et al. 1987; Butler et al. 1988; Nishida et al. 2000; Markovits et al. 1983; Ostlund et al. 2001; Borchelt et al. 1990). Several investigators have described various biochemical and, at best, only subtle phenotypic differences in scrapie-infected cells. In addition, both increases and decreases in the rates of cell proliferation have been reported in infected cell lines. Unfortunately, it is not clear that the changes described were necessarily only due to the scrapie agent as opposed to clonal differences or to other factors present in the inoculum used to infect the cells. In addition, since the concentration of PrP has been shown to influence infectability, replication, and transmissibility of the prion agent in vivo, using an overexpressing cell line, such as the murine N2a neuroblastoma, allows these cells to be readily infected by the three mouse-adapted scrapie strains, Chandler, 139A, and 22 L (Nishida et al. 2000).

A common feature of susceptible cell lines is that they only support the propagation of TSE strains that have been experimentally adapted to rodents. Recently, Vilette et al. developed a new heterologous model for naturally occurring sheep scrapie. This model was obtained by stable expression of the ovine PrP gene in a rabbit epithelial cell line (RK13) (Vilette et al. 2001). The authors showed that the expression of heterologous PrP in an otherwise refractory system, such as the rabbit system, is sufficient to cross the species barrier ex vivo. The use of gene-edited mouse cell lines with murine prions has now been shown to have the potential to express bank vole and cervid PrP genes following CRISPR–Cas9 editing and lentiviral transduction, allowing for greater insight into chronic wasting disease and cross species comparisons (Walia et al. 2019). Further use of such methods may allow for diverse cellular protein platforms, providing cellular infection models with greater compositional diversity. Still, such models demonstrate the primacy of rodent models, where conventional editing techniques serve to modify such models rather than compete with this standardized modality.

Infected cell culture models have provided some valuable insights into the biogenesis of PrP^{Sc} in terms of conversion, subcellular localizations, physiopathological consequences, and species-barrier determinants. They have also contributed to the screening and the study of possible therapeutic compounds and to the development of new strategies for the investigation of TSE-specific biomarkers. Studies with infected cell cultures have shown that PrP^{C} and PrP^{Sc} are associated with the cell surface differently, since only the former can be released by phosphatidylinositol-specific phospholipase C treatment of intact infected cells (Caughey et al. 1990; Lehmann and Harris 1996). Analysis of several types of infected cells, including N2a, GT1, and HaB (Schatzl et al. 1997; Taraboulos et al. 1990), made it clear that PrP^{Sc} resides within the cell and accumulates in late endosomes and/or lysosomes (McKinley et al. 1991; Pimpinelli et al. 2005), where amino terminal trimming of PrP^{Sc} may occur (Caughey et al. 1991).

Furthermore, although PrP^{C} is rapidly synthesized and degraded, while the abnormal PrP^{Sc} isoform is relatively stable (Borchelt et al. 1990; Caughey et al. 1989; Nunziante et al. 2003), the infected cells do have the capacity, processing functions, and proteases to degrade PrP^{Sc} (Beringue et al. 2004; Enari et al. 2001; Feraudet et al. 2005; Peretz et al. 2001; Perrier et al. 2004).

The information obtained from the use of infected cell cultures to study events associated with neurodegeneration have been limited. Replication of the prion agent in cultured cells can result in specific alterations in cellular metabolism, some of which can affect cell survival. For instance, infection with several murine prion strains impairs the cellular response of GT1 and N2a cells to oxidative stress (Milhavet et al. 2000), presumably through a decrease in superoxide dismutase activity. It is interesting to note that prion-infected cell lines accumulating infectious titers similar to those in brain tissue do not show any obvious cytopathic effect, with the possible exception of RML-infected GT1 cells that undergo apoptosis inconsistently. The use of primary cultures may lead to a better understanding of the effect of prion agent replication on neuronal death. For example, infection of

primary cultures of neurons and astrocytes by a sheep scrapie agent resulted only in neuronal apoptosis involving JNK-c-Jun signaling (Cronier et al. 2004).

Numerous compounds have been used successfully to inhibit PrP^{Sc} formation in vitro, but the results in vivo have been disappointing. An example is provided by cellular heparan sulfates, which are sulfated linear polysaccharides typically linked to proteins to form heparan sulfate proteoglycans located at the cell surface (Turnbull et al. 2001). A number of studies suggest that heparan sulfates are involved in the biogenesis of PrP^{Sc} possibly by bringing together components involved in the conversion process, such as PrP^{C}, PrP^{Sc}, and other possible cofactors. A variety of sulfated glycans, including pentosan polysulfate (Birkett et al. 2001; Caughey and Raymond 1993), dextran sulfate 500 (Barret et al. 2003; Beringue et al. 2004; Caughey and Raymond 1993), and heparin (Gabizon et al. 1993), are potent inhibitors of PrP^{Sc} accumulation in several cell lines infected with murine prions presumably by competitive inhibition of cellular heparan sulfates for the binding to PrP^{C} (Gabizon et al. 1993).

The use of cell culture models to determine the therapeutic value of compounds in vivo has been disappointing. A large number of compounds have been found to inhibit PrP^{Sc} accumulation in prion-infected cultures, mainly in N2a cells (Kocisko et al. 2003); however, most of them showed no or very limited effects when subsequently tested in infected animals (Trevitt and Collinge 2006). This does not necessarily mean that infected cell models are not adequate to screen for anti-prion drugs, but rather indicates that prion propagation in organisms is a complex biological process. In addition to drugs, passive immunization with anti-PrP antibodies (Abs) has been tested in cell culture models (Enari et al. 2001; Peretz et al. 2001; Perrier et al. 2004; Gilch et al. 2003). These Abs significantly reduced prion agent replication in cell culture by preventing the conversion of PrP^{C} into PrP^{Sc} through blockage of PrP^{C}–PrP^{Sc} binding and/or by stabilizing the PrP^{C} on the cell surface. Although infected animals injected with antibodies did, under certain circumstances, show a modest increase in survival times, it did not reflect the extent demonstrated in cell culture (Sigurdsson et al. 2003; White et al. 2003).

The utilization of a cell culture system as a replacement for the expensive and time-consuming animal bioassay has been explored. However, this has been hampered because of low sensitivity due, in part, to the small percentage of cells actually infected (Race et al. 1987). The isolation of N2a subclones with higher permissiveness (Bosque and Prusiner 2000; Enari et al. 2001), along with improved detection of PrP^{Sc}, allowed the development of a quantitative, highly sensitive scrapie cell-based infectivity assay (SCA) for the RML murine prion strain (Klohn et al. 2003). Although the SCA is almost as sensitive as the mouse bioassay while being much less expensive and ten times faster, it is limited in that N2a cells are not permissive to natural strains of the infectious agents.

Further research is still needed for the development of better cell culture models. These models will be important tools to dissect the properties of the prion agents, including their molecular composition, the basis of cell permissiveness, and the identification of the biochemical and molecular mechanisms causing neuronal death. Some interesting studies along these lines have been reported. Weissmann's

group recently demonstrated that the composition of the glycan can affect infection efficiency (Browning et al. 2011). In another study, sialyation of the glycosylphosphatidyl inositol anchor was shown to play a significant role in PrP aggregation, which is associated with neurodegeneration (Bate and Williams 2012).

In addition, there has been progress through the use of unconventional nonmammalian models. Insight within evolutionary distant models has demonstrated the use of *Saccharomyces (S.) cerevisiae* as an effective, affordable screening method within the induction and propagation of prions (Ishikawa 2021). Traditionally, such models utilize [PSI+] and [URE3] yeast prions, leading to the identification of relevant therapeutics, allowing for the potential repurposing of conventional medications, such as the TLR7 agonist imiquimod or the calcium antagonist flunarizine (Oumata et al. 2013; Bamia et al. 2021). The use of these screening modalities has identified the relationship between yeast [PSI+] and the evolutionarily conserved protein folding activity of ribosome, demonstrating the continued value of utilizing nonmammalian cell models to elaborate upon the prion life cycle and potential corresponding treatments (Blondel et al. 2016; Banerjee and Sanyal 2014). Together, this understanding can present as a lower risk, lower cost means to identify homogeneity between eukaryotic prion cell models, granting alternative forms of modeling and treatment through enhanced screening methods.

14.2 Cell Models of Pathogenic Mutations in the Prion Protein

Following the discovery that PrP was a normal cellular protein, pathogenic mutations associated with familial prion diseases were discovered (Hsiao et al. 1989). This provided the opportunity to study the metabolism of the mutant protein in cell culture models with the hope that this might shed light on the conditions that lead to pathogenic conversion of the prion protein. Cell culture models had been used to study the metabolism of PrP in infected cells, which included a detailed study of the synthesis and processing of PrP^C (Caughey et al. 1989). PrP^C is modified in the endoplasmic reticulum by the addition of a glycosylphospatidyl inositol (GPI) anchor and the nonobligatory addition of *N*-linked glycans; there are two N-linked glycosylation sites in the prion protein (Robakis et al. 1986; Locht et al. 1986). The N-linked glycans serve as a major source of heterogeneity in the prion protein (Rudd et al. 1999). As mentioned above, the glycans appear to influence infection (Browning et al. 2011) and may provide the basis for strain determination, as previously conjectured (Rudd et al. 2001).

To study the effect of point mutations on the synthesis and metabolism of PrP^C, a variety of cell models have been established. (The insert mutation in the octapeptide repeat is not included based on the complexity of the clinical phenotype, see Solomon et al. (2010) for a review.) Some of the models use heterologous pairings of cells and homologues of the pathogenic human mutations (Lehmann and Harris

1995, 1996; Ma and Lindquist 2001). An alternative model, in which the human PrP-coding region was placed under the control of a strong promoter in an episomal vector (Petersen et al. 1996) and transfected into a human neuroblastoma cell line (M17), is the only cell model that has been validated by comparison with human tissue. The use of an episomal vector eliminates problems of copy number and integration site effects. In addition, the instantaneous rate of protein synthesis is the same for all constructs, so that modifications that may be concentration dependent, i.e., glycosylation, GPI anchoring, etc., are unaffected. In general, however, similar results have been obtained in all systems.

Detailed studies examining the metabolic defects associated with expression of mutant PrP, PrP^M, suggest that the effects of the mutations fall into two general categories (see Fig. 14.1). The first category includes mutations around the normal amino terminal cleavage site at residue 111/112, which includes those at codons 102, 105, and 117. In the human neuroblastoma cell models, these mutations do not appear to affect overall metabolism, but seem to cause an altered cleavage of PrP (Mishra et al. 2002) (RBP, unpublished). The truncated fragments are generally associated with Gerstmann–Straussler–Scheinker syndrome, a familiar form of prion disease that presents with prion plaques. Novel PrP fragments have been found in cells expressing the F198S, E200K, and Q217R mutations, indicating an alteration in protein processing (Zaidi et al. 2005; Capellari et al. 2000a; Singh et al. 1997).

The second category of mutations is clustered in and around the site of post-translational modifications that include nonobligatory addition of two *N*-linked glycans and the formation of a disulfide bridge. These mutations include those at codons D178N (Petersen et al. 1996), T183A (Capellari et al. 2000b), F198S (Zaidi et al. 2005), E200K (Capellari et al. 2000a), and Q217R (Singh et al. 1997). In this category, the mutations shared four common alterations in metabolism or processing: (1) PrP^M is unstable and degraded. This is particularly evident in the unglycosylated form of the PrP^M, which is virtually absent in some of these mutants. In addition, treatment with tunicamycin, which inhibits glycosylation, results in the rapid degradation of PrP^M compared to PrP^C in the secretory pathway. These results

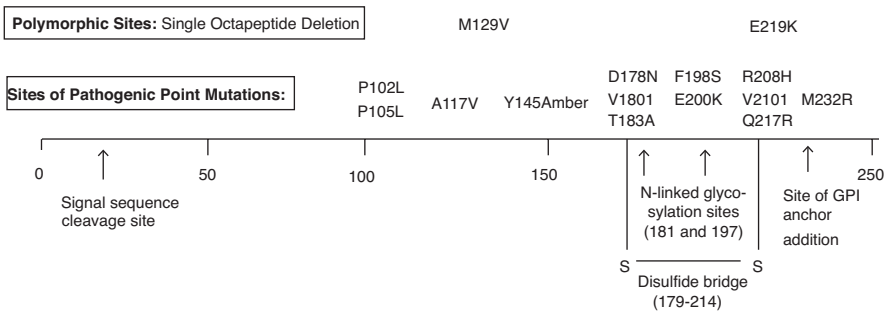

Fig. 14.1 Linear map of the prion protein showing a limited number of the pathogenic mutations in the human PrP as well as the sites of major post-translational modifications

support the role of *N*-linked glycans in facilitating protein folding. An early study suggested that loss of the first glycosylation site blocks transport of the mutant protein (Lehmann and Harris 1997); however, it was later established that the T183A mutation results in a structural change in the protein (Capellari et al. 2000b). (2) As a result of the decreased stability of the PrP^{M}, less PrP^{M} is found on the surface of cells expressing PrP^{M} and the ratio of glycoforms found at the cell surface parallels that found in the cells with marked underrepresentation of the unglycosylated form. Approximately 90% of PrP^{C} reaches the cell membrane, indicating that 10% of the normal protein fails to fold properly (Cohen and Taraboulos 2003). (3) A greater proportion of PrP^{M} partitions in a detergent insoluble fraction, indicating that PrP^{M} is aggregated in cells. (4) Most of these defects can be alleviated, in part, by incubating the cells at reduced temperature, 24 °C, suggesting that the processing defects arise due to misfolding of the PrP^{M}; misfolding has been shown to be partially corrected by reduced temperature (Singh et al. 1997). It is interesting to note that biophysical studies using recombinant PrP indicated that, with the exception of mutations at codons 183 and 198, these mutations do not appear to affect the physical properties of PrP^{M} versus PrP^{C} (Liemann and Glockshuber 1999).

In addition to the common changes resulting from the mutations, some of the mutants exhibited their own specific alterations. First, the Q217R mutation results in the production of a 32-kDa PrP lacking the GPI anchor, which attaches PrP to the cell surface (Singh et al. 1997). The F198S mutation results in the most profound reduction in the unglycosylated form of PrP^{M} (Zaidi et al. 2005). This arises for two reasons. First, the unglycosylated form is unstable, and second, the mutation replaces the phenylalanine residue, which is in the middle of the second glycosylation site, with a serine residue that is known to produce a more efficient glycosylation site. The F198S mutation also established that while the protein can achieve a normal conformation when expressed in a cell, after denaturation, it fails to refold into a native conformation (Zaidi et al. 2005). The E200K mutation, which is just beyond the second N-linked glycosylation site, results in the delayed maturation of PrP^{M} and the production of an abnormally modified glycan that is observed by its abnormal migration in SDS gels (Capellari et al. 2000a). Finally, comparison of the D178N/129 M mutation (FFI) and D178N/129 V mutation (CJD^{178}) did not exhibit specific differences, although the reduction of the unglycosylated form was more pronounced in the FFI expressing cells (Petersen et al. 1996). It is interesting to note that the prion disease referred to as sporadic fatal insomnia is also linked to codon 129 methionine, indicating that methionine may be required for the initiation of the disease process in the thalamus (Parchi et al. 1999).

Although some of the mutations studied resulted in general and specific changes in the metabolism of PrP^{M}, such as aggregation, none of the transfected human neuroblastoma lines produced bona fide protease-resistant PrP (PrP^{Sc}), as assessed by the gel migration pattern or infectivity. In fact, using an antibody that recognizes the carboxyl terminal region of PrP, wild-type PrP is as resistant to proteinase K treatment as PrP^{M} (Capellari et al. 2000a). The carboxyl terminal region of PrP is inherently resistant to protease digestion, and mutations in the region of post-translational modification appear to extend the tertiary structure through residues 90–112 that are typically unstructured. Thus, the weak protease resistance that has been associated

with PrP^{M} expressed in cell culture probably reflects a conformational change of the protein. In retrospect, it is not surprising that the cell culture models expressing the mutant PrP failed to produce PrP^{Sc}. The inherited human prion diseases are age-related diseases, so while the mutations may be necessary for the development of disease, they are clearly not sufficient. This suggests that some age-related deficit in the cellular repair/defense mechanisms is required to enable the initiation of the disease process.

While the cells expressing the mutant PrP grow normally, inhibition of the proteasomal degradation has been observed to result in neuronal cytotoxicity. The first observation of PrP^{M} accumulation was in cells expressing the nonsense mutation at codon 145, Y145Stop (Zanusso et al. 1999), in which the mutant protein accumulated in the cell after inhibition of the proteasome with lactacystin. Subsequent studies demonstrated that the codon 177 murine homologue of the human D178N mutation also accumulated in cells, even in the absence of proteasome inhibition (Ma and Lindquist 2001), and that this resulted in neurotoxicity (Ma et al. 2002).

In addition to conventional cell models, cerebral organoid (CO) creation has appeared as a frontier within prion modeling (reviewed in Walters and Haigh 2022). Utilizing induced pluripotent stem cell technology, complex, three-dimensional organoids now illustrate prion pathology, metabolism, and pharmacology within neuronal lineage cells (Groveman et al. 2021). Through their ability to model structures directly mimicking functional neuroanatomy, CO demonstrate the potential for personalized drug screening within familial prion diseases, offering increased therapeutic relevance within such schemes (Pineau and Sim 2021). Yet, despite their conservation of functional–structural relationships, these models face challenges seen within conventional cell culture models, such as the inability of mutations to induce a disease state independent of relevant aging processes or genetic modifiers. Such limitations were demonstrated by the insufficiency of CO generated from E200K asymptomatic donors to present disease isoforms 12 months post-differentiation (Foliaki et al. 2020). Furthermore, issues with diffusion and the lack of non-neuronal lineage cells present challenges in accurately representing human anatomical composition, where further developments, such as 3D-printed vasculature and regional specification, may alleviate current compositional concerns (Groveman et al. 2021). Collectively, these notions demonstrate the therapeutic potential seen in the correspondence between CO models and human tissue while still acknowledging the reality that mutations independent of aging or genetic determinants fail to reproduce the clinical entity demonstrating the need to further refine CO models.

14.3 Conclusion

Cell models have been invaluable for studying the infectious process at a cellular level. The infected cell model is currently facilitating studies that will help clarify the origin of prion strains. The cell models of the pathogenic prion mutations

indicate that the mutations are not sufficient to produce the disease-associated form of PrP, but show the potential for chronic stress in the secretory pathway that may facilitate the disease process.

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Chapter 15
Transgenic Mice Modelling in Prion Diseases

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Abstract Although the prion protein (PrP) was discovered in the early 1980s, there is still a lack of knowledge of the normal function of the PrP protein and its precise role in the infectious process of transmissible spongiform encephalopathies (TSEs) or prion diseases. The production and use of a multitude of transgenic mice expressing different forms of PrP has enabled us to increase our knowledge of PrP in health and disease. Using mice expressing PrP from different species, we are able to define the strain of TSE agent infecting a wide range of hosts and model the transmission potential of each agent within and between species. Transgenic mouse models are also utilised in investigating the normal function of PrP, the impact of differential glycosylation in PrP biology and the genetics underlying disease susceptibility. Advances in transgenic technologies have enabled us to control both spatial and temporal expression of PrP, allowing us to define the mechanisms and routes of disease pathogenesis. Transgenic mice also play a vital role in understanding the mechanisms of neurodegeneration in the TSEs, which may also lead to a better understanding of the other protein misfolding diseases, such as Alzheimer's disease.

Keywords Creutzfeldt · Jakob disease · Gene targeting · Prion transmission · Prnp · PrP · PrP^C knockout · Species barriers · Transgenic models · Transmissible spongiform encephalopathies (TSE) · TSE strains

15.1 Introduction

Transgenic mice have been at the forefront of research into the transmissible spongiform encephalopathies (TSE) (or prion diseases) since 1989 when the first transgenic mice were produced which overexpressed the hamster prion protein (PrP) via insertion of the hamster gene (*Prnp*) into the murine genome (Scott et al. 1989). Since then, transgenic mice have added a wealth of knowledge to the field. The number of transgenic mouse models constructed to assess the role of PrP in health

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and disease increases each year with new technologies available and a greater understanding of disease mechanisms. This review will concentrate on the contribution of transgenic mouse models in identifying and characterising strains of infectious agent, defining transmission within and between hosts and modulating disease pathogenesis. It will focus on models in which the *Prnp* gene has been altered, in particular where gene targeting has been used to alter the PrP-coding sequence, ranging from changing of a single amino acid to complete replacement of the mouse protein sequence with that of a different species.

15.2 Host PrP and Susceptibility to TSEs

The hypothesis that a misfolded form of PrP was responsible for TSE diseases led to the development of PrP null mice referred to as *Zurich I* and *Npu,* respectively (Bueler et al. 1992; Manson et al. 1994a). These have been followed by a number of PrP null mice constructed on a range of genetic backgrounds, predominantly 129Ola, and using both gene targeting and TALEN-based gene editing, such as the *Zurich III* model (Nuvolone et al. 2016). No overt phenotype was observed in these mice, thus allowing their use in TSE transmission studies. PrP null mice were shown to be resistant to a range of TSE agents (Weissmann et al. 1994a; Manson et al. 1994b). The heterozygous null mice in these studies were shown to have longer incubation times than the wild-type mice (Manson et al. 1994b; Weissmann et al. 1994b). This demonstrated, as had a number of previous experiments with mice overexpressing the *Prnp* gene, that the expression levels of host cellular PrP (PrP^C) altered incubation time, with overexpression in general shortening incubation periods (IP) and reduced expression leading to longer IP (Scott et al. 1989; Westaway et al. 1991).

Early mouse studies revealed that susceptibility to disease and IP could be influenced by the PrP genotype. The first transgenic mouse studies by Scott et al. (Scott et al. 1989) used mouse models which overexpressed hamster PrP in a background of endogenous murine PrP expression. These mice were susceptible to hamster scrapie and gave a significantly shorter IP than control mice (Scott et al. 1989). This led to the hypothesis that sequence identity between the host and donor PrP is important in determining disease susceptibility and IP; the greater the similarity between PrP sequences the greater their susceptibility to disease and the shorter the IP. Differences in sequence identity were proposed to form the basis of the 'species barrier'; the inefficient transmission of a TSE agent to a new host species, often with long incubation times which decrease upon subsequent passage in the new host species (Kimberlin et al. 1987; Kimberlin and Walker 1979). In general, identity between PrP sequences often shortens incubation time, but this is not always the case. Gene-targeted[1] mice in which the murine *Prnp* gene has been replaced by a

[1] Gene targeting is a technique that uses homologous recombination to alter an endogenous gene.

bovine *Prnp* gene in a 129Ola background inoculated with bovine spongiform encephalopathy (BSE) have a longer IP than their wild-type equivalent despite the increase in sequence homology between the PrP in the inoculum and the host gene (Fraser et al. 1992; Bishop et al. 2006). The same is also true for variant Creutzfeldt–Jakob disease (vCJD) transmitted to 129Ola gene-targeted mice expressing human PrP (Bishop et al. 2006). Thus, increased identity between host and donor PrP can either decrease or increase incubation times, suggesting that sequence homology plays only a part of determining transmission of disease across the species barrier and that other factors are present.

Single polymorphisms in the *Prnp* gene can have important consequences for incubation time of TSEs. Murine *Prnp* has three naturally occurring alleles: *Prnp*-a (Leu-108, Thr-189), *Prnp*-b (Phe-108, Val-189), and *Prnp*-c (Phe-108, Thr-189) (Westaway et al. 1987; Lloyd et al. 2004). Gene targeting was used to construct mice in which the endogenous *Prnp*-a allele was modified to express *Prnp*-b rather than *Prnp*-a (Moore et al. 1998). These experiments established that these polymorphisms have a major influence on incubation time of disease in mice. However, it is also evident from other studies that there are other factors involved, since TSE IP can vary by more than 100 days in different strains of mice possessing identical *Prnp* sequences (Fraser et al. 1992; Lloyd et al. 2001; Kingsbury et al. 1983). Genetic factors mapping to four chromosomal regions and environmental factors, namely, age and x-cytoplasmic interactions in the host were shown, were shown to modify disease IP on cross species transmission of BSE to mice (Manolakou et al. 2001).

Bishop et al. (Bishop et al. 2010) used gene-targeted mice expressing variants different alleles of human PrP possessing either methionine or valine at codon 129 at endogenous levels of and under the control of normal gene expression modifiers of murine *Prnp*. This allowed direct comparison between the three lines each representing a different human codon 129 genotype (methionine homozygous; HuMM, methionine/valine heterozygous; HuMV and valine homozygous; HuVV). Bishop et al. showed that not only did sporadic CJD (sCJD) transmit more efficiently to these transgenic mice than wild-type mice, but that transmission rates were higher and IP shorter when the donor and host codon 129 genotype matched, i.e., type MM1[2] sCJD transmitted to HuMM mice in 446 days versus 588 days in HuVV mice, whereas type VV2 sCJD transmitted to HuVV mice in 274 days versus 582 days in HuMM mice (Table 15.1).

Single polymorphisms can have unpredictable consequences in host susceptibility. Gene-targeted mice were produced in which a proline-to-leucine polymorphism was introduced into codon 101 in the murine PrP sequence (101LL). Inoculation with the human genetic form of prion disease, P102L Gerstmann–Sträussler–Scheinker disease produces disease in 288 days with 100% susceptibility, suggesting the importance of the proline to leucine change in determining susceptibility

[2] Sporadic CJD is sub-classified via the codon 129 genotype of the host and typed by biochemical properties.

Table 15.1 Primary inoculation of TSE strains in three transgenic mouse lines

	HuMM		HuMV		HuVV	
Strain of agent	IP	TSE Pathology	IP	TSE Pathology	IP	TSE Pathology
vCJD	>401	11/17	>600	11/16	–	1/16
sCJD ($M1^{CJD}$)	446	29/29	457–475	31/32	588-603	29/34
sCJD ($M2^{CJD}$)	–	0/16	–	2/18	–	3/17
sCJD ($V1^{CJD}$)	–	2/16	557	9/14	568	7/14
sCJD ($V2^{CJD}$)	563–582	25/31	450–575	27/32	274–288	32/32
BSE	–	0/18	–	0/23	–	0/22
Sheep BSE	>750	16/23	>708	0/24	>650	0/23
CWD	–	–	–	–	–	–

Primary passage data for incubation period (IP) and TSE pathology confirmed by either immunocytochemistry or lesion profile

– Indicates no clinical signs. Sheep BSE data from (Plinston et al. 2011). sCJD data from (Bishop et al. 2010). vCJD data from (Bishop et al. 2006). BSE data from (Bishop et al. 2006)

(Manson et al. 1999). More unexpected, however, was that when these mice were inoculated with hamster-passaged scrapie (263 K) or a pooled natural scrapie strain (SSBP/1), the IP was dramatically reduced when compared with wild-type mice: 374 days versus 707 days and 346 days versus over 400 days, respectively (Barron et al. 2001). Both these strains of TSE are associated with PrP from different species and carry a proline at the equivalent codon 101 position. In contrast, ME7, a murine strain from a 101PP host, shows a longer IP in 101LL mice compared with wild-type mice despite being of the same species (Manson et al. 1999; Barron et al. 2001). These studies suggest that the proline-to-leucine mutation in mice can significantly alter incubation time across three species barriers and the host/donor sequence homology is not the most important criteria for determining transmissibility of disease.

If sequence compatibility between host and donor PrP is not sufficient to explain host susceptibility, other factors should be considered. PrP glycosylation may be an important factor in determining the susceptibility of the host to different TSE sources. This was previously suggested by in vitro experiments, where the removal of sugars abolished the species barrier (Priola and Lawson 2001). To address in vivo whether PrP glycosylation is a major factor in influencing TSE infection, three gene-targeted inbred lines of mice were produced carrying mutations at the first (residue 180) or second (residue 196) N-linked glycosylation site in PrP, in which the first, second, or both glycosylation sites were removed: N180T (G1), N196T (G2), and N180T–N196T (G3), respectively. Initial studies showed that the lack of glycans altered the cellular location of the G3 mutant to mainly intracellular PrP, whilst G1 and G2 PrP appeared mainly on the cell surface similar to wild-type PrP (Cancellotti et al. 2005). Mice devoid of the first or both glycosylation sites (G1 and G3) have either total resistance or reduced susceptibility (indicated by extended IP) to a number of agents, including ME7, 79A, 263 K, sCJDMM2, and vCJD (Tuzi

et al. 2008; Wiseman et al. 2015). When these mice were inoculated intracerebrally with mouse ME7, 79A, 263 K, vCJD, or sCJDMM2, G3 mice were only susceptible to 79A and exhibited a significantly longer IP than G1 or G2 mice. G1 mice were only susceptible to 79A and 263 K with extended IP or sub-clinical disease (Tuzi et al. 2008; Wiseman et al. 2015). In contrast, the absence of the second N-glycan site appears to facilitate transmission of disease, with all strains inoculated showing evidence of transmission, even sCJDMM2 which wild-type mice are resistance to (Tuzi et al. 2008; Wiseman et al. 2015). Interestingly, in vitro studies using G2 mouse brain homogenate as a substrate in protein misfolding cyclic amplification (PMCA) showed that G2 PrP^{C} was able to convert into PrP^{Sc} when seeded by mouse protein only recombinant PrP^{Sc}. This property was not shared by G1 or G2 mice (Burke et al. 2020). Using intraperitoneal inoculation (i.p.) to study the effects of peripheral transmission of infectivity, it appears that host PrP glycosylation can influence the timing of neuroinvasion. Following i.p. inoculation of 79A, both G1 and G2 mice showed increased incubation times when compared to wild-type mice, whereas G3 mice showed no signs of clinical disease. Inoculation of ME7 resulted in only a slight lengthening of incubation time in G2 mice, but showed no transmission in either G1 or G3 mice (Cancellotti et al. 2010). Transmission of TSE agents to these mice thus established that glycosylation of host PrP has a major influence on the outcome of disease and that it may provide a route to blocking the disease process (Tuzi et al. 2008; Wiseman et al. 2015; Cancellotti et al. 2010).

15.3 Transmission of Agent Within a Host

Peripheral routes of infection are most relevant for natural TSE transmission in humans and animals, e.g., orally through contaminated food, through blood as has been the case with vCJD, and potentially also nasally or through lesions to skin or mucous membranes (Bruce et al. 1997; Peden et al. 2004; Llewelyn et al. 2004; Mabbott 2017). Experimental studies in the 1960s revealed that after peripheral (intraperitoneal) injection, TSE agents accumulate to high levels in tissues of the lymphoreticular system (LRS), suggesting that the many decades later, the use of *Prnp*-transgenic and *Prnp*-deficient mice has been instrumental in defining the tissue and cellular that periphery may play an important role in the disease pathogenesis (Eklund et al. 1967). Decades later, the use of *Prnp*-transgenic and *Prnp*-deficient mice has been instrumental in defining the tissue and cellular route that TSE agents take after peripheral exposure to establish disease in the CNS.

Following peripheral exposure to a TSE agent, there is an early accumulation of disease-associated PrP (PrP^{Sc}) in tissues of the LRS, such as the spleen and lymph nodes, before the disease spreads to the CNS (Muramoto et al. 1993; McBride et al. 1992).

15.4 Transport into the LRS

Orally acquired TSE agents must first cross the gut epithelium to infect the host (Fig. 15.1). The epithelium covering the gut-associated lymphoid tissues (GALT), such as the Peyer's patches, contains specialised phagocytic epithelial cells, termed M cells. These cells constitutively transport particulate antigens and microorganisms from the lumen of the intestine into Peyer's patches and other GALT (Mabbott et al. 2013). The initial uptake of TSE agents from the gut lumen into the Peyer's patches also occurs via M cells, and is essential to establish infection via the oral route (Heppner et al. 2001; Donaldson et al. 2012; Donaldson et al. 2016). The uptake and transport of certain microorganisms and particulate antigens by M cells is mediated via the express of a range of distinct receptors on their apical surfaces (Mabbott et al. 2013). This raises the possibility that the sampling of TSE agents by M cells is also mediated via a particular receptor. For example, M cells PrPC and this may be exploited by pathogenic bacteria such as *Brucella abortus* to establish host infection (Nakato et al. 2012). However, studies using PrP-deficient mice, or mice with *Prnp*-deficiency restricted to the gut epithelium, show that prion uptake from the gut lumen into Peyer's patches occurs independently of PrPC expression the M cells and the gut epithelium (Kujala et al. 2011; Marshall et al. 2018).

The specialised basolateral pocket beneath the M cells contains lymphocytes and mononuclear phagocytes (a heterogeneous population of macrophages and conventional dendritic cells, cDC) that sample the particles that pass through the M cells. Once the TSE agents are delivered across the gut epithelium by M cells, they are subsequently acquired by cDC, and used by them as "Trojan horses" to establish infection in the Peyer's patches (Raymond et al. 2007; Bradford et al. 2017). The cellular PrP is expressed on the surfaces of most immune cell populations, raising the hypothesis that the interaction of mononuclear phagocytes with TSE agents is PrP^C-dependent. Mononuclear phagocytes, such as lymphocytes, derive from haematopoietic precursors in the bone marrow. However, the delivery of prions into the SLO is not affected if *Prnp*-expression is ablated in bone-marrow-derived cells (Brown et al. 1999; Klein et al. 1998), demonstrating that the uptake of TSE agents by cDC occurs independently of PrP^C-expression.

Once the TSE agents have been successfully delivered into LRS tissue, they are acquired by follicular dendritic cells (FDC) in the B cell follicles. The accumulation and amplification of the TSE agents upon the FDC is essential for their efficient spread to the CNS, as disease susceptibility is reduced or blocked in their absence (Mabbott et al. 2000; Montrasio et al. 2000). FDC are long-lived stromal-derived cells that trap and retain native antigens on their cell surfaces for long periods, and are important for the effective induction of antibody responses to the trapped antigens by B cells (Aguzzi et al. 2014). Studies have used the non-bone marrow origin of FDC to determine whether FDC are sites of TSE agent amplification. Briefly, mismatches were created in *Prnp* gene expression between the FDC-containing stroma and the surrounding lymphocytes and mononuclear phagocytes in the LRS. These studies showed that TSE agent accumulation and amplification was

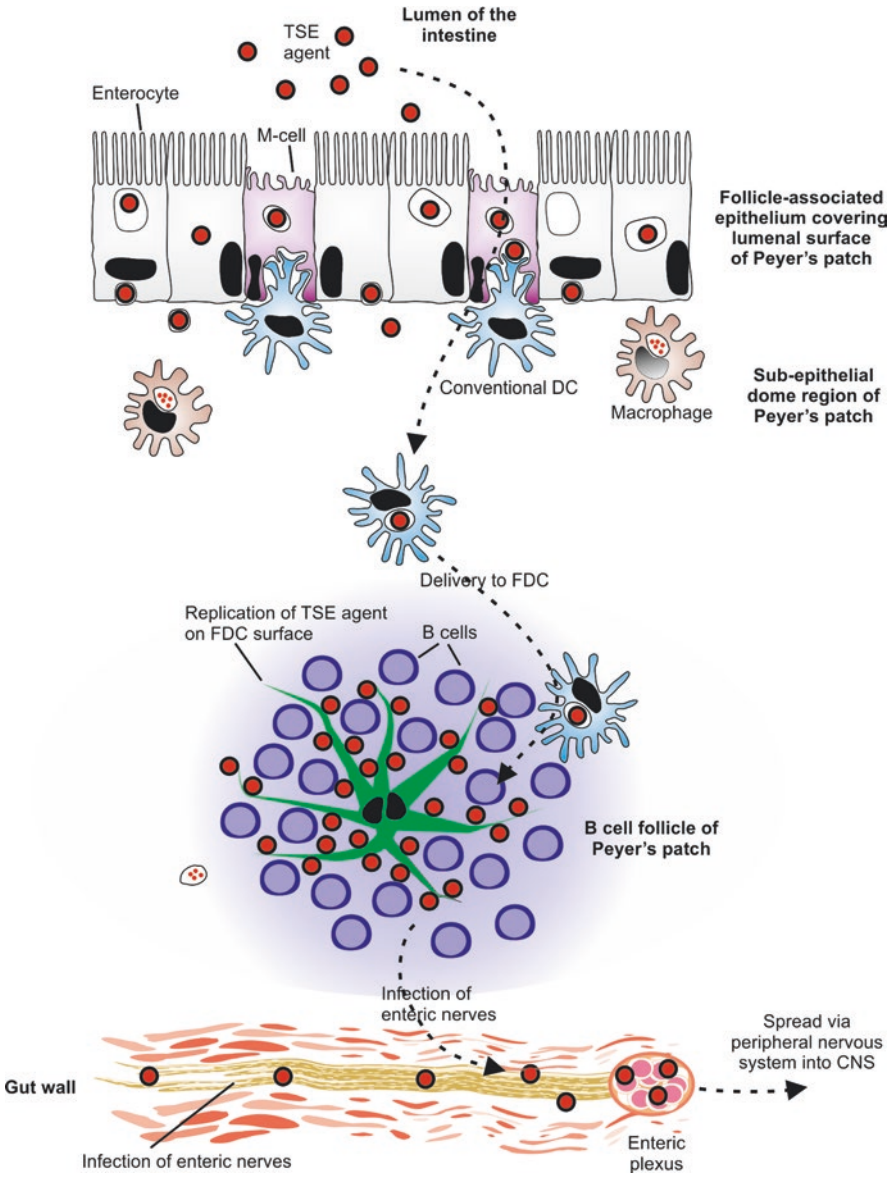

Fig. 15.1 Cells involved in the spread of prions from the intestine to the central nervous system (CNS). After oral exposure, the replication of prions upon follicular dendritic cells (FDC) in the Peyer's patches in the intestine is essential to establish host infection. With the Peyer's patches, the prions exploit an elegant cellular relay to make their way from the lumen of intestine to the nervous system. [Originally published in Mabbott (2017). Figure copyright owned by NAM]

only detected in association with the FDC when the stroma expressed *Prnp*. (Brown et al. 1999; Klein et al. 1998). Subsequent studies used the Cre-LoxP system to conditionally "switch on" or "switch off" *Prnp* expression only in mouse FDC. Data from this study similarly showed that prion accumulation and amplification upon FDC was only detected when the FDC expressed PrP^C on their surfaces (McCulloch et al. 2011).

Highly immunodeficient SCID mice lack mature FDC in their SLO and are refractory to infection with TSE agents via peripheral routes. However, these mice succumb to infection with mouse-passaged scrapie agents when injected directly into the brain (intracerebral injection) (Fraser et al. 1996). In contrast, SCID mice are refractory to infection with primary BSE agents even after intracerebral injection (Brown et al. 1997). These contrasting data from transmission in FDC-deficient SCID mice raise the hypothesis that the early accumulation of TSE agents upon LRS tissues is essential to establish infection after cross-species transmission. Indeed, studies have compared the ability of brain and SLO tissues of transgenic mice expressing ovine and human PrP^C isoforms to accumulate TSE agents after cross-species transmission. Beringue and his colleagues showed that the SLO tissues of transgenic mice were consistently more permissive than the brain to the accumulation and replication of TSE agents such those causing bovine spongiform encephalopathy after cross-species transmission (Béringue et al. 2012). Taken together, data from this study and those using SCID mice (above) suggest that the FDC may play an important role in the initial adaptation of the TSE agent to the new host to enable it to subsequently infect the nervous system. Experiments using transgenic mice with FDC expressing PrP^C from different host species may help to understand the role of the FDC in the cross-species transmission of TSE agents.

Together, data from the above studies clearly demonstrate that FDC are the essential sites of TSE agent accumulation in LRS tissues. This has raised the hypothesis that after infection peripheral route the FDC play an important role in the amplification of TSE agents above the threshold required to establish infection in the nervous system. However, little is known of how the TSE agents are subsequently propagated from the FDC to the nervous system.

Expression of PrP^C is required for replication of the agent and its transport to the CNS. When neurografts from either wild-type or PrP over-expressing (*Tga*20) mice were placed in *Prnp* knockout mice and a TSE agent inoculated via the peripheral route, neither clinical symptoms of disease nor neuropathology were observed in the recipients of the neurografts, indicating that the spread of disease from the spleen to the brain was impaired (Blattler et al. 1997). To understand the role that PrP expression in the LRS plays in neuroinvasion, neurografted *Prnp* knockout mice were lethally irradiated and reconstituted with lymphohematopoietic stem cells derived from *Tga*20 or wild-type mice. Following i.p. or intravenous (i.v.) inoculation with scrapie, these mice failed to show any signs of pathology, indicating that a non-haematopoietic PrP-expressing tissue is required for the transfer of infectivity between the spleen and brain (Blattler et al. 1997).

If PrP^C is indeed a requirement for transport of infectivity from the periphery to the CNS, then removing PrP in a tissue specific and temporal manner should

establish the importance of particular cell types in the disease process. Models in which PrP^C expression is selectively removed from various cells have, therefore, been developed. Peripheral nerves are thought to be a major routing of infectivity from the periphery to the CNS (McBride et al. 2001) and Schwann cells were implicated in this transport (Follet et al. 2002). A model was developed using the Cre/*LoxP* system in which PrP expression was removed from Schwann cells (Bradford et al. 2009). This resulted in a 90% reduction in the level of PrP^C including loss of all glycosylated forms in peripheral nerves, with no adverse effects reported in myelin morphology or integrity. This model was challenged with two well-characterised mouse-passaged scrapie agent strains, ME7 and 139A via peripheral routes of infection. Removal of PrP expression from Schwann cells had no effect upon TSE neuroinvasion and no statistically significant differences in IP were observed between Schwann cell PrP knockout mice and controls. Thus, whilst Schwann cells express the majority of PrP in the peripheral nerves, this expression is not required for TSE neuroinvasion. This raises further questions as to the role that different cells play in the transport of infectivity.

15.5 Crossing the Species Barrier and Strain Adaptation

Transgenic mice expressing heterologous protein often allow us to overcome the species barrier and assess the risk of a TSE crossing from one species to another and to model intraspecies transmission. This is of particular importance in assessing the risk to human health from TSEs. To achieve this, both overexpressing and gene targeted mice expressing human PrP^C have been produced which carry each of the codon 129 genotypes. Codon 129 is of particular interest in humans as this codon has been shown to play a role in susceptibility, IP length and phenotypic variation of sCJD and acquired CJD, fatal familial insomnia, and variably protease-sensitive prionopathy (Collinge et al. 1991; Palmer et al. 1991; Saba and Booth 2013; Zhang et al. 2022; Baiardi et al. 2022; Baiardi et al. 2021). Hill et al. (1997) showed that BSE could transmit to mice overexpressing human PrP (129VVTg-152); however, incubation times were relatively long (602 days compared with 371 days for FVB wild-type mice) and transmission rates were low (38%). These initial studies showed that there was potentially a significant barrier between BSE and human PrP. Using gene-targeted mice expressing human PrP^C, Bishop et al. (2006) failed to transmit BSE to mice expressing human PrP, whereas the same inoculum gave 100% positive transmission to transgenic mice expressing bovine PrP. The combination of these two sets of data suggests a significant species barrier between BSE and humans; this may explain why despite the extensive exposure of the UK population to BSE; only 178 UK vCJD cases have occurred so far (https://www.cjd.ed.ac.uk/surveillance/data-and-reports).

In comparison with BSE, vCJD has been shown to transmit to both overexpressing and gene-targeted mice expressing human PrP with varying susceptibility depending on the host genotype at the 129 codon of the human *PRNP* gene (Bishop

et al. 2006; Hill et al. 1997; Asante et al. 2002). This demonstrated that human-to-human transmission of vCJD was possible with all genotypes having the potential to be infected. The results from the mouse studies suggest that MV and VV genotypes may have a longer IP or may not develop clinical disease (Bishop et al. 2006). These findings have been borne out by four blood transfusion-associated cases of vCJD, including an asymptomatic case of 129MV vCJD (Peden et al. 2004) and, in 2016, the first clinical 129MV vCJD case (Mok et al. 2017). Both 129MV cases have been shown to be infectious via wild-type or transgenic mouse transmission studies (Boyle et al. 2020; Diack et al. 2019). Additionally the results of three UK retrospective studies aimed at estimating prevalence of PrP^{Sc} in the UK population found positive appendix samples in all three *PRNP* codon 129 genotypes (Gill 2013, 2020; Hilton et al. 2004). These are now the subject of ongoing transmission studies.

There are ongoing concerns that animal TSE diseases, such as chronic wasting disease (CWD), classical and atypical scrapie, sheep, and/or goat BSE, and H-type and L-type BSE could be transmissible to humans. CWD tissue homogenates have so far failed to transmit clinically and pathologically to mice overexpressing human PrP: 129MM Tg40 and 129MM Tg1 (expressing one- and twofold, respectively) (Kong et al. 2005), 129MM Tg35, 129MM Tg45, and 129VV Tg152 [expressing 1, 2, 2, 4 and 6 (Kong et al. 2005)], 129MM Tg35, 129MM Tg45 and 129VV Tg152 (expressing one-, two-, two-, four- and sixfold, respectively), which are susceptible to both human and BSE prions (Sandberg et al. 2010), Tg(HuPrP)440 (expressing twofold) (Tamguney et al. 2006), and gene-targeted mouse models: HuMM, HuMV and HuVV (Wilson et al. 2012). However, studies using a combination of PMCA and bioassays now demonstrate that PMCA-generated CWD-derived human PrP^{Sc} can transmit to 129MM Tg40 and TgVV mice with signs of clinical disease and neuropathology, thus suggesting the potential for zoonotic transmission (Wang et al. 2021). The zoonotic potential of atypical and classical scrapie has been tested in a number of studies with contrasting results dependent on the mouse models used. Gene-targeted mice challenged with natural sheep and goat scrapie or atypical sheep scrapie failed to show signs of disease at primary passage (Wilson et al. 2012; Plinston et al. 2011). Transgenic mice overexpressing human PrP have shown negative results at primary passage (Tg35, Tg152); however, in later studies using 129MM Tg340, 129VV Tg361 (both fourfold), the F1 cross, and 129MM Tg650 (sixfold), subclinical disease was detected in the F1 cross and tg650 mice. Subpassage in the same moues lines gave rise to clinical disease and PrP^{TSE} distribution identical to that of sCJD in the mice (Cassard et al. 2014). These data indicate that although a significant species barrier exits, it is possible to transmit scrapie to mice expressing human PrP. In terms of BSE, using tg650 mice which are fully susceptible to vCJD, Beringue et al. showed that classical BSE transmits relatively inefficiently (4/25 mice), whilst L-type BSE shows 100% transmissibility and H-type BSE does not transmit at all to this mouse model. These results were replicated by Marín-Moreno et al. (2020) using different transgenic mouse lines. Both sheep and goats are experimentally susceptible to classical BSE, and confirmed and suspected cases of goat BSE have been reported (Jeffrey et al. 2006; Eloit et al. 2005). There is potential that following passage through another species, BSE strain

characteristics could alter and become more virulent to man. In order to model this, experimental sheep BSE was transmitted to humanised mice with 70% of HuMM mice showing pathological signs of TSE disease, and no other genotype of mice was affected (Table 15.1). Padilla et al. (2011) later showed similar results with sheep and goat BSE using two lines of methionine homozygous overexpressing mice (tg650 and tg340). These results would suggest that ovine passaged BSE and L-type BSE pose a greater zoonotic risk than classical BSE.

15.6 Defining Strains of TSE Agents

Many TSE strains are characterised by a range of phenotypic properties in vivo following experimental transmission of the infectious agent into wild-type mice. Upon serial passage, the characteristics of a given strain or isolate stabilise, resulting in highly reproducible combinations of the IP of disease, PrP^{Sc} biochemical profile as assessed by western blot and the amount and distribution pattern of vacuoles, PrP^{Sc} deposition in the brain, and reactive astrocyte heterogeneity in CD44 upregulation (Bruce et al. 1999; Ritchie et al. 2009; Dickinson et al. 1968; Bradford et al. 2019). However, some strains of agent do not readily transmit to wild-type mice, i.e., sCJD, and in these cases, transgenic mouse panels using mice in which the murine PrP sequence has been replaced by that of another species have proved to be useful (Bishop et al. 2006; Bishop et al. 2010). Inbred gene targeted lines prove particularly useful in this respect as the mice are genetically identical except for the replaced PrP-coding sequence.

Using a panel of transgenic humanised-PrP mice, Bishop et al. (2010) sought to establish whether there were different strains of sCJD. Clinico-pathological phenotypes of sCJD can be sub grouped according to host codon 129 genotype and the biochemical characteristics of PrP^{Sc} (Parchi et al. 1999; Brown et al. 1994a; Hill et al. 2003; Cali et al. 2006). A typical case from each of the six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2) was inoculated into HuMM, HuMV, and HuVV

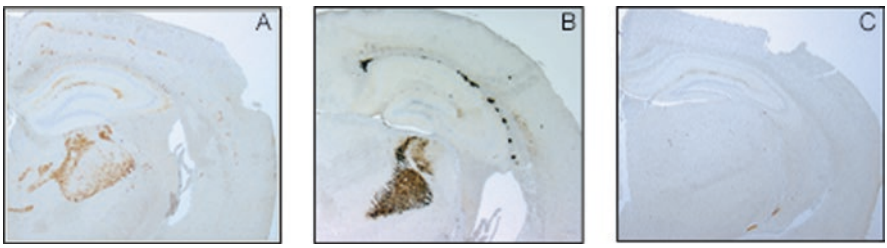

Fig. 15.2 Comparison of immunocytochemistry for PrP in transgenic mice expressing the human 129MM genotype (HuMM) challenged with A) $M1^{CJD}$ (MV1), B) $V2^{CJD}$ (MV2) and C) $V1^{CJD}$ (VV1). V2CJD did not transmit to HuMM mice. Immunocytochemistry with ant-PrP antibody (6H4) was performed on histological sections of the mouse brains. Representative sections are shown through the hippocampus/thalamus (Magnification: 2.5×)

mice and four distinct strains emerged: $M1^{CJD}$ (MM1, MV1); $M2^{CJD}$ (MM2); $V1^{CJD}$ (VV1); and $V2^{CJD}$ (MV2 and VV2) (Table 15.1, Fig. 15.2). MM1 and MV1 ($M1^{CJD}$) isolates showed identical transmission characteristics based upon IP, vacuolation profiles, western blot profile, and PrP^{Sc} deposition patterns. MV2 and VV2 ($V2^{CJD}$) isolates showed similar characteristics, whilst MM2 ($M2^{CJD}$) and VV1 ($V1^{CJD}$) isolates behaved differently from each other and other isolates (Bishop et al. 2010). Thus, four strains of sCJD were identified in this study. Similar conclusions for the number of strains of sCJD were also reached using an in vitro study (Uro-Coste et al. 2008). The in vivo strain typing approach is now being utilised to define new human and animal strains of disease identified through surveillance programmes (Table 15.1) and is also being used to assess whether vCJD cases from different countries arise from a single strain of agent.

It is important to establish whether human-to-human transmission of vCJD, i.e., through blood transfusion, could lead to strain modification particularly if is transmitted to individuals carrying different alleles of *PRNP*. These studies can be carried out in two ways: (1) by studying cases where it has been established that the vCJD has arisen by human-to-human transmission or (2) by modelling such transmission in transgenic mice carrying the different *PRNP* alleles. In the first instance, cases of human-to-human transmission such as the blood associated cases can be inoculated into both the humanised mice panel and a wild-type strain typing panel. The resulting data can then be compared between the donor and recipient of the contaminated blood and with vCJD cases associated with transmission from BSE. This comparison allows us to define whether the human-to-human passage has caused any strain modifications or changes in virulence of the disease. Initial studies by Bishop et al. (Bishop et al. 2008) from an MM donor to an MM recipient have shown that there is no change in the transmission efficiency of the vCJD agent following human-to-human transmission modelled in this manner (Bishop et al. 2006). Further studies will assess the effect of different *PRNP* genotypes on strain characteristics where possible. The second approach uses humanised mice to model human-to-human passage by carrying out serial passage of the vCJD agent. This allows us to study which TSE agents can adapt to which hosts and whether certain genotypes are more susceptible to human-to-human transmission. At present, a study performing second and third passage of vCJD in humanised mice is showing that there is no adaptation to the host and that virulence is decreasing with each passage (Diack et al., unpublished).

15.7 Mechanisms of Neurodegeneration

At the clinical endpoint of TSE disease, there is characteristic vacuolation, PrP^{Sc} deposition, and neuronal and synapse loss in various areas of the brain. The targeting of these pathologies is modulated by both strain and host factors. It has been shown that PrP^{Sc} is not directly neurotoxic and (Benilova et al. 2020) and it is still to be determined if loss of function of PrP^{C} plays a role in rendering neurons

susceptible to degeneration. Neurodegenerative diseases have traditionally been considered as cell-autonomous processes in which the damage within a population of affected neurons alone is sufficient to produce disease. However, much evidence now exists to suggest that other populations of cells within the CNS may contribute to the process of neurodegeneration for many of these diseases. These non-neuronal mechanisms are described as non-cell autonomous neurodegeneration. Gliosis is evident early in the preclinical phase of disease and transcriptomic investigation reveals changes in glia and specifically astrocytes much earlier than neuronal changes; prion-mediated neurotoxicity has been associated with astrocyte activation (Ilieva et al. 2009; Kushwaha et al. 2021).

Disease IP in the prion diseases is related to the amount of total PrP^C in the brain (Scott et al. 1989; Manson et al. 1994b) and predicted by astrocyte activation (Makarava et al. 2021). PrP is found throughout neuronal cells of the brain but with variable levels in different neuronal populations (Kretzschmar et al. 1986). *Prnp* mRNA and PrP protein have also been described in non-neuronal cell types in the CNS (Baker et al. 2002; Moser et al. 1995; van Keulen et al. 1995). However, the high levels of expression in the neuronal cells of the CNS have been the focus in defining mechanisms of neurodegeneration in the prion diseases. Template-induced misfolding of PrP^C to PrP^{Sc} is thought to occur on the neuronal cell surface or within neuronal cells and lead to neurodegeneration through accumulation of the misfolded protein in and around the neuronal cell (Bruce et al. 1994; Jeffrey et al. 1994). Strong evidence for a cell autonomous neurodegenerative mechanism has been provided from in vivo studies with transgenic mice designed to express PrP^C in neurons only, which were shown to be susceptible to TSEs and from in vitro studies, where cultured neurons which do not express PrP have been shown to be resistant to neurodegeneration from toxic fragments of PrP (Brown et al. 1994b). Moreover, further evidence for cell autonomous processes was provided using a model in which PrP^C expression was removed from neurons at a specific timepoint during the course of disease. The disease process appeared to be blocked by the removal of neuronal PrP with the reversal of TSE spongiform pathology and behavioural deficits (Mallucci et al. 2007).

In support of non-cell autonomous neurodegeneration, it has been demonstrated that transgenic mice expressing PrP in astrocytes experienced neurodegeneration (Jeffrey et al. 2004) and succumbed to TSE disease. More refined models, however, reveal that astrocyte-specific expression of PrP^C can support prion replication without glial activation, neuroinflammatory responses or neurodegeneration (Lakkaraju et al. 2021a). This study revealed a non-autonomous mechanism by which prion-infected neurons instruct astrocytes (and microglia) into a neurotoxic state that drives prion neuropathogenesis. Similarly, investigation into transgenic mice expressing PrP in a range of cell types has suggested multiple neurodegenerative mechanisms in brain and retina (Chesebro et al. 2005; Kercher et al. 2004, 2007), dependent upon which cell types are expressing PrP. Both astrocyte and neuronal primary cultures have been shown to sustain prion infection (Cronier et al. 2004; Taraboulos et al. 1990).

A triple transgenic knockout model removing TNF-α, IL-1α, and C1qa was used to determine the role of microglial activation of neurotoxic astrocytes. Prion infection in these mice led to an accelerated disease course with early dysregulation of microglia and abolishment of complement $C3^+$ astrocytes . In order to address the roles of glial cells in prion neurodegeneration, we have produced a model in which the Fms intronic regulatory element (FIRE) was removed from the colony stimulating factor 1 receptor (CSF1R) gene via CRISPR/Cas9 technology. The resultant mice completely lack microglia within the CNS, but develop normally. Prion challenge within these mice led to much shorter survival times without impacting upon the rate of PrP^{Sc} accumulation within the CNS. Accelerated astrocyte activation was observed including enhanced unfolded protein responses (UPR) and synaptic engulfment by reactive astrocytes. These data reveal that microglia do not play a role in uptake and removal of PrP^{Sc} but instead function to restrict astrocyte activation and neuro- and synapto-toxic activities .Prion infection in these mice led to an accelerated disease course with early dysregulation of microglia and abolishment of complement $C3^+$ astrocytes (Hartmann et al. 2019). In order to address the roles of glial cells in prion neurodegeneration, we have produced a model in which the Fms FIRE was removed from the CSF1R gene via CRISPR/Cas9 technology. The resultant mice completely lack microglia within the CNS but develop normally (Rojo et al. 2019). Prion challenge within these mice led to much shorter survival times without impacting upon the rate of PrP^{Sc} accumulation within the CNS. Accelerated astrocyte activation was observed, including enhanced UPR and synaptic engulfment by reactive astrocytes. These data reveal that microglia do not play a role in uptake and removal of PrP^{Sc} but instead function to restrict astrocyte activation and neuro- and synapto-toxic activities (Bradford et al. 2021).

UPR-reactive astrocytes have been shown to fail to support synapses due to altered secretome constituents. Targetted genetic modification of the UPR pathway in astrocytes was performed using a lentiviral vector to overexpress an active fragment of the Growth Arrest and DNA damage-inducible protein GADD34. GADD34 inhibition of astrocyte UPR responses prior to synapse loss during prion infection resulted in profound synaptic and neuroprotective responses and mild prolongation of survival time comparable to neuronally targetted UPR interventions (Moreno et al. 2013). This intervention had no impact on PrP^{Sc} accumulation (Smith et al. 2020).UPR upregulation has also been shown to drive prion-specific vacuolation by impaired acylation and degradation of the phosphoinositide kinase PIKFyve and delocalisation of PIKfyve-specific acyltransferases resulting in the enlargement of endolysosomes and vacuole formation (Lakkaraju et al. 2021b). Prion-induced vacuole formation is impaired in lysosomal trafficking regulator (LYST) protein mutant animals that phenocopy Chediak–Higashi syndrome, including LYST/Beige mice (Marsh et al. 1976).

In conclusion, the conversion of PrP^{C} to PrP^{Sc} in neurons, possibly at synapses, results in the activation of astrocytes, upregulation of the UPR, synapse loss, vacuolation and ultimately neuronal loss in prion diseases. Microglia play a role in restricting astrocyte activation and thus preserving synapses and neurons in the early stages of prion disease.

15.8 Conclusion

Transgenic mouse models have made a major contribution to our understanding of TSEs particularly in the assessment of zoonotic potential and modeling intraspecies transmission, where the host species may be large animals or humans. Using gene targeted or knock out mice to understand the pathogenesis of TSE disease allows us to unravel the mechanisms of prion replication and the infective process whilst also providing a model for other neurodegenerative protein misfolding diseases. As new techniques in transgenic production are implemented in these studies and the use of other transgenic models involving non-*Prnp* factors to investigate prion pathogenesis and replication, we can only expect our knowledge and understanding of the TSEs to increase and move towards defining intervention and treatment strategies for these devastating diseases.

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Chapter 16
Stem Cell Models in Prion Research

Cathryn L. Haigh

Abstract Stem cells have the capacity to differentiate into the mature cells of any organ within the body. For this reason, they offer an interesting opportunity to model many cellular systems and their associated diseases. Prion diseases (PrDs) are a family of fatal neurodegenerative diseases caused by mis-folding of the prion protein (PrP), a protein lacking consensus on its native function. Stem cell models have been used for investigating PrP function and PrDs for over two decades. Within this time, the models and the understanding of their use have been substantially expanded. Herein, the utilizations of stem cell models and the contribution to knowledge that has emerged from their use are summarized.

Keywords Prion · CJD · Stem cells · Cerebral organoid · Neurosphere · Neural stem cell · Progenitor

16.1 Introduction

Prion diseases (PrDs) or the transmissible spongiform encephalopathies (TSEs) are a family of transmissible neurodegenerative diseases affecting humans and animals. In humans, as well as being acquired, they can occur due to mutation within the prion protein (PrP) gene (PRNP). Hereditary human diseases include Creutzfeldt–Jacob Disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), and fatal familial insomnia. These diseases can also occur with no known cause, referred to as sporadic disease. Sporadic CJD (sCJD) is the most common sporadic PrD and also accounts for the majority of all human PrDs (Knight 2017). Transmissible diseases that occur due to infection of an individual include variant CJD (vCJD), which

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arose after meat contaminated with bovine spongiform encephalopathy (BSE) entered the food chain in the UK in the 1980s, and kuru, arising from ritualistic cannibalism during funeral practices by the Fore tribe of Papua New Guinea (Asher and Gregori 2018; Knight 2017). Additionally, iatrogenic CJD has occurred by introduction of infection through contaminated blood transfusion, surgical instruments, and human-derived products, such as growth hormone (Asher and Gregori 2018; Douet et al. 2021; Kobayashi et al. 2018; Uttley et al. 2020). Despite the concerns over transmissibility, such events are fortunately rare.

The family of PrDs is caused by the mis-folding of the host-derived PrP into infectious isoforms known as prions. Prions have specific biochemical behaviors including the production of protease-resistant species (PrP^{Res}) and the ability to seed the formation of more of themselves. These characteristics allow the mis-folded isoforms to be detected in vitro. PrP^{Res} can be detected by western blotting for PrP following protease digest and the self-propagating attributes can be detected by amyloid seeding assays, such as Real-Time Quaking-Induced Conversion (RT-QuIC) assays (Atarashi et al. 2011; Peden et al. 2012; Wilham et al. 2010). Another interesting feature of prions is that slight variations in the mis-folded structure cause the emergence of prion strains that may manifest with different symptoms and incubation times. In sCJD, the variations in mis-folding cause different subtypes of the disease, which can be identified by changed mobility of PrP^{Res} when examined by western blotting. It is thought that these small changes in the mis-folded structure lead to selective neuronal vulnerability causing different brain regions to be attacked disproportionately in different PrDs (Jackson 2014; Sakaguchi et al. 2020).

Many models of PrD have been developed, including in vivo, in vitro, and ex vivo models (Krance et al. 2020; Pineau and Sim 2021; Watts and Prusiner 2017). Stem cell-derived cultures present an in vitro system with some properties that differ from traditional cell culture models. There are numerous characteristics of stem cells that can be utilized for PrD research. The ability of progenitors to differentiate into their downstream cell types allows consideration of prion biology and disease within the terminal cell types. Stem cells can also be used to understand the role of host PrP in development and its cellular level function within progenitors. Most recently, the development of human-induced pluripotent stem cell (iPSC) technology has paved the way for the development of human models of prion infection and disease in living neuronal tissues. This chapter aims to summarize the contribution of stem cell models in our discoveries to date.

16.2 Stem Cell Models Used in Prion Research

Herein, the focus will be on three stem cell models that have featured in CNS prion research, although others have been used and will be referred to where relevant. These three models include murine 1C11 cells, murine stem cells, and human iPSCs. The 1C11 model was developed by manipulation of F9 multipotent embryonic carcinoma cells (Mouillet-Richard et al. 2000b). 1C11 cells behave as a

neuroectodermal progenitor line and their differentiated neuronal progeny may assume serotonergic or noradrenergic phenotypes. Mouse stem cells can be harvested from embryonic, post-natal, or adult brain tissue. Embryonic stem (ES) cells are pluripotent (able to generate cells of any germ cell linage: endoderm, ectoderm, or mesoderm), whereas adult stem cells are multipotent, confined to producing cells of a specific lineage. Adult stem cells are usually referred to as progenitor cells or, in the case of neural progenitors, neural stem cells (NSCs) to reflect their commitment to a lineage (Gage et al. 1995). NSCs are constrained to produce cells of neural lineage, including neurons, astrocytes, and oligodendrocytes, and are often grown in suspension culture, as small cell aggregates called neurospheres (Belenguer et al. 2016; Deleyrolle and Reynolds 2009; Gil-Perotín et al. 2013). Neurospheres are not pure NSC cultures but also contain some transit amplifying cells and committed progenitors (Obermair et al. 2010; Belenguer et al. 2016). Human iPSCs are 'induced' by reprogramming an alternative cell type collected from a donor (Takahashi et al. 2007; Yu et al. 2007); typically, in prion research, fibroblasts have been used (Matamoros-Angles et al. 2018; Foliaki et al. 2020; Gonzalez et al. 2018). Human iPSCs are pluripotent; they can become cells of any lineage allowing the study of various cell types by modulating the conditions under which the cells are differentiated.

16.3 Stem Cell Models for Elucidating Prion Cell Biology and Function

One application of stem cell models in prion research is for generating terminally differentiated cultures, which can be used to investigate PrP function in different neuronal cells. Substantial insights into the influence of PrP within different cell types have been generated using these systems. For example, studies using the 1C11 differentiated progeny cells identified the role of PrP in signal transduction through NADPH oxidase and ERK1/2 via Fyn kinase (Mouillet-Richard et al. 2000a; Schneider et al. 2003). It was additionally shown that PrP-dependent activation of the Fyn signal transduction cascade occurs only in mature neuronal cells, not in immature progenitor cells, and was localized within neurites (Mouillet-Richard et al. 2000a). In the noradrenergic and serotonergic progeny, this pathway required caveolin as part of the Fyn recruitment and signaled through NADPH oxidase and ERK1/2 (Schneider et al. 2003). The location of the signaling activation within the neurites and the involvement of membrane domains in recruitment suggest that the location of PrP within the cell membrane could be critical in influencing its signaling functions.

In similarity with the 1C11 cells, NSC cultures can be differentiated into their terminal cell types. Using cultures of neurons, astrocytes and oligodendrocytes differentiated from murine NSCs in combination with primary cultures of microglia, it was found that a cleavage fragment derived from the N-terminus of PrP, designated

the N1 fragment, could modulate the phenotype of the differentiated NSCs and both the phenotype and interactions of the microglia, especially with astrocytes (Carroll et al. 2020). The N1 fragment mediated its effect through changing GM1-rich membrane domains at the contact sides of microglia with surrounding cells and increasing secretion of the cytokine CXCL10, without the latter reaching toxic levels. Adding exogenous CXCL10 alone was sufficient to cause the phenotypic changes in the differentiated NSC cultures. These data showed a new role for the N1 fragment in brain cell homeostasis and further underscored the importance of cell membrane interactions in normal PrP signal transduction.

16.4 Stem Cell Models for Understanding Normal PrP Function in Progenitors

As well as providing terminally differentiated models for elucidating intra- and inter-cellular interactions, stem cells offer an opportunity to investigate the role of PrP in progenitor growth, migration, and differentiation. During differentiation, pluripotent stem cells slow their self-renewal and become committed to a lineage pathway. At this stage, these progenitors are multipotent; although committed to a pathway, they can become any cell within that lineage. Committed progenitors can then differentiate and mature into the terminal cell type. Adult neurogenesis has been extensively investigated in mice. In the mouse brain, there are two well-characterized stem cell niches, the subgranular zone of the dentate gyrus and the sub-ventricular zone (SVZ) (Nogueira et al. 2021). Further areas of neurogenic activity have additionally been identified, although some controversy exists over whether stem cells originate within these areas or migrate from the SVZ (Jurkowski et al. 2020). Progenitors from the dentate gyrus replenish hippocampal neurons and SVZ progenitors the olfactory bulb. When neuronal differentiation begins in the SVZ, the progenitors migrate out of the SVZ into the rostral migratory stream and from here track to the olfactory bulb, where they mature (Bressan and Saghatelyan 2020; Kaneko et al. 2017). Many chemical signals and proteins control this progression to ensure that it proceeds to meet the demand for mature cells without depleting critical progenitor cell pools. PrP has been identified as one protein that can modulate these processes, and this is illustrated in Fig. 16.1.

Using human ES cells incubated with recombinant PrP, folded into the predominantly alpha-helical conformation associated with the normal cellular form of PrP, it was found that the increased concentration of soluble PrP maintained the ES cells in a highly proliferative state and delayed spontaneous differentiation (Lee and Baskakov 2010). Follow-up studies found that normal PrP influenced the function of the most immature stem cells with the neural progenitors being less affected by the presence or absence of PrP (Lee and Baskakov 2014). Data from in vivo studies support that extracellular, possibly secreted soluble PrP, may be responsible for controlling NSC proliferation and differentiation. Studies by Steele et al. found that PrP

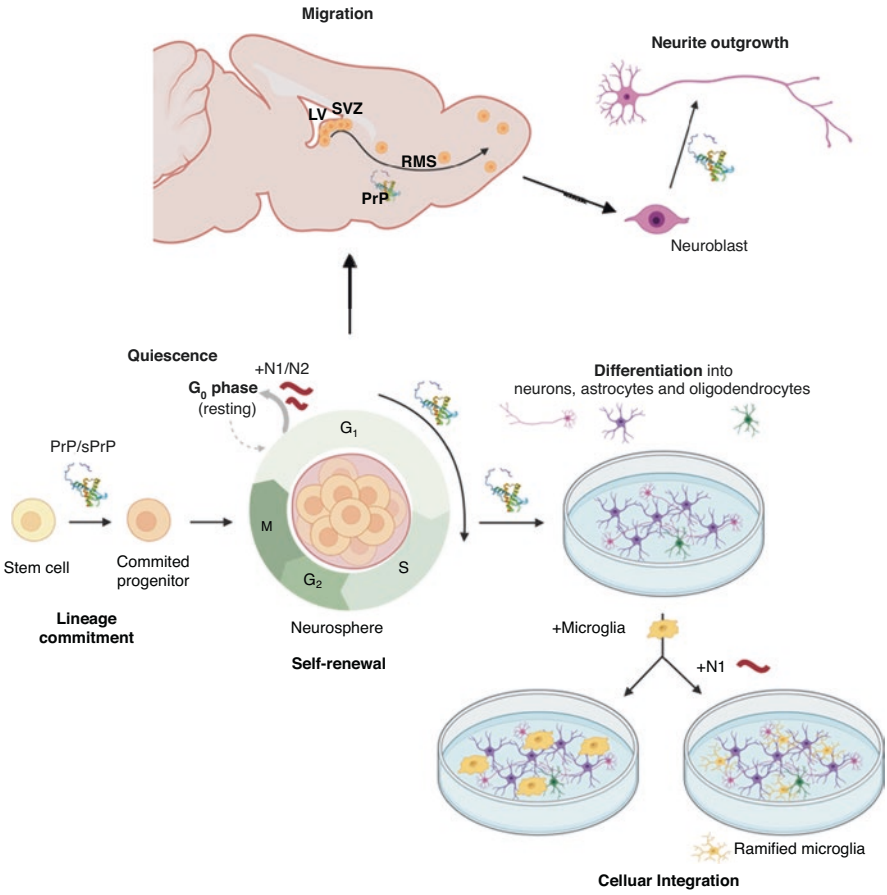

Fig. 16.1 *Schematic diagram showing PrP modulation of neurogenesis.* PrP and N-terminal cleavage fragments derived from PrP have been shown to influence most aspects of neurogenesis. From the earliest phases of stem cell lineage commitment through increasing cell cycling/self-renewal of NSCs and balancing this push for self-renewal, by antagonizing cell cycling through stimulation of quiescence by the cleaved soluble N-terminal fragments, N1 and N2. PrP further influences migration through the rostral migratory stream (RMS) to the olfactory bulb and enhances differentiation, cellular integration, and neurite outgrowth. The N1 fragment additionally modulates microglia integration into differentiating cultures and interaction between cell types

expression levels enhanced cellular proliferation and positively influenced neuronal differentiation in both embryonic and adult neurogenesis (Steele et al. 2006). PrP expression was adjacent to but not detectable within the proliferating cells of the SVZ, suggesting PrP's influence was mediated by a soluble signal, possibly secreted PrP itself (Steele et al. 2006). A functional influence of secreted soluble PrP is further supported by studies, demonstrating that ADAM10, the enzyme responsible for PrP secretory cleavage (Altmeppen et al. 2011, 2012; Linsenmeier et al. 2018; Taylor et al. 2009), is also involved in neurogenesis. ADAM10 is known to cleave

several other proteins that influence neurogenic pathways, including intermediates in the Notch signaling pathway (Hartmann et al. 2002; Muraguchi et al. 2007; Bozkulak and Weinmaster 2009), which plays a major role in controlling the regulation of embryonic development and adult neurogenesis (Engler et al. 2018).

Further studies have investigated the PrP-signaling pathways that influence NSC self-renewal. It was found that PrP expression positively regulates NSC proliferation through the NANOG (Miranda et al. 2011) and the aforementioned Notch (Martin-Lannerée et al. 2017) signaling pathways. The Notch pathway was linked with controlling expression of epidermal growth factor receptor (EGFR). EGFR expression and EGF production control several aspects of neurogenesis, including NSC proliferation, progenitor pool enlargement, progenitor migration, and the lineage of the differentiated cells (Wong and Guillaud 2004). Studies in cancer cells also support a role for PrP in controlling the cell cycle from G1 to S phases (Liang et al. 2007) and expression of PrP with a mutation associated with hereditary PrD (four extra octarepeat insertions) within rabbit kidney cells stalls cell cycle progression at G2/M phase (Martín et al. 2006). Together, this indicates a functional role of PrP in stem cell proliferation.

It has been found that PrP can act both as a receptor and a ligand in modulating NSC growth. Membrane bound PrP is known to be a receptor for the amyloid-beta (Aβ) peptides associated with Alzheimer's disease (Brody and Strittmatter 2018; Laurén et al. 2009; Salazar and Strittmatter 2017; You et al. 2012), and in turn, NSC self-renewal is modulated by beta-amyloid peptides in a PrP-dependent manner (Collins et al. 2015). Aβ was found to suppress growth in NSCs expressing normal levels of PrP but to stimulate self-renewal in PrP knock-out NSCs.

As a ligand, PrP also has a role in the maintenance of quiescence. Adult NSCs exist in a mostly quiescent state until stimulated to grow and control of this is important for their preservation (Kokovay et al. 2012). Cleavage fragments derived from the N-terminus of PrP, N1 and N2, induced NSC quiescence by reducing intracellular reactive oxygen species (ROS) signaling from the NADPH oxidase family, which through induction of mitochondrial fission resulted in up-regulation the antioxidant protein SOD2 (Collins et al. 2018). Redox balance is essential for maintaining NSC quiescence and a redox cycle occurs with the cell cycle with fluctuating SOD2 levels controlling progression and exits to a quiescent G0 state (Sarsour et al. 2008, 2012 Menon et al. 2003). Mitochondrial fission also plays a role regulating the cell cycle, with mitochondrial morphology varying depending upon cell cycle stage (Horbay and Bilyy 2016; Spurlock et al. 2020). It should be noted that the enzyme responsible for the α-cleavage event that produces N1 is still unknown despite being a matter of debate for years (Altmeppen et al. 2011; Taylor et al. 2009). However, the production of N2 is known to be induced by heightened ROS (Mcmahon et al. 2001) and also by calpains (Yadavalli et al. 2004), both of which are implicated in control of neurogenesis (Dickinson et al. 2011; Baudry et al. 2021; Santos et al. 2012).

Many of the studies looking at the role of PrP in stem cell self-renewal also examined its role in directing differentiation. When PrP was knocked down in immature stem cells, their ability to differentiate into neural progenitors was

compromised (Peralta et al. 2011; Lee and Baskakov 2013, 2014). Differentiation into all three germ layer lineages was suppressed, but this was especially pronounced for differentiation into the ectoderm lineage that forms NSCs (Lee and Baskakov 2013, 2014). Prodromidou and colleagues found that more proliferating NSCs remained within the SVZ of PrP knock-out mice, because less entered the rostral migratory stream to become mature neurons compared with control mice (Prodromidou et al. 2014). The authors linked this with a direct binding of PrP to neural cell adhesion molecule (NCAM), which is a known PrP binding partner (Schmitt-Ulms et al. 2001). It has been proposed that other members of the PrP family share its neurogenic function, with combined knock-down of PrP and the related protein shadoo producing a lethal phenotype in mouse embryos (Young et al. 2009).

In addition, PrP influences neurite outgrowth, a critical part of neuronal maturation. This has been shown in numerous studies, and although there is no consensus on a central pathway, diverse interactions and signaling intermediates are implicated, including laminin, metabotropic glutamate receptors, LRP1, NMDA receptors, β1 integrin, Fyn, NCAM, caveolin-1, and stress-inducible protein 1 (Beraldo et al. 2011; Loubet et al. 2012; Mantuano et al. 2020; Santuccione et al. 2005; Graner et al. 2000; Lopes et al. 2005; Pantera et al. 2009). When considered altogether, these studies show that PrP influences all aspects of neurogenesis from the direction of the most immature cell to the final stages in neuronal maturation.

16.5 Murine Stem Cell Models for Studying Prion Disease

Using stem cell models for production of infected cultures is a desirable research direction for several reasons. First, the influence of infection on both the progenitor cells and their differentiated progeny can be examined, and second, stem cells can potentially propagate infection long term over many passages. Additionally, infected stem cell systems can be cryopreserved for long-term storage. Several murine stem cell models have been found to be permissive to prion infection.

Infection of the 1C11 progenitors showed no change in their ability to differentiate into their mature lineage cells, but changes were observed in the neurotransmitter levels within the mature cultures (Mouillet-Richard et al. 2008). Prion infection was further found to change normal cellular signaling, causing constitutive activation of the Fyn and ERK1/2 signal transduction intermediates and the CREB transcription factor. Additionally, overactivation of two other central signaling intermediates, p38 and JNK, was observed. The authors linked these overactivation events with increased Aβ detection, which positively correlated with the severity of prion infection in CSF taken from infected mice (Pradines et al. 2013). Recent work using the 1C11 model has found that PrP signaling through protein kinase A controls the expression of pyruvate dehydrogenase 4 and that this becomes corrupted during infection causing an imbalance in glucose metabolism (Arnould et al. 2021). These findings support a shift in signal transduction during disease that may result in toxic outcomes.

Propagation of prion infection has also been demonstrated in mouse neurospheres with the kinetics of PrP^{Res} production correlating with PrP expression of the NSCs (Giri et al. 2006). The authors found that they could propagate infection for at least 12 passages, substantially beyond the persistence of the initial infecting inoculum (Giri et al. 2006). However, a later study found that no PrP^{Res} could be detected by passage 20, and therefore, infection may be lost over time (Herva et al. 2010). This might be influenced by the density of passaged cells as has been reported for standard cell lines with low-density passaging resulting in faster loss of infection (Ghaemmaghami et al. 2007). Together, the studies suggest that mouse neurosphere cultures are permissible to prion infection and can remain infected for several passages.

Prion infection can also be established in cultures differentiated from neurospheres (Herva et al. 2010; Milhavet et al. 2006; Pradines et al. 2013). Herva et al. exposed the NSCs to infectious inoculum at the same time as beginning their differentiation and found propagation of prions up to 18 dpi. Propagation was dependent upon the infecting prion strain with mouse-adapted scrapie strains (22 L and RML) propagating well, but no propagation of the BSE derived 301C strain. Milhavet et al. only continued their cultures to 12 dpi but found that the increase in PrP^{Res} that they observed was likely influenced by cell fate, as modulation of the differentiation protocol altered the efficiency of infection. Prion replication in differentiated cultures causes significant cytotoxicity (Iwamaru et al. 2013; Haigh et al. 2011; Sinclair et al. 2013) with increased caspase activation (Iwamaru et al. 2013) and oxidative stress (Haigh et al. 2011). The differentiated NSC culture system has also been further modified to produce cultures from transgenic mice expressing the elk PrP sequence. These cultures were shown to propagate CWD prions from both elk and deer (Iwamaru et al. 2017), thus establishing the utility of the system for studying different animal diseases of zoonotic importance.

Further advancements of the prion-infected neurosphere model considered the three-dimensional (3D) nature of brain tissue. NSCs can be differentiated from neurospheres in 3D suspension cultures. In the 3D cultures, the differentiated neurons and astrocytes produce layers of cells with the astrocytes and remaining immature cells surrounding the outside of the culture and the neurons within the core (Collins and Haigh 2017). These 3D cultures, like their two-dimensionally cultured counterparts, also showed significant cytotoxicity when challenged with prion infection. Recent developments have shown that this 3D model can be infiltrated with primary microglia for a closer replication of brain tissue (Carroll et al. 2021), thus allowing neuroinflammation during prion infection to be investigated. Overall, the differentiated NSC system offers a versatile tool for investigating both infection of prions from different species in vitro and for studying the mechanisms of prion neurotoxicity.

16.6 Human Stem Cell Models of Prion Disease

16.6.1 Infectious Disease

A major limitation in the field of human prion research has been the lack of a completely human model system. Various systems have been tested over many years, with only one ever showing a promising result until recently. This first system, where Sh-SY5Y human neuroblastoma cells were exposed to sCJD prions and PrP^{Res} detected after 12 passages (Ladogana et al. 1995), ultimately was never widely adopted very likely due to inconsistencies inherent within the system (Kovalevich et al. 2021). A later study using human ES cells challenged with BSE, vCJD, and sCJD prions showed that these cells could internalize the prions and would then clear the infected material very quickly (Krejciova et al. 2011). The authors did not observe any de novo prion production but only followed the infections for 3 day post-removal of the infectious homogenates. An exciting development in human PrD models arose when Krejciova et al. discovered that astrocytes differentiated from human iPSCs could be infected with human prions (Krejciova et al. 2017). The astrocytes replicated the subtypes of the original infecting inoculum based upon their codon 129 genotype. This model represented the first on-demand human cell infections in vitro.

Our group was able to advance this model using a newly developed technology called human cerebral organoids (Lancaster et al. 2013; Lancaster and Knoblich 2014). Cerebral organoids are small, up to pea sized, free-floating cultures of human brain tissue with a limited ability to form self-organizing structure. They can be differentiated from iPSCs, gradually populating with different cerebral neuronal subtypes and with astrocytes and oligodendrocytes by approximately 5 months of age (Giandomenico et al. 2021; Lancaster and Knoblich 2014; Renner et al. 2017). We found that human cerebral organoids could be infected with prions from brain homogenate taken from people who had died of sCJD (Groveman et al. 2019). The organoids showed uptake and clearance of the original inoculum, followed by emergence of de novo prions. Both the efficiency of infection and organoid health were influenced by the infecting inoculum. We additionally found that infections within organoids could be used to monitor the efficacy of putative therapeutic molecules (Groveman et al. 2021a). In this context, the model offers some versatility. For example, treatment regimens can be designed to mimic prophylactic treatment, as might be used for people carrying hereditary mutations within PRNP or who have been exposed to a source of infectivity, or therapeutic treatment, which is required once symptoms have onset in a patient. The organoid model has substantial potential to be developed further, with potential applications including understanding the way different human prion subtypes manifest disease differently, how different cell types are affected and interact with each other, and cellular factors that modulate infection (Groveman et al. 2020, 2021b). A summary of organoid infection studies so far is included in Fig. 16.2a.

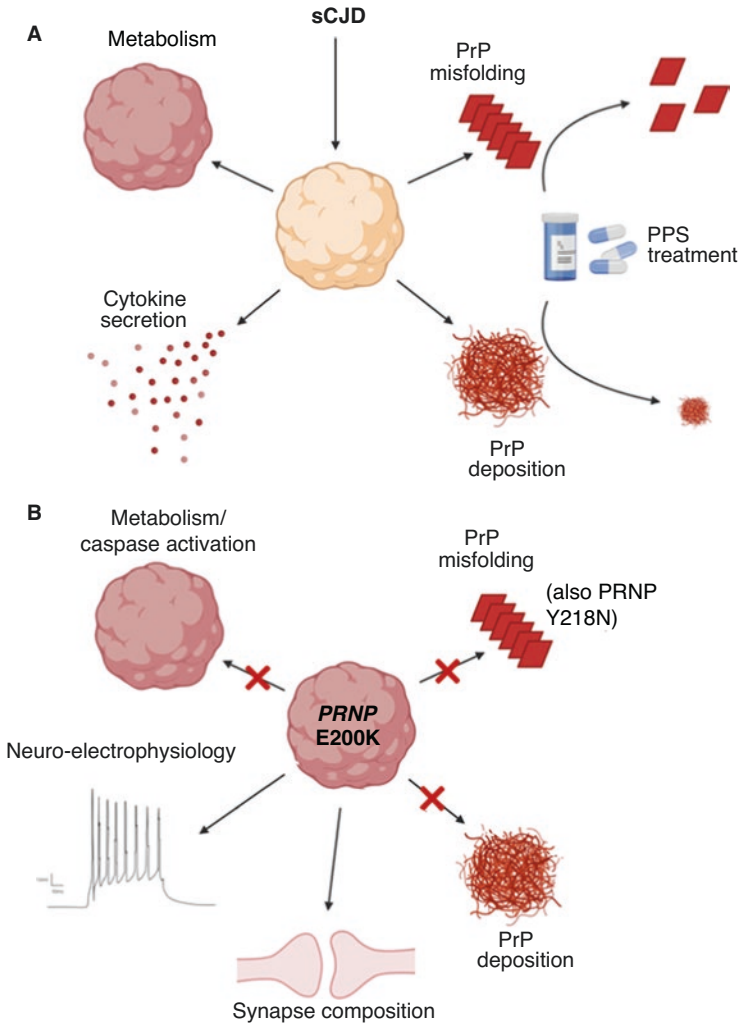

Fig. 16.2 *Summary of human cerebral organoid model findings.* (**a**) sCJD infection of cerebral organoids results in production of mis-folded prions that display RT-QuIC seeding activity and protease resistance, which can deposit within the organoids. Infected organoids demonstrated changed metabolism and cytokine secretion. Each of these factors may be influence by the infecting sCJD subtype. Treatment with pentosan polysulphate (PPS) can reduce PrP^{Res} and RT-QuIC seeding activity detection. (**b**) Organoids generated from donors with the *PRNP* E200K mutation that causes genetic CJD show no disease-associated prion isoforms. The absence of detection is indicated with an X and the finding that protein mis-folding was also not observed in *PRNP* Y218N spheroids noted. However, neuro-electrophysiological disruption is observed accompanied by changes in synapse composition

16.6.2 Genetic Disease

Human iPSCs can be made from any consenting donor. This permits cells to be collected from donors that carry disease causing mutations. There are a number of PrD-associated mutations that have been modeled in human iPSC-derived cell cultures. The first of these was the *PRNP* Y218N mutation that causes GSS with Tau pathology (Matamoros-Angles et al. 2018). Spheroid cultures, made using a differentiation protocol that is very similar to the one used for generating cerebral organoids, showed that neuronal tissue made from a *PRNP* Y218N donor did reproduce the Tau pathology observed during disease, but did not demonstrate any mis-folded PrP accumulation. Gonzalez et al. also produced cerebral organoids from two further mutations associated with hereditary CJD, *PRNP* E200K, and an octarepeat expansion mutation (10 octameric repeats) within the PrP N-terminal region (Gonzalez et al. 2018). These organoids were used as negative controls in a study of Alzheimer's Disease and Down Syndrome organoid pathology. Neither mutation showed evidence of disease pathology (Aβ reactive staining or phospho-Tau accumulation), but the authors did not specifically examine PrP mis-folding. The findings overall suggested that mutation within PrP itself is insufficient to trigger prion mis-folding in human cerebral organoids within the lifetime of the organoid.

We additionally examined the *PRNP* E200K mutation from two further donors within the cerebral organoids and found no evidence of mis-folded, protease resistant, or RT-QuIC seeding positive protein species within these organoids (Foliaki et al. 2020). However, the *PRNP* E200K organoids did demonstrate a neuro-electrophysiological phenotype, evident in older organoids (>6 months), showing that the mutation does influence neuronal function within the organoid (Foliaki et al. 2021). These organoids showed overall reduced electrophysiological activity, changes comparable with organoids generated from Down Syndrome and Parkinson's Disease donors (Foliaki et al. 2021). A shift in the excitatory and inhibitory balance was observed with changes occurring in receptor expression levels, synapse composition, and neurotransmitter production and release (Fig. 16.2b). Specific changes were identified within the GABAergic system, agreeing with previous studies showing that this is one of the first systems to become damaged in sCJD and animal models of PrD (Belichenko et al. 1999; Guentchev et al. 1998, 1997). While this phenotype suggests that the presence of the mutation is sufficient to cause functional changes in the absence of disease-associated PrP mis-folding, it must be appreciated that these neurons are within a model system. Organoids lack a blood brain barrier and non-neuronal lineage cells; therefore, the phenotype cannot be presumed to be a complete representation of what is occurring within the brain in human diseases, but it does provide insight into the potential causes of dysfunction linked to mutations within human PrP.

16.7 Other Stem Cell Models

While this chapter has mainly focused on the most published upon stem cell models relating specifically to PrP CNS function and PrD research, a number of other models have been developed. Most notably, the role of PrP in cellular proliferation has been investigated in cancer stem cell models and these studies further support a role for PrP in cell growth and differentiation, as well as a role for PrP in promoting malignancy and metastases. These attributes have been extensively reviewed elsewhere (Martin-Lannerée et al. 2014; Mouillet-Richard et al. 2021; Ryskalin et al. 2021). Additionally, studies utilizing dental pulp stem cells have further confirmed that PrP signaling drives neuronal differentiation through ERK1/2, AKT, and EGFR (Martellucci et al. 2018), with soluble recombinant PrP able to drive this signaling in the presence of endogenous PrP (Martellucci et al. 2019). Finally, in an expansion of the models available for studying animal PrDs, ovine neurosphere cultures have been produced (Duittoz and Hevor 2001). These offer the possibility of investigating scrapie strains in a completely ovine model. No subsequent studies have utilized these cultures to date, but their generation further shows that stem cell models can be extended to the study of animal diseases.

16.8 Summary

Stem cell models have contributed substantially to the understanding of PrP function within differentiated neurons and its role in progenitor growth and differentiation. Furthermore, stem cell models of PrD offer some advantages and features that differ from secondary cell lines and primary cultures when investigating infection and toxic pathways. Development of stem cell models is ongoing. Numerous improvements have been and continue to be applied to these models in an attempt to create better representations of the human brain. As these cultures become more sophisticated, they will provide the opportunity to examine human disease in vitro in a way that has not previously been possible.

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Chapter 17
Drosophila Models of Prion Diseases

Ryan R. Myers and Pedro Fernandez-Funez

Abstract Prion diseases encompass a heterogeneous group of fatal brain disorders associated with the accumulation of misfolded isoforms of the prion protein (PrP) in brain neurons. Modeling these diseases in rodent models in the laboratory have led to fundamental advances in our understanding of prion transmission and pathogenesis. Genetically tractable animal models such as the fruit fly *Drosophila melanogaster* have made significant contributions to neurodegenerative disorders, including prion diseases. When we wrote the original version of this chapter 9 years ago for the inaugural book series in 2013 (written in 2012), very little work had been done in *Drosophila* models expressing mammalian PrP. In fact, so little had been done that we included work on non-mammalian models of PrP biology and pathophysiology. Since then, the amount of work on *Drosophila* models has expanded considerably. Over the last 10 years, the number of transgenic fly models and their application has continued to grow. These recent models have addressed fundamental aspects of PrP function and physiology, mechanisms of neurotoxicity, identification of residues mediating PrP misfolding and toxicity, and progress on the development of prion bioassays for surveillance that can reduce the use of rodents for that purpose. This updated chapter describes the main advances in *Drosophila* models expressing mammalian PrP over the last 10 years.

Keywords *Drosophila* · Fruit fly · Prion disease · PrP expression · Transgenic models · Neurotoxicity · Transmission · Modifiers · Protective residues

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Abbreviations

Ctm	C-terminal region of PrP
Cyt	Cytosolic PrP
DEG	differentially expressed genes
GPI	glycosyl-phosphatidyl-inositol anchor
PMCA	Protein misfolding cyclic amplification
Prnp	Prion protein gene
PrP	Prion protein
PrP^{res}	resistant PrP
PrP^{Sc}	scrapie PrP
WT	Wild-type allele

17.1 The Prion Protein in Disease

Prion diseases are rare and incurable degenerative conditions with broad effects in the brain. In humans, prion diseases are highly heterogeneous at presentation with diffuse perturbations of cognitive, motor, or sleep domains followed by fast progression into other brain regions and death months after diagnosis (Aguzzi et al. 2008; Colby and Prusiner 2011). Prion diseases are rare yet highly unique due to their mechanism of transmission. Pathologic, genetic, and biochemical evidence identify the prion protein (PrP) as the causative agent in the pathogenesis of prion diseases. PrP is a 230 amino acid long membrane-anchored glycoprotein highly expressed in the brain and other tissues. PrP has an unstructured N-terminus and a globular domain in the C-terminus. The globular domain contains three alpha helices and a short beta-sheet; the conformational dynamics of this domain is proposed to determine PrP solubility and the initiation of pathogenesis. PrP undergoes conformational changes from its normal "cellular" (PrP^{C}) isoform into a variety of misfolded conformations, including soluble (oligomers and protofibers) and insoluble (fibers) species. Conversion into the "scrapie" (PrP^{Sc}) or "resistant" (PrP^{res}) isoform is proposed to be the main molecular event leading to the formation of pathogenic and transmissible prions (Prusiner 1998). This conformational conversion is associated with dramatic changes in the biochemical properties of PrP (misfolded, insoluble, aggregated, resistant to denaturing agents, and proteases) and it is based on alterations of its secondary, tertiary, and quaternary structures (Soto and Satani 2010). Thus, the infectious agent in prion diseases contains misfolded conformations of PrP, making these disorders extraordinary to study and understand mechanistically.

Unfortunately, the precise molecular and cellular mechanisms mediating PrP conformational conversion remain largely unknown. Moreover, our current understanding of the mechanisms responsible for neurotoxicity and neuronal loss is still enigmatic. The standard model posits that PrP^{Sc} is the agent responsible for transmission and neurotoxicity (Prusiner 1998; Prusiner et al. 1998). Some alternatives

have been proposed over the years that identify PrP^{Sc} as the transmissible agent and assign neurotoxicity to other conformations, transitional states, or assemblies (Sandberg et al. 2011; Lasmezas et al. 1997; Brandner et al. 1996; Sandberg et al. 2014). Multiple PrP isoforms have been identified biochemically and some were proposed as neurotoxic species, including transmembrane (Ctm) PrP, cytosolic (cyt) PrP, and transitional (intermediate) states of PrP conversion, such as PrP* and PrP^{L} (lethal) (Hill and Collinge 2003; Hegde and Rane 2003; Saa et al. 2016). Despite this wealth of conformations, the nature of the neurotoxic species and their mechanisms of action remains to be elucidated.

Another unique feature of prion diseases is that a few mammals, mainly ruminants, suffer endemic forms of prion diseases that are direct pathological correlates of the human disorder. In addition, several other animals have shown susceptibility to prions, including small rodents. This makes modeling prion diseases in the lab easier and more accurate mechanistically than for other related protein misfolding disorders, such as Alzheimer's and Parkinson's diseases. Alternative model organisms (non-mammalian) can play an important role in answering fundamental questions in PrP biology due to their fast and flexible manipulation, and the access to optimized research tools, including an expanded genetic toolbox.

17.2 Investigating Biological Processes in Animal Models

Rodents (mostly mice and rats) play a fundamental role in biomedical research owing to their anatomical, genetic, and physiological similarities to humans. Mice and hamsters played critical roles in elucidating the nature of prions, isolating different strains, and discovering the species barrier (Groschup and Buschmann 2008). Both mice and hamsters develop prion diseases that share key features with human prion diseases. In fact, rodent models of prion diseases are better correlates of the human disease than mouse models for other brain disorders. Mice are easier to breed, cheaper, and more amenable to transgenesis that any other mammalian laboratory model, making them the top choice for foundational and preclinical research. In addition, small, non-mammalian models such as zebrafish, fruit flies, and worms have also contributed to elucidate fundamental biological and medical processes over the last 50 years. These models expand the experimental opportunities thanks to their short life cycles, low cost, and their vast and dynamic experimental toolbox. Zebra fish is another non-traditional model that bridges the gap between invertebrate and mammalian models, with faster generational time than mice and more conserved anatomy and physiology than invertebrates. The tiny nematode *C. elegans* emerged as an ultrafast animal model for genetic discoveries assisted by its asexual reproduction. The fruit fly *Drosophila melanogaster* is the oldest of these non-mammalian models. This little golden fly provides an all-inclusive experimental system for investigating a vast array of topics, from genome architecture to complex behaviors (Bellen et al. 2010). *Drosophila* has contributed to the elucidation of biological principles in genetics (chromosomes and mutations), the development of

the body plan, the identification of odor receptors and innate immunity factors, and the genetics basis of behavior, work that was recognized with six Nobel Prizes from 1933 to as recent as 2017. Importantly, flies and humans show a high degree of evolutionary conservation that can be recognized in the basic cellular machinery critical for neuronal function and the development of a basic body plan organization. Flies have a small but complex tripartite brain homologous to the mammalian brain suggesting a shared origin hundreds of millions of years ago (Reichert 2005). The fly brain contains 10^5 neurons, an estimated 10^6 synapses, and well-characterized centers that control sophisticated behaviors, providing a robust system for studying neurodegenerative diseases (Bellen et al. 2010; Simpson 2009; Zheng et al. 2018). This chapter discusses in some detail the prowess and versatility of fruit flies toward uncovering the molecular bases of PrP-related pathologies.

17.3 Modeling Prion Diseases in Drosophila

17.3.1 Modeling Neurodegeneration in Flies

Over the last 20 years, *Drosophila* has been established as a remarkable model to study neurodegenerative diseases despite its small size and brain (Pandey and Nichols 2011; Rincon-Limas et al. 2012; Jaiswal et al. 2012). The expansive fruit fly genetic toolbox includes easy mutagenesis and transgenesis, highly curated genome and transcriptome, and vast collections of mutants (Graveley et al. 2011; Matthews et al. 2005; Venken and Bellen 2007; Pfeiffer et al. 2010). The UAS/GAL4 bipartite expression system is a widely used tool and is well-suited for studying genes with deleterious effects, because the transgenes controlled by UAS are silent until they are combined with the transcriptional activator GAL4, resulting in expression in highly specific spatiotemporal patterns (Brand and Perrimon 1993; Jenett et al. 2012; Elliott and Brand 2008).

Around the year 2000, several *Drosophila* models of human neurodegenerative diseases caused by aggregating amyloids were published demonstrating the suitability of fruit flies to study these complex conditions (Jackson et al. 1998; Warrick et al. 1998; Fernandez-Funez et al. 2000; Feany and Bender 2000). These first models of polyglutamine expansion and tau showed protein aggregation and neuronal loss, two key features of a growing class of neurodegenerative disorders that includes prion diseases. The success of these early models led to the development of models for other proteinopathies, such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia–amyotrophic lateral sclerosis, and many others (Rincon-Limas et al. 2012). These models have yielded major advances toward the identification of the molecular mechanisms mediating neurodegeneration. Some of the most significant findings include the regulation of amyloid aggregation by molecular chaperones, the role of protein phosphorylation and other post-translational modifications in the neurotoxicity of amyloids, and the degradation of large aggregates by autophagy (Chen et al. 2003; Warrick et al. 1999; Ravikumar

et al. 2004; Chen and Feany 2005; Steinhilb et al. 2007). One of the largest contributions to understanding the mechanisms underlying protein misfolding diseases in fly models has been the completion of multiple genetic screens trying to identify functional interactions with the offending protein. Genetics screens have been completed for several polyglutamines (Huntingtin, Ataxins 1 and 3), tau, amyloid-beta, TDP-43, and others. Genetic screens can be limited focusing on candidate genes selected from known mechanisms (e.g., Tau phosphorylation) or predicted protein function (RNA-binding proteins, such as TDP-43 and FUS). When little preliminary information is available, unbiased genetic screens have the potential to uncover novel mechanism, sometimes identifying unsuspected targets and pathways. These screens look for functional interactions based on easy to score assays, mainly aberrant eye morphology induced by amyloids. Of these interactions, those genes that enhance the toxicity of amyloids identify factors that contribute to toxicity, but also identify genes with important functions in eye development or cell survival. On the other hand, the rare finding of a gene that suppresses amyloid toxicity can have a deeper impact in understanding amyloid pathophysiology as well as identify potential therapeutic targets. *Drosophila* has powerful genetic tools in its expansive toolbox for these screens and the resources have continued to improve, since we published the first genetic screen in a proteinopathy model in the year 2000 using flies expressing Ataxin-1 with 78 polyglutamine repeats (Fernandez-Funez et al. 2000). There are currently several independent collections of flies expressing RNAi against almost all endogenous genes (genome-wide) and fast progress is being made on equivalent collections for gene overexpression. CRISPR/Cas9 technology has made a recent impact on the generation of these large-scale genetic tools and provides additional resources for conduct genetic screens or validates other collections (Gratz et al. 2014). Overall, these resources and their application to specific disease models place *Drosophila* at the forefront of research on neurodegenerative diseases.

17.3.2 Early Fly Models of Prion Disease

Over 75% of genes involved in human disease are conserved in flies (Adams et al. 2000), but some genes are not. That is the case with PrP, which is a gene well-conserved in vertebrates but not in invertebrates. Flies do not possess endogenous copies of two close PrP homologues, Doppel and Shadoo, providing a clean background for studying the pathogenic consequences of the heterologous expression of PrP. Thus, *Drosophila* is well-suited to study the molecular and cellular perturbations caused by human or mammalian PrP.

In 1995, before the publication of several successful *Drosophila* models of proteinopathies, S. Prusiner and colleagues generated transgenic flies expressing PrP-WT from Syrian golden hamster under the control of the heat shock promoter (Table 17.1). Surprisingly, these flies displayed no signs of pathology (Raeber et al. 1995), likely because the promoter system used did not result in sustained high levels of PrP. Eight years later, S. Supattapone and colleagues created new

Table 17.1 Drosophila models of prion protein

Reference	Years	PrP transgene	Reported phenotype/application
Raeber et al. (1995)	1995	Heat shock-hamster PrP, random insertions	Low levels of PrP, No phenotypes
Deleault et al. (2003)	2003	UAS-mouse PrP-WT; PG14, random insertions	Detection of PrP, No phenotypes
Gavin et al. (2006) and Robinson et al. (2014)	2006	UAS-mouse PrP-WT (3F4), P101L, random insertions	Locomotor dysfunction, brain degeneration and pathogenic conformations by 15B3 antibody Aberrant synaptic activity
Fernandez-Funez et al. (2009)	2009	UAS-hamster PrP-WT, random insertions	Locomotor dysfunction, vacuolar degeneration, insolubility, guanidinium resistance and 15B3+
Fernandez-Funez et al. (2010)	2010	UAS-mouse PrP-WT UAS-rabbit PrP-WT random insertions	Rodent PrP: Locomotor dysfunction, no brain degeneration, insolubility and 15B3+ Rabbit PrP: No phenotypes
Robinson et al. (2014)	2010	UAS-mouse PrP-WT (3F4); P101L, random insertions	P101L: Locomotor dysfunction, vacuolar degeneration, abnormal synaptic architecture in NMJ
Thackray et al. (2012a) and Thackray et al. (2012b)	2012	UAS-ovine PrP WT (ARQ, AHQ, VRQ, VHQ), attP-51D	Sporadic toxicity, infectivity model
Sanchez-Garcia et al. (2013)	2013	UAS-Hamster PrP-M203S, M203S–M2013S, random insertions	Mutations induce aberrant biogenesis resulting in Ctm conformations
Thackray et al. (2014b)	2014	UAS-Ovine PrP-Cyt Ovine PrP-GPI(−), attP-51D	Sporadic toxicity of membrane bound, secreted (GPI^{-}) and cytosolic forms of PrP. WT and Cyt support prion transmission, prions can be propagated into mice
Sanchez-Garcia et al. (2016)	2016	UAS-Mouse PrP-N159D, random insertions	Single amino acid substitution rescues locomotor deficits and decreases the amount of pathogenic PrP conformations
Thackray et al. (2017)	2017	Mouse PrP-WT (3F4), hamster WT 178, E200K, attP-51D	Mouse PrP carrying pathogenic mutants support replication of mouse prions, prions can be propagated into mice
Fernandez-Funez et al. (2017)		UAS-Human PrP-WT (V129), random insertions	Human PrP is more toxic than hamster PrP and induces a new eye phenotype
Sanchez-Garcia and Fernandez-Funez (2018)		UAS-Horse PrP-WT, S167D Dog PrP-D159N, rabbit PrP-S174N, random insertions	PrP WT from dog, horse and rabbit are not toxic; dog D159N and horse S167D are toxic; rabbit S174N has no effect
Thackray et al. (2021)		UAS-Bovine PrP, attP-51D	Bovine PrP support replication of BSE prions, prions can be propagated into mice; assays more sensitive than those in mice

(continued)

Table 17.1 (continued)

Reference	Years	PrP transgene	Reported phenotype/application
Rincon-Limas et al. (2012)		Human PrP-WT (M129), random insertion Mouse and hamster PrP-WT, attP2 Human PrP-WT (V129, M129), attP2 Human PrP-(V129)–N159D; D167S; N174S, attP2 N159D–D167S; N159D–D167S–N174S (triple), attP2	Novel phenotypes induced by human PrP in the eye and in the brain Direct comparison of rodent and human PrP shows heightened toxicity of human PrP from equivalent expression levels Expression of single, double, or triple amino acid replacements have mild to moderate effect on the toxicity of human PrP

transgenic flies expressing mouse PrP either WT or carrying a mutation in the repeat region (PG14) under the control of the UAS regulatory sequence (Deleault et al. 2003). Strangely, pan-neural expression of PrP-PG14 was detected in the eyes, but not in the brain, suggesting that *Drosophila* brain neurons prevented the accumulation of mutant PrP. Following up on these studies, Supattapone and colleagues created new transgenic flies expressing mouse PrP carrying the Gerstmann–Sträussler–Scheinker-linked mutation P102L (P101L in mouse PrP) (Gavin et al. 2006). These flies showed brain degeneration, progressive locomotor deficits, shortened lifespan, and misfolded PrP isoforms recognized by the PrP^{Sc} conformational antibody 15B3. Still, older flies expressing mouse PrP-P101L accumulated no detergent-insoluble or protease-resistant PrP conformers. Further work from a different group using flies expressing mouse PrP-P101L reported similar observations (Choi et al. 2010). Overall, these results indicate that flies expressing WT or mutant mouse PrP reproduce some of the PrP-associated neuropathological features, including PrP misfolding and progressive neuronal dysfunction and neurodegeneration. These early models also illustrate the growing pains associated with developing novel disease models.

17.3.3 Modeling Sporadic Prion Diseases: Neurotoxicity of PrP-WT

Sporadic Creutzfeldt–Jakob disease is the most common form of prion disease, affecting ~80% of all patients. Sporadic prion diseases are explained by the intrinsic propensity of PrP-WT to transition from its native structure into pathogenic conformers. This intrinsic dynamics is, in turn, encoded in its amino acid sequence, which modulates the stability of the secondary domains and the flexibility of the loops (van der Kamp and Daggett 2009). We hypothesized that sporadic PrP neurotoxicity could be modeled in flies by expressing PrP from the appropriate animal species conferring high toxicity. We originally decided against using human PrP to

avoid the risk of creating prions on two wings with the potential of transmissibility to human experimenters. We used the same logic against bovine or sheep PrP because of their potential transmissibility to humans. Instead, we selected PrP from Syrian golden hamster, the initial choice by Prusiner's team: hamsters exhibit an aggressive disease course along with a high PrP^{Sc} titer, suggesting that hamster PrP is more prone to misfold than mouse PrP. Warned by previous difficulties detecting PrP expression in fly brains, we selected Gal4 strains driving robust expression in the brain. Flies expressing hamster PrP-WT in brain neurons displayed progressive locomotor dysfunction and vacuolar degeneration (Fernandez-Funez et al. 2009). We also found progressive changes in the biochemical and structural properties of hamster PrP-WT, including sarkosyl-insolubility, resistance to denaturing agents, and immunoreactivity to the conformational antibody 15B3. These are key features of pathogenic forms of PrP in human patients and in mammalian models of the disease. Despite these significant structural changes, flies did not accumulate the 19 kDa proteinase K-resistant fragment typical of PrP^{Sc}. Thus, hamster PrP-WT can induce progressive neuronal dysfunction and spongiform degeneration in the absence of PrP^{Sc}. This finding is consistent with the prominent role of other neurotoxic isoforms PrP^{L} or PrP* described in mammalian models (Hill and Collinge 2003; Hegde and Rane 2003; Saa et al. 2016).

A report by Park et al. revealed additional deleterious effects of PrP-WT (Park et al. 2011). Whereas mouse PrP-WT had no effect in the *Drosophila* eye, it enhanced the toxicity of mutant Ataxin-3 in the eye, which is a polyglutamine protein responsible for spinocerebellar ataxia type 3. Moreover, mouse PrP-WT increased the susceptibility to toxins, such as the reducing agent DTT (dithiothreitol) and the oxidative stressors H_2O_2 and paraquat.

To continue with the fly models expressing rodent PrP, the Bujdoso group generated flies expressing mouse and hamster PrP WT, with mouse PrP carrying the 3F4 epitope to detect both mouse and hamster with the same 3F4 antibody (Thackray et al. 2017). They also developed flies carrying mouse and hamster PrP with the D178N and E200K mutations associated with inherited forms of prion disease. All these flies were generated with newer PhiC31 recombinase technology (Bischof et al. 2007) that allowed for the transgenes to be inserted in the same precise locus, thus eluding the confounding consequences of position effects in gene expression. Subjecting these flies to behavioral assays revealed locomotor deficits of all transgenes (WT and mutant) that were more robust in flies expressing the E200K mutant in some conditions (Thackray et al. 2017). However, flies expressing hamster PrP at 25 °C showed essentially the same locomotor deficits for WT, D178N and E200K supporting our previous observation that hamster PrP-WT is highly toxic in flies (Fernandez-Funez et al. 2009, 2010).

The Bujdoso group had generated flies expressing ovine PrP before they handled the murine models. In this case, they generated ovine PrP carrying four natural amino variants or polymorphisms at residues 136, 141, and 154 (ARQ, AHQ, VRQ, and VHQ) which confer different susceptibility to different strains (Goldmann et al. 1994; Moum et al. 2005; Clouscard et al. 1995). All these lines were inserted into flies using the above-mentioned PhiC31-recombinase with the purpose of creating comparable lines (Thackray et al. 2012a). The ARQ and VRQ variants showed

higher expression than the other two lines, but in the brain, ARQ was significantly higher than VRQ, making this a preferred line for follow-up studies in this and following papers. All variants produced insoluble isoforms, but ARQ accumulated more insoluble PrP. Consistent with the expression levels, only the ARQ line demonstrated deficits in a locomotor assay, whereas ARQ, AHQ, and VHQ showed reduced survival. This original paper by the Bujdoso lab established the ovine PrP model by showing the toxicity of the WT allele and showing the sensitivity of the *Drosophila* host to natural variations among ovine PrP.

17.3.4 Physiological Functions PrP

PrP is a highly expressed protein in the mammalian brain, both in neurons and glia (Bendheim et al. 1992). PrP is also highly expressed in gut, lymphatic tissues, and other tissues which can promote PrP conversion in peripheral tissues (Castle and Gill 2017). This broad expression would suggest that PrP is a critical protein for the function of the brain and other tissues and for survival. Yet, mice devoid of endogenous PrP ($Prnp^{-/-}$) are viable and only seem to display mild behavioral changes (Bueler et al. 1992; Manson et al. 1994). Goats and cattle devoid of endogenous PrP are also viable and healthy, although they have not been characterized in detail (Yu et al. 2006; Benestad et al. 2012; Richt et al. 2007). Elucidating the function of this abundant protein has been puzzling so far and contributions have come from simplified systems such as cell culture and animal models, including mice, zebrafish, worms, and fruit flies. Potential roles for PrP include stress protection, cellular differentiation, neuronal excitability, myelin maintenance, circadian rhythm, metal ion homeostasis, mitochondria homeostasis, and cell signaling (reviewed in (Castle and Gill 2017; Wulf et al. 2017; Panes et al. 2021)). In the nervous system, PrP has been detected along axons and in presynaptic and post-synaptic terminals, although it is more abundant in presynaptic terminals, including neuromuscular junctions (Herms et al. 1999; Moya et al. 2000). Work with PrP knockout (KO) mice ($Prnp^{-/-}$) has been confounded by genetic background and the contribution of the PrP paralog Doppel. All PrP KO mouse models agree that these animals are resistant to prion infection (Bueler et al. 1992; Manson et al. 1994; Sakaguchi et al. 1996). Rigorous studies of these animals seem to agree on the role of PrP in preserving circadian rhythm and long-term potentiation, which is a key neuronal function underlying memory and learning (Senatore et al. 2012; Collinge et al. 1994; Maglio et al. 2006; Rangel et al. 2009; Tobler et al. 1996). Studies in fish have proposed a role for PrP in regulating cell adhesion during nervous system development, but these studies are complicated by the presence of three copies of PrP in zebrafish with different temporal and spatial expression patterns (Malaga-Trillo et al. 2009; Fleisch et al. 2013). Numerous studies have proposed that PrP^C can modulate neuronal excitability based on behavioral alterations and direct molecular interactions. PrP interacts with several neurotransmitter receptors, including acetylcholine, kainite, and glutamate (Kleene et al. 2007; Carulla et al. 2011; Khosravani et al. 2008; Beraldo et al. 2011). The interaction with NMDA and AMPA receptors seems to inhibit them and may exert

protective function by preventing excitotoxicity and reducing the likelihood of seizures (Ratte et al. 2011). These interactions could underlie LTP perturbations along with behavioral alterations (Khosravani et al. 2008), although weakening of inhibitory potentials through GABA receptors have also been implicated in the LTP perturbations (Lledo et al. 1996). In contrast, PrP2 KO in zebrafish increased susceptibility to seizures (Fleisch et al. 2013), revealing a confusing scenario with several confounding genetic variables. PrP has also been reported to regulate Ca^+ and K^+ currents by modulating the trafficking and/or function of voltage-dependent Ca^+ channels (VGCC or CaV) or the voltage-gated K^+ channel Kv4 (Senatore et al. 2012; Mercer et al. 2013) (reviewed in (Panes et al. 2021)). Overall, PrP has multiple partners in the brain and seems to be involved in multiple physiological processes. At this time, the function of PrP appears to be fragmented into multiple disconnected pieces and a unifying model for PrP function has not yet emerged.

Drosophila models expressing mammalian PrP have added to this knowledge. Expression of mouse PrP-P101L in brain neurons resulted in accumulation in presynaptic terminals of motor neurons along with behavioral defects and early death (Choi et al. 2010). This study found that PrP-P101L but not the WT allele disrupted the highly stereotyped synapses, including increased sprouting and decreased expression of active zone markers. A later study of the electrophysiological properties of fly NMJs revealed that mouse PrP-WT but not PrP-P101L disrupts synaptic activity (Robinson et al. 2014). Although PrP-WT had no effect on bouton morphology and active zone density in the larval motor neurons, it resulted in increased synaptic activity and enlarged presynaptic vesicles. These findings are consistent with PrP interactions with synaptic proteins, such as scaffolding (laminin B), ion channels, and synaptic trafficking proteins (synapsin), among others (reviewed in (Castle and Gill 2017; Wulf et al. 2017; Panes et al. 2021)). Strikingly, these two studies in flies differ in the consequences of expressing PrP-WT vs P101L. The later study is more detailed, but both include contrasting behavioral studies that could be explained by different expression levels from loosely controlled transgenic lines based on protein expression levels. PrP may play a role in vesicle fusion, recycling, and/or storage), a physiological function relevant in the development, maturation, and activity of brain synapses. The function could also be relevant in pathogenic processes, because the progressive misfolding of PrP would lead to a loss-of-function and a reduction in synaptic activity. This mechanism could further support the functional connections proposed between PrP and several amyloids (e.g., amyloid-β peptide, tau, α-synuclein, and TDP-43), in turn, explaining the contribution of PrP to several brain proteinopathies. It is clear, though, that much work is still needed to understand the biological functions of this complex protein.

17.3.5 Topological Variants of PrP

In addition to its high conformational dynamics, PrP can also exist in several topological conformations by normal physiological processes. PrP is a secreted protein with well-preserved signal peptide for targeting its synthesis to the secretory

pathway and a GPI anchor that keeps PrP attached to the extracellular aspect of the plasma membrane. These structural elements result in most PrP accumulating in the membrane. Transmembrane, fully secreted (unbound), and cytosolic isoforms can also be detected in brains and in laboratory models (Hegde and Rane 2003; Ashok and Hegde 2009; Hegde et al. 1998). Secreted PrP isoforms originate from a failure to form the GPI anchor but can also accumulate in the cytosol following retrotranslocation (Ma et al. 2002). The Bujdoso group compared the toxicity of ovine PrP in transgenic flies expressing constructs that accumulate in different compartments: membrane-bound, secreted, and cytosolic (Thackray et al. 2014a, b). In this experimental setup, all the transgenes were inserted in the same genomic locus to ensure comparable expression levels. Secreted ovine PrP showed the highest toxicity, whereas cytosolic PrP accumulated at lower levels due to higher turnover (Thackray et al. 2014a; b). The heightened toxicity of secreted PrP is consistent with the identification of pathogenic mutations in PrP in familial forms of prion disease lacking the C-terminus (e.g., Y145X, Y163X). These variants are highly amyloidogenic and share molecular and cellular features with the amyloid-β peptide causative of Alzheimer's disease.

To study other PrP topologies, we replaced two highly conserved methionines (Met, M) at 206 and 213 with polar serines (Ser, S) in hamster PrP. Met are hydrophobic residues; these two Met are deeply buried in the hydrophobic core and maintain helices 2 and 3 in close contact. Introducing M206S alone or together with M213S had a dramatic effect on the biogenesis of hamster PrP (Sanchez-Garcia et al. 2013). The polar substitutions disrupted the cysteine (Cys, C) bridge between helices 2 and 3, exposing a weak transmembrane domain spanning amino acids 111–135. Exposure of this weak transmembrane resulted in PrP insertion into the membrane as a transmembrane protein with the C-terminus into the ER lumen (Ctm topology) and the N-terminus in the cytosol. The inverse topology with the N-terminus in the ER (Ctm topology) has also been observed. Ctm and Ntm PrP accumulate in small amounts due to errors in PrP translation and folding that can be more common during aging, further adding more challenges to PrP biology (Hegde and Rane 2003; Ashok and Hegde 2009; Hegde et al. 1998). In flies, Ctm PrP is stuck in the ER and shows signs of aberrant post-translational maturation but shows no toxicity (Sanchez-Garcia et al. 2013). Yet, Ctm PrP promotes the conformational change and toxicity of PrP-WT, suggesting an interaction between age-related aberrant PrP biogenesis and sporadic PrP pathogenesis.

17.3.6 PrP Conformational Dynamics: The PrP Zoo

PrP is a highly conserved protein in mammals at both sequence and structural levels; small changes in its sequence across species can impact the disease potential of PrP. In fact, wild animals reveal a wide spectrum of TSE susceptibility, with a few animals suffering endemic forms of prion diseases and others showing natural resistance. This susceptibility spectrum may help uncover key insight into PrP

conformational dynamics and pathogenesis. Only a handful of mammals demonstrates endemic forms of prion diseases: scrapie in sheep and goats, and chronic wasting disease in cervids. The infamous bovine spongiform encephalopathy (BSE or mad-cow disease) seemed to originate from scrapie-contaminated bone meal, although anecdotal cases of endemic BSE have been reported. In addition to these ruminants, rodents, felines (cats), and mustelids (ferret family) are susceptible to prions transmitted in the lab or in zoos during the "mad-cow" epidemics of the 1980s. Conversely, rabbits, dogs, horses, and pigs seem to be resistant to prion diseases under similar conditions. Rabbits were directly inoculated with prions in classic studies, whereas dogs and horses were exposed to the same contaminated feed as other domestic and farm animals that developed prion diseases.

To examine the impact of the primary amino acid sequence of PrP on its structural dynamics and toxicity, we generated transgenic flies expressing PrP-WT from mammals susceptible (hamster and mouse) and resistant (rabbit) to prion diseases (Fernandez-Funez et al. 2010). We already showed that hamster PrP induced progressive toxicity in flies from our previous work. Expression of mouse PrP produced mixed results, inducing early locomotor dysfunction, but neither spongiform degeneration nor PrP aggregation, which we found with hamster PrP. Conversely, rabbit PrP did not convert into pathogenic conformations nor induced neurotoxicity. Thus, three highly conserved PrP sequences exhibited prominent differences in their conformational dynamics and toxicity in transgenic flies. These results were highly valuable toward establishing *Drosophila* as a valid model to study mammalian PrP: expression of PrP from different animals results in the preservation of structural and biological features demonstrated by the different misfolding and toxicity of rodent and rabbit PrP. These results provided confidence to express other variants, including other animals and mutations that introduce structural perturbations on PrP. In a follow-up study, we generated transgenic flies expressing PrP-WT from rabbit, dog, and horse, three animals resistant to prion diseases. Neither of these PrPs caused neurodegeneration in flies confirming the hypothesized conformational stability of these disease-resistant PrPs (Sanchez-Garcia and Fernandez-Funez 2018). On the other extreme of this continuum of PrP toxicity, expression of human PrP in flies leads to extremely high toxicity, including a new eye phenotype (Fernandez-Funez et al. 2017; Myers et al. 2022). Overall, these experiments support that the spectrum of PrP toxicity is due to changes in PrP sequence.

17.3.7 Protective Residues from Resistant Animals

Mammals show different susceptibility to TSEs. Thus, small amino acid changes on the PrP sequence play a key contribution in modulating its conformational dynamics and, therefore, its propensity to cause disease. The next challenge is to determine how specific amino acids encode the conformational dynamics of the globular domain. Fortunately, PrP sequences and structures from many different animals are available for comparative studies, providing unparalleled resources for addressing

this critical question. It is thoroughly accepted that the *N*-terminal domain (residues 1–94) does not drive PrP conversion (Fischer et al. 1996; Lawson et al. 2001; Rogers et al. 1993). Thus, the attention focuses on the sequence and dynamics of the globular domain. Despite the high PrP sequence and structure conservation in mammals, many small changes in the amino acid sequence are evident throughout the PrP globular domain in mammals (Fig. 17.1). Amino acid alignments identify 10–15 changes between human PrP and other animals within the globular domain. Of these, most changes are conservative and, hence, are not expected to largely impact its dynamics. The alignment indicates that the variation accumulates in the loop between the second β-sheet and helix 2 (β2–α2 loop, residues 166–170) and in the terminal portion of helix 3 (residues 219–229). Interestingly, these two regions are close to each other in the 3D conformation. Several H+-bonds stabilize this interaction, indicating that these sub-domains form, in fact, a 3D domain (Fig. 17.2a). This domain has been implicated in the initiation of PrP^{C} conversion to PrP^{Sc} by classic studies (Telling et al. 1995; Kaneko et al. 1997), indicating the importance of the intramolecular dynamics within this domain. In contrast, a lack of conservation in distal helix 3 is assumed to mainly be related to the formation of the GPI anchor, which may underestimate the importance of this region. Sequence and structural

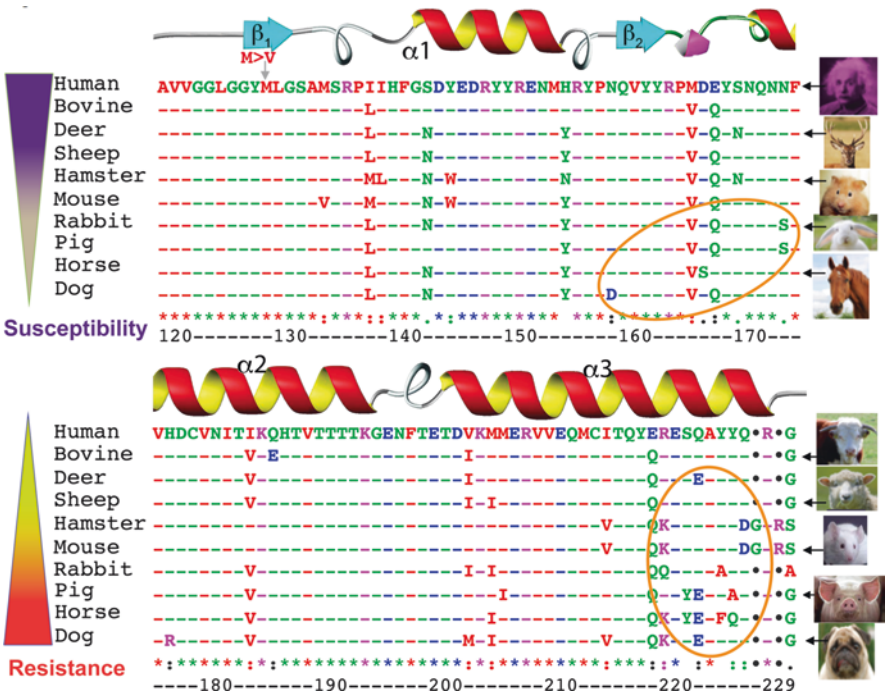

Fig. 17.1 Sequence differences between human and animal PrP. Sequence alignment of the C-terminal globular domain of PrP from human and several animals relevant in prion research. Amino acid numbering corresponds to human PrP throughout. The alignment shows high overall conservation with most variation clustered in the β2–α2 loop and distal helix 3 (circles)

alignments identified three prominent amino acid differences between animals that are susceptive vs highly resistant to prion diseases. At a structural level, the globular domain from human PrP looks similar to the same domain from animals resistant to prion diseases (Fig. 17.2b, c). It is proposed that these changes likely encode the differences in conformational stability of PrP: D159 in dog, S167 in horse, and S174 in rabbit and pig (Fig. 17.2c, d). We will discuss next the work we have done in transgenic flies to examine the impact of each of these residues on PrP toxicity.

Dog PrP: D159

Most mammals, including humans, have an asparagine (N) at position 159, but dogs and other Canids (wolf, coyote, fox) have either an aspartic acid (D) or a glutamic acid (E) (Stewart et al. 2012; Fernandez-Borges et al. 2017). Two mustelids, the wolverine and the marten, also share this acidic residue at 159 but other mustelids carry N159. The NMR structure of dog PrP displays a highly conserved globular domain with subtle changes compared to human PrP (Fig. 17.2b–d).

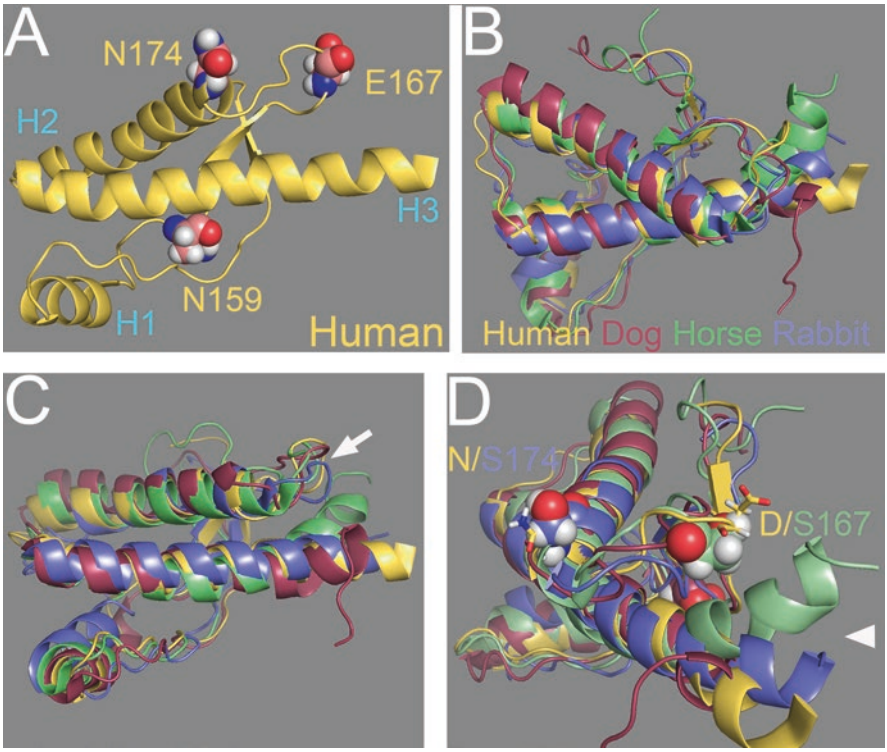

Fig. 17.2 Structural similarities and differences between human and animal PrP. (**a**) 3D visualization of human PrP indicating the position of N159, E167, and N174. (**b**, **c**) 3D alignment of human (gold), dog (maroon), horse (green), and rabbit (violet) PrP globular domains. The three proteins share the same structural elements, with three helices (H1–3) and a short β-sheet. The local differences around D/S167 and N/S174 are indicated (**d**)

Dogs have no β-sheet, and the surface charge around D159 is more negative and shows increased solvent exposure due to the acidic replacement (Lysek et al. 2005). This change in surface charge is likely to affect the interaction with other proteins. It is unclear how these changes in surface charge affect the dynamics of dog PrP, but there appear to induce mid-distance perturbations in the β2–α2 loop. D159 flanks the β-sheet and may prevent its formation by stiffening the loop and preventing the proper alignment of the two sections of the β-sheet. Eliminating the probability of forming the β-sheet could be sufficient to increase the stability of the entire globular domain. To test the ability of D159 to stabilize PrP structure and lower its toxicity, we generated transgenic flies carrying mouse PrP with the N159D substitution (Sanchez-Garcia et al. 2016). Expression of mouse PrP-N159D in *Drosophila* motor neurons improved locomotor performance compared to flies expressing mouse PrP-WT. This improvement correlated with lower levels of pathogenic conformations of PrP identified by a conformational antibody (Sanchez-Garcia et al. 2016). We next introduced the reverse D159N substitution in the dog PrP backbone. Dog PrP-WT is not toxic in behavioral and anatomical assays in flies. In contrast, flies expressing dog PrP-D159N exhibit age-dependent degeneration of brain neurons and locomotor dysfunction (Sanchez-Garcia and Fernandez-Funez 2018). These results probing the reverse substitutions in mouse and dog PrP strongly support the critical role of D159 in encoding higher PrP conformational stability and lower toxicity. These results generated in transgenic flies are consistent with experiments in transgenic mice expressing murine PrP-N159D, which showed resistance to mouse prions (Otero et al. 2018). Mice expressing bank vole PrP are highly susceptible model to prion transmission. Introduction of the N159D mutation in bank vole PrP resulted in significant delay in the clinical onset of prion disease (Otero et al. 2019). The Castilla group also developed the reverse experiment in which mice express dog PrP-WT or E159N. In this case, expression of dog PrP-WT resulted in no propagation of prions, but the expression of the E159N mutant allowed the replication of prions (Vidal et al. 2020). Overall, these experiments in flies and mice are consistent in demonstrating the protective effect of D/E159, although the exact mechanisms mediating this effect are unknown.

Horse PrP: S167

The NMR structure of horse PrP revealed high structural stability of the β2–α2 loop compared to mouse PrP (Perez et al. 2010). Since S167 is in the center of the β2–α2 loop, this substitution exclusively found in horse PrP has the potential to reveal important clues about how the dynamics of the loop are encoded (Fig. 17.2b–d). The S167 substitution was introduced in recombinant mouse PrP along with other horse-specific substitutions (Q168E, and N173K) for NMR studies. Mouse PrP carrying the horse single or double substitutions showed that D167S alone increased the structural definition of the β2–α2 loop and increased the long-distance interactions between the loop and helix 3 (Perez et al. 2010). We used this information to generate flies carrying WT and mutant horse PrP. Horse PrP-WT shows no toxicity when expressed in *Drosophila* motor neurons, whereas expression of horse

PrP-S167D showed degeneration of brain neurons and aggressive locomotor dysfunction (Sanchez-Garcia and Fernandez-Funez 2018). Curiously, horse PrP-S167D induced a phenotype in degenerating brain structures not seen previously with other PrP: the cell bodies swelled up causing an expansion of the cell body clusters (Sanchez-Garcia and Fernandez-Funez 2018). Toxic hamster PrP induces shrinking, not expansion, of cell body clusters, indicating different pathogenic mechanisms. Transgenic mice expressing murine PrP-D167S developed spontaneous disease due to high expression levels, something described for PrP-WT (Sigurdson et al. 2011). Expression of murine PrP-D167S at lower levels and infected with mouse prions resulted in lower levels of insoluble PrP, suggesting a partial inhibition of prion replication.

Rabbit PrP: S174

Structural, biochemical, cell culture, and cell-free evidence identified S174 as a key residue mediating the stability of rabbit PrP (Vorberg et al. 2003; Khan et al. 2010; Wen et al. 2010). S174 was proposed to form a unique helix-capping domain that extends and stabilizes helix 2 (Khan et al. 2010) (Fig. 17.2b–d). Introducing the S174N substitution in rabbit PrP disrupts its stability in vitro and increases the dynamics of the β2–α2 loop. We generated transgenic flies expressing WT and mutant rabbit PrP-S174N expecting to detect an increase in toxicity as shown above for dog and horse PrP mutants. Instead, flies expressing rabbit PrP-S174N in brain neurons exhibited no changes in locomotion nor in brain architecture (Sanchez-Garcia and Fernandez-Funez 2018). This puzzling result is inconsistent with the impact of S174N substitution on in vitro replication of rabbit PrP (Vorberg et al. 2003; Erana et al. 2017). Moreover, a single amino acid change shows a limited impact on the structural dynamics of rabbit PrP in vivo, suggesting that additional residues contribute to the high stability of rabbit PrP. It is still unclear which amino acids encode the conformational stability of rabbit PrP. It is likely that multiple amino acids in the β2–α2 loop and helix 3 cooperate to increase the stability of the CT3D domain. The conservative nature of the amino acid variants found in this domain complicates the experimental demonstration of which residues and structural features confer conformational stability to rabbit PrP. The N/S174 residue has been studied extensively in in vitro assays (Khan et al. 2010; Erana et al. 2017), although the original chimeric mouse studies indicated the protective nature of the N174S substitution in mice (Vorberg et al. 2003).

The evidence gathered in several experimental systems including *Drosophila* agrees that rabbit, horse, dog, and pig PrP are comparatively more resistant to conversion and less toxic than PrP from naturally susceptible animals. Yet, the evidence for the role of specific amino acids in encoding the conformational stability is mixed. There seems to be more evidence at this time for the protective role of D/E159 and S167, whereas the evidence for S174 is not as robust. So far, these results indicate that single amino acids may introduce moderate changes into the dynamics of the globular domain.

17.3.8 New Drosophila Models Expressing Human PrP

17.3.8.1 Human PrP Exhibits Heightened Toxicity

The development of *Drosophila* models expressing mammalian PrP demonstrated their utility in several applications, including examining the in vivo consequence of introducing pathogenic or protective amino acid replacements. The phenotypes identified in the models described above are detected through brain expression, aging, and time-consuming biochemical, microscopic, and behavioral techniques. This makes fly models expressing PrP quite inefficient and limits the experimental applications of these models, including the number of sequence perturbations that can be tested and performing screens to identify genetic or pharmacologic modifiers.

One of the main differences between the fly models of prion diseases and those for other proteinopathies is that the later almost universally displays robust eye phenotypes (Rincon-Limas et al. 2012; Fernandez Funez and Myers 2020). Moreover, the eye phenotypes are quite unique for each model, supporting the disruption of specific pathways in the development of the *Drosophila* eye. The perturbation of specific cellular and molecular pathways provides opportunities to identify disease-specific mechanisms underlying disease pathogenesis. Why are eye phenotypes so important for *Drosophila* disease models? Because the fly eye is easy to observe under a simple stereoscope and the lattice formed by the 600 units of each compound eye facilitates the detection of small changes in its organization. Genetic perturbations resulting in flies with abnormal eyes are ideal for efficient genetic discoveries thanks to this easy-to-score phenotype. In the year 2000, we reported the first large-scale genetic screen in a fly model of a proteinopathy, which identified multiple novel mechanisms mediating expanded Ataxin-1 toxicity, a polyglutamine disease (Fernandez-Funez et al. 2000). Many similar screens have been published over the last 20 years that have revealed critical cellular and molecular mechanisms underlying multiple human proteinopathies.

In contrast, none of the *Drosophila* models expressing WT or mutant mouse, hamster, or sheep PrP show eye perturbations. Our work with flies expressing PrP from animals with low susceptibility to prion disease inspired us to focus on identifying a PrP on the other spectrum of susceptibility: a highly toxic PrP. We settled on human PrP for several reasons. (1) Humans are the only mammals that have naturally occurring prion diseases with all three etiologies: infectious, sporadic, and genetic. (2) Human prion diseases are highly heterogeneous clinically, suggesting the involvement of several strains with distinct neurotropism. (3) Inherited prion diseases involve more than 50 different mutations in a mature protein that is around 230 amino acids long. Some of the mutations causing dominant forms of inherited prions diseases arise from highly conservative changes (V180I, V210I). These observations support an elevated conformational dynamic for human PrP relative to other PrPs. Following this logical argument, we created transgenic flies carrying human PrP-WT in a BSL3 environment. As we predicted, human PrP induced a novel and robust eye phenotype: eyes were small, highly disorganized (glassy). This eye phenotype was distinct from other amyloids (Rincon-Limas et al. 2012; Fernandez Funez and Myers

2020), suggesting the perturbation of specific pathways. We further examined the toxicity of the new transgenic human PrP by expressing the construct in brain neurons. Expression of human PrP pan-neutrally resulted in developmental lethality (Myers et al. 2022). Using a conditional expression system to bypass this developmental toxicity, expression of human PrP in adult neurons caused a rapid loss of locomotor activity. This work confirmed our rationale that we could create new fly models expressing mammalian PrP with more robust and easier to score phenotypes. The new transgenic flies expressing human PrP were created with random insertions, which are compared by examining protein expression levels. The human PrP constructs had another innovation: we codon-optimized the constructs to make translation more efficient in a heterologous system. This innovation along with the inherent problems of comparing expression levels from random insertions creates a problem when comparing the toxicity of human PrP to other mammalian PrPs.

To address this issue, we created a new suite of PrP constructs using the same technologies to make them highly comparable. We first generated codon-optimized human, mouse, and hamster PrP-WT constructs. Next, we integrated each construct in the same molecularly defined landing site using attP/C31 integrase technology (Bischof et al. 2007). Then, we expressed these constructs in the *Drosophila* eye: only human PrP disrupted the eye. Even adding a second dose of the mouse and hamster PrP constructs did not result in abnormal eyes, whereas two copies of human PrP resulted in smaller and more disorganized eyes (Myers et al. 2022). This experiment confirmed our hypothesis that human PrP is intrinsically more toxic than mouse and hamster PrP. This is not simply a change in the intensity of the phenotype, and it is rather a qualitative difference, since rodent PrP does not alter the eye organization. Importantly, this new eye phenotype dramatically expands the experimental potential for unraveling the mechanism-mediating PrP toxicity. Two applications for this new model are a) conducting genetic screens for modifiers and b) identifying the structural features that make human PrP highly toxic by introducing protective mutations.

17.3.8.2 Examining Protective Amino Acids into Human PrP

The other application for the new fly model expressing highly toxic PrP is the identification of protective amino acids in vivo. *Drosophila* has well-established techniques for generating transgenic animals that are highly economical and accessible over the last 2 decades. Commercial services can efficiently generate transgenic lines for small fees, making fruit flies a well-suited system for structure–function studies that require the generation of multiple constructs carrying single, double, or multiple residue substitutions. Since the cDNA for PrP is small, it can be fully synthesized ready for ligation in an expression vector, saving time and effort. Finally, using the attP/C31 integrase technology described above, constructs are inserted in the same molecular locus in transgenic flies, resulting in comparable expression. With this technology, the toxicity and biochemical behavior of WT and mutant PrP can be easily compared in many assays without concerns over the comparability of the constructs.

Taking advantage of the robust phenotypes induced by human PrP, we identified candidate residues that could suppress this toxicity. We started by examining the protective activity of D159, S167, and S174, the residues proposed to be responsible for the high conformational stability of dog, horse, and rabbit PrP, respectively. We generated transgenic flies expressing human PrP-N159D, D167S, or N174S. In a recent publication, we showed that human PrP-N159D does not suppress toxicity, whereas PrP-D167S and -N174S are mildly protective (Myers et al. 2022). These results were surprising, because the reverse mutations showed increased toxicity of dog PrP-D159N and horse PrP-S167D (Sanchez-Garcia and Fernandez-Funez 2018). The limited effect of the single substitutions in human PrP suggests a high conformation dynamic that cannot be stabilized by the subtle structural changes introduced by each substitution. We also introduced double (N159D–D167S) and triple substitutions (N159D–D167S–N174S) to examine if these residues showed cooperative effects, but we still observed partial rescue similar to S167D alone. The impact of D/E159, S167, and S174 on PrP structure has been tested in multiple systems; of these three, S174 is the only associated with a new structural feature. So far, the evidence supporting the protective activity of S174 is mixed (Sanchez-Garcia and Fernandez-Funez 2018; Vorberg et al. 2003; Khan et al. 2010; Erana et al. 2017). Comparing our reciprocal substitutions in human PrP vs dog, horse, and rabbit PrP, only the D167S/S167D reciprocal mutations showed effects in both directions, suppressing human PrP toxicity and inducing a novel horse PrP toxicity. The residue at 167, located in the center of the β2–α2 loop, seems to exert a critical impact on toxicity, a conclusion that correlates with structural studies identifying a key role for this loop in PrP dynamics (Myers et al. 2022; Myers et al. 2020). It is also likely that D/E159, S167, and S174 cooperate with other amino acids in the vicinity. The reason why this cooperation has escaped curious examination is because conservative changes rarely receive attention. However, in a molecule like PrP with high intrinsic conformational dynamics, small changes in the charge or size of side chains have large effects locally and over long distances by altering subdomains and H+-bonds.

These partially supportive results have piqued our interest in continuing to unravel the rules governing PrP misfolding. The next steps will include introducing double or triple mutations in coordinated combinations, meaning coming from the same animal to add the cooperative effect of multiple small changes. It is also possible that we have been looking at the wrong residues. We have started to identify residues contributing to the unique structural features of human PrP, including conservative changes. We have generated new transgenic flies carrying single and double mutants to examine their potential cooperative effect. We are carrying structural studies in parallel to determine the predicted consequences of the substitutions on human PrP and focusing on the most promising ones. Identifying combinations that eliminate the eye phenotype induced by human PrP will also reveal critical changes in structural dynamics. These studies have the potential to reveal new insight into PrP structure–toxicity relationships and identify the structural motifs that make human PrP so unique among the PrP Zoo.

17.3.9 Genetic Interactions of PrP in Drosophila

Genetic analyses are the main experimental advantage of model organisms with short generation time and easy to manipulate, including yeasts, the nematode *C. elegans, Drosophila*, and zebrafish. Genetic analysis can help identify the functional interactions of genes regulating the same biological process. The two main approaches for identifying genetic interactions are a candidate approach and an unbiased genetic screen. We will describe here progress made so far with the candidate approach due to the limitations imposed by the time-consuming assays that have prevented thus far large-scale unbiased screens. However, the eye phenotype in our new human PrP model is the ideal tool to conduct genetic screens in the future.

Molecular Chaperones: Hsp70

Using the fly model expressing hamster PrP-WT, we examined the ability of the heat shock protein 70 (Hsp70) to regulate PrP misfolding and neurotoxicity. Hsp70 is a molecular chaperone that recognizes misfolded proteins and promotes their refolding. Hsp70 demonstrated protective activity in *Drosophila* models of intracellular amyloids, such as Ataxin-3 and α-Synuclein (Warrick et al. 1999; Auluck et al. 2002). Remarkably, flies co-expressing human Hsp70 and PrP showed lower levels of misfolded PrP and improved locomotor activity (Fernandez-Funez et al. 2009; Auluck et al. 2002). Moreover, Hsp70 physically interacted with PrP in lipid rafts, a specialized and highly dynamic plasma membrane domain. We later replicated the protective activity of recombinant Hsp70 in an in vitro PrP conversion assay with a mammalian brain homogenate (Rincon-Limas et al. 2010). More is on the functional interaction of PrP and Hsp70 later in this chapter.

Now that we have a PrP model with strong eye phenotypes, we can use this phenotype to identify the fly genes that either cooperate with or counteract PrP toxicity. To examine the sensitivity of the robust eye phenotype induced by human PrP, we first introduced perturbations in known genetic modifiers of PrP that can help validate the mechanisms mediating PrP toxicity in flies. We expanded the above work on the Hsp70 family. *Drosophila* studies on Hsp70 function in developmental and disease context have produced Hsp70 variants to modulate its function. We combined some of the existing Hsp70 constructs in flies with the expression of human PrP in the fly eye to examine their functional interactions. Overexpression of cytosolic (wild-type) human Hsp70 (HSPA1L) (Warrick et al. 1999) alone in the eye results in normal eyes (Fig. 17.3a, b). Co-expression of HSPA1L has no significant impact on the toxicity of human PrP (Fig. 17.3e, f). Similarly, overexpression of *Drosophila* Hsp70 (Hsc70-4^{WT}) alone has no effect in the eye (Fig. 17.3c) and has no effect on human PrP toxicity (Fig. 17.3g). In contrast, expression of dominant negative Hsp70 lacking ATPase activity (Hsc70-4^{K71S}) alone causes severe eye depigmentation but has no effect on the eye size and organization, indicating an endogenous function in the development of pigment (Fig. 17.3d). Co-expression of human PrP and Hsc70-4^{K71S} results in very small eyes (Fig. 17.3h), indicating a strong functional interaction. Although increasing the activity of Hsp70 does not suppress human PrP toxicity, the exacerbation of the eye phenotype by the dominant

negative Hsp70 strongly suggests a protective role for Hsp70 against PrP. Since PrP is synthesized in the ER and later secreted and Hsp70 is cytosolic, this functional interaction is likely mediated by direct interaction with PrP retro-translocated to the cytosol for degradation or by indirect mechanisms linking cytosolic and ER dyshomeostasis.

PrP is a secreted protein anchored to the extracellular aspect of the membrane; thus, modulating Hsp70 activity in the ER or in the secretory pathway is expected to directly modulate PrP toxicity. Overexpression of the ER-resident chaperone Hsc70-3^{WT} (BiP/Grp78 in mammals) alone has no effect in the eye (Fig. 17.3i). Hsc70-3^{WT} has no effect on the toxicity of human PrP in the eye (Fig. 17.3m). Silencing Hsc70-3 (RNAi allele) alone results in smaller, glassy, depigmented eyes (Fig. 17.3j), showing the critical function of the ER chaperone in eye development. Co-expression of human PrP and Hsc70-3-RNAi results in synthetic lethality (Fig. 17.3n), a dramatic phenotype considering that there is just leaky expression in a few brain neurons. Expression of a dominant negative Hsc70-3 (ATPase-dead Hsc70-3^{K97S}) alone results in slightly disorganized eyes (Fig. 17.3k), a mild phenotype compared to the RNAi allele. Co-expression of Hsc70-3^{K97S} with human PrP results in very small and disorganized eyes (Fig. 17.3o). Finally, misexpression of an engineered HSPA1L carrying a signal peptide for secretion (secHsp70) (Fernandez-Funez et al. 2016) alone has no effect on the eye (Fig. 17.3l). Co-expression of secHsp70 and human PrP suppresses PrP toxicity, albeit partially (Fig. 17.3p). These results show that human PrP demonstrates strong genetic (functional) interactions with cytosolic, ER-bound, and secreted forms of Hsp70. We propose that human PrP induces proteostatic dyshomeostasis in the ER and in the cytosol that engages endogenous heat shock proteins to prevent cell death. Although WT Hsp70 in the cytosol and the ER shows no suppression of human PrP toxicity, loss-of-function of Hsp70 in either compartment results in a dramatic boost of PrP toxicity, identifying functional interactions. The suppression of PrP toxicity by secreted Hsp70 illustrates the potential benefits from boosting extracellular chaperone activity. We followed these studies by describing the ability of a two-drug cocktail (17-DMAG and dexamethasone) to pharmaceutically stimulate Hsp70 activity. We found that the combined drug treatment, but not individual drugs, decreased PrP misfolding and neurotoxicity (Zhang et al. 2014), suggesting that this could be a potential therapeutic approach for prion diseases and other proteinopathies.

ER Stress and the Unfolded Protein Response

One of the best understood pathways mediating PrP toxicity is the unfolded protein response (UPR) pathway. It has been known for some time that brain tissue from human patients and mouse models of prion diseases accumulates markers of ER stress and activation of the UPR pathway (Hetz et al. 2003; Moreno et al. 2012; Hughes and Mallucci 2019). The UPR consists in three sensors of protein folding in the ER: PERK, Ire1alpha, and ATF6. Activation of PERK (Protein Kinase RNA-like Kinase) results in the phosphorylation of eIF2α, which disrupts the assembly of the ribosome. This acute global repression of translation prevents ER overload and dysfunction and helps restore proteostasis. Conditions causing chronic ER stress result

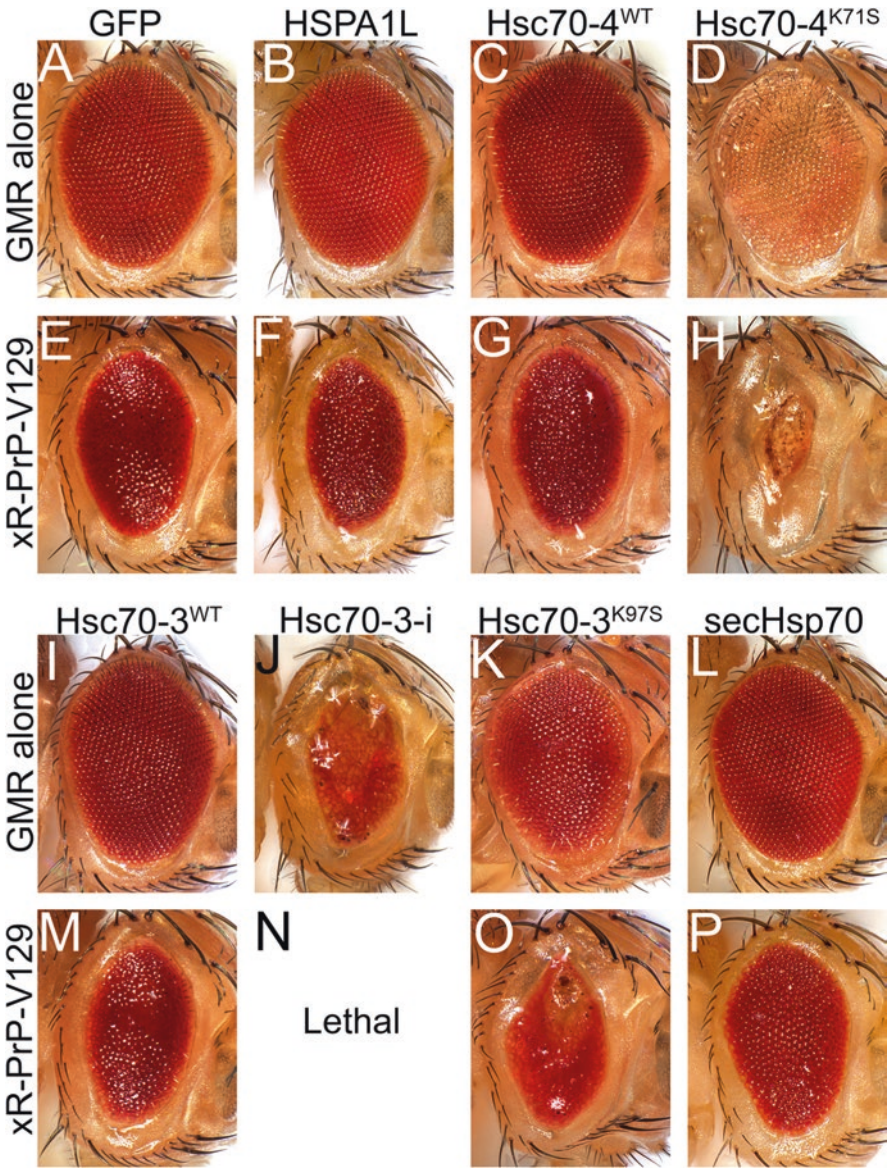

Fig. 17.3 HSP activity modulates PrP toxicity. (**a**–**p**) Micrographs of fresh eyes expressing mCD8-GFP or Hsp70 alleles alone (A–D and I–L), or in combination with human PrP-V129 (E–H and M–P) in the eye under the control of *GMR-Gal4* at 27 °C. (**a**) Control eyes from flies expressing mCD8-GFP (*GMR-Gal4/UAS-mCD8-GFP-attP2*). (**e**) Human PrP-V129 (random) co-expressed in the eye with mCD8-GFP (*GMR-Gal4/UAS-mCD8-GFP-attP2/UAS-human PrP-V129*). These eyes are small and disorganized. (**b**, **c**) Flies expressing human HSPA1L alone (*GMR-Gal4/UAS-Hsp70*) or fly Hsc70–4 alone (*GMR-Gal4/UAS-Hsc70–4^{WT}*) have normal eyes. (**f**, **g**) Flies co-expressing PrP-V129 with human HSPA1L (*GMR-Gal4/UAS-HSPA1L/UAS-human PrP-V129*) or fly Hsc70–4 (*GMR-Gal4/UAS-Hsc70–4^{WT}/UAS-human PrP-V129*) show mildly

in sustained translational repression that is deleterious by limiting the availability of critical proteins. A seminal paper elegantly showed that genetic expression of the eIF2α phosphatase GADD34 can rescue prion-induced pathogenesis in mice (Moreno et al. 2012). Similarly, pharmacological inhibition of PERK also delayed the symptoms in prion-infected mice (Halliday et al. 2014). The UPR pathway is highly conserved in flies and can be easily manipulated with genetic tools. In flies, PERK overexpression prevents eye development on its own (Myers et al. 2022; Malzer et al. 2010), whereas PERK loss-of-function (RNAi) has no effect in the eye. Interestingly, PERK RNAi alleles robustly suppressed the abnormal eye induced by human PrP (Fernandez-Funez et al. 2017; Myers et al. 2022). Activating transcription factor 4 (ATF4) is a PERK effector that can activate the expression of protective genes. Remarkably, ATF4 RNAi alleles also suppress human PrP toxicity (Myers et al. 2022). These results support (1) the key role of the two main PERK effectors, eIF2α and ATF4, in the toxicity of human PrP, (2) the conserved cellular and molecular mechanisms of PrP pathogenesis from mice to flies, and (3) the feasibility of a genetic screen, since known mediators of PrP toxicity rescue human PrP toxicity in flies.

The Amyloid-β Peptide and Alzheimer's Disease

Multiple reports support the direct interaction of PrP and the amyloid-β42 (Aβ42) peptide in biochemical assays (Lauren et al. 2009; Chen et al. 2010; Zou et al. 2011; Gimbel et al. 2010; Gunther and Strittmatter 2010; Balducci et al. 2010). PrP may be required for the manifestation of Aβ phenotypes in brain neurons in mouse models, suggesting a functional link between Alzheimer's and prion diseases. Aβ42 oligomers bind PrP with high affinity and this interaction results in synaptotoxicity, exacerbation of the LTP disruptions, and neurotoxicity (Gimbell). These findings suggest that PrP behaves as a critical Aβ42 receptor mediating the pathophysiology of Alzheimer's disease. The fruit fly can be used as a system in which two exogenous proteins coming from mammals can be examined for functional interactions in a naïve genetic background. Viles and colleagues co-expressed ovine PrP-VRQ (Thackray et al. 2012a) with three forms of the Aβ peptide: Aβ40, Aβ42, and Aβ42 with the Arctic mutation that exacerbates aggregation and toxicity (Crowther et al.

Fig. 17.3 (continued) improved eyes. (**d**) Flies expressing dominant negative Hsc70–4 alone (*GMR-Gal4/UAS-Hsc70–4^{K71S}*) have large but depigmented eyes. (**h**) Flies co-expressing PrP-V129 and dominant negative Hsc70–4 (*GMR-Gal4/UAS-Hsc70–4^{K71S} 4^{WT}/UAS-human PrP-V129*) exhibit very small eyes. (**i**) Flies expressing Hsc70–3 (BiP) alone (*GMR-Gal4/UAS-Hsc70–3^{WT}*) have normal eyes. (**m**) Flies co-expressing Hsc70–3 and PrP-V129 (*GMR-Gal4/UAS- Hsc70–3^{WT}/UAS-human PrP-V129*) show no changes. (**j**) Silencing Hsc70–3 alone (*GMR-Gal4/UAS-Hsc70–3-RNAi*) results in small, glassy, and depigmented eyes. (**n**) Silencing Hsc70–3 in flies expressing PrP-V129 (*GMR-Gal4/UAS-Hsc70–3-RNAi/UAS-human PrP-V129*) results in pupal lethality. (**k**) Flies expressing dominant negative Hsc70–3 alone (*GMR-Gal4/UAS-Hsc70–3^{K97S}*) have large slightly disorganized eyes. (**o**) Flies co-expressing PrP-V129 and dominant negative Hsc70–3 (*GMR-Gal4/UAS-Hsc70–3^{K97S} 4^{WT}/UAS-human PrP-V129*) exhibit small, glassy eyes. (**l**) Flies expressing secHsp70 alone (*GMR-Gal4/UAS-secUAS-Hsp70*) exhibit normal eyes. (**p**) Flies co-expressing secHsp70 and PrP-V129 (*GMR-Gal4/UAS-secUAS-Hsp70/UAS-human PrP-V129*) exhibit larger, well-organized eyes than PrP-V129 alone

2005). All combinations of PrP and Aβ reduced longevity, with Aβ42 and Aβ42-Arctic showing the stronger effects (Younan et al. 2018). These combinations also showed a robust effect on disrupting circadian rhythms. These robust interactions are explained by direct interaction as illustrated by co-immunoprecipitation and stabilization of Aβ42, which increases its toxicity (Younan et al. 2018). Taking advantage of the phenotypic differences between flies expressing human and rodent PrP, we examined the genetic interactions of Aβ42 with human and rodent PrP. We co-expressed human or rodent PrP with our own construct expressing high levels of Aβ42 (Casas-Tinto et al. 2011). This Aβ42 construct induces a robust eye phenotype, so we raised the progeny at a low temperature to induce lower levels of the transgenes and weaker eye perturbations. At this low temperature, human PrP had no visible effect on the external organization of the eye (Myers et al. 2022). We found that co-expressing rodent PrP and Aβ42 had no effect on Aβ42 toxicity in the eye; in contrast, co-expressing human PrP and Aβ42 induced robust disorganization of the eye (Myers et al. 2022). These two studies highlight the specific interactions of PrP and Aβ42 and the utility of fly models to study the potential contribution of these interactions to human disease.

17.3.10 Prion Transmission Studies in Flies

Transmission into Flies Expressing Ovine PrP

The most salient feature of prion diseases is their unique transmissibility mechanism mediated by a proteinaceous material that contains specific misfolded conformations of PrP that are highly resistant to denaturing agents: PrP^{Sc} or PrP^{res}. The Bujdoso lab has put a significant effort in demonstrating that *Drosophila* is an appropriate environment to replicate mammalian prions. This is an interesting question from a mechanistic point of view, but it can be applied toward replacing rodent-based bioassays for disease surveillance in animal and human populations. The Bujdoso lab started by generating flies expressing ovine PrP (see above) (Thackray et al. 2012a) and selected flies expressing ovine PrP–ARQ and VRQ for infectivity experiments. These flies were exposed to scrapie brain extracts from sheep in the growth media. Flies exposed to sheep prions exhibited faster locomotor dysfunction and reduced survival compared to ovine PrP flies grown on normal media (Thackray et al. 2012b). These flies accumulated pathogenic PrP conformations recognized by the 2G11 conformational antibody, yet PrP was sensitive to protease digestion and the flies showed no spongiform pathology (Thackray et al. 2012b). These results suggest that the sheep prions mediated the conversion of fly-expressed transgenic ovine PrP, although the biochemical properties do not agree with a complete conversion to PrP^{Sc}. A later study showed that exposure to sheep prions increased the toxicity of membrane-bound and cytosolic but not fully secreted ovine PrP (Thackray et al. 2014a, b). Flies expressing cytosolic PrP demonstrated the largest increase in toxicity in the presence of prions with no accumulation of PK-resistant PrP. A

follow-up study utilized the sensitive PMCA (protein misfolding cyclic amplification) assay (Saborio et al. 2001) to detect the presence of PK-resistant PrP in transgenic flies. PMCA can amplify a small number of PrP^{Sc} units by an in vitro seeding-polymerization process through cycles of incubation and sonication to create new seeds that exponentially grow over time. This technique amplifies the amount of PrP^{Sc} in the sample making it detectable by western blot which otherwise would be under the detection limit of this technique. Flies expressing membrane bound PrP, but not cytosolic or secreted PrP, show the presence of PrP^{Sc} by PMCA. These results suggest that limited amounts of membrane-bound PrP-WT can be converted into PrP^{Sc} in transgenic flies. However, the robust effect of scrapie extract enhancing the toxicity of cytosolic PrP is apparently not due to prion conversion in flies.

Another key feature of infectious agents is the ability to continue to infect successive generations with similar symptoms. To further examine whether the accumulation of small amounts of PrP^{Sc} in flies was infectious, the Bujdoso lab performed fly-to-fly transmission studies (second passage) (Thackray et al. 2014a, b). Extracts from 30-day-old flies expressing membrane-bound, cytosolic, or secreted ovine PrP that were exposed to scrapie prions (first passage) were homogenized and incorporated into the media into which flies expressing cytosolic ovine PrP were grown. Remarkably, all extracts enhanced locomotor dysfunction in host flies expressing either cytosolic or membrane bound PrP. Additionally, extracts from flies expressing secreted PrP not exposed to prions also accelerated locomotor dysfunction in host flies expressing cytosolic PrP, which was interpreted as the sporadic generation of prions. These studies suggest the potential for prion transmission in transgenic flies.

Further work on prion transmissibility with flies expressing ovine PrP focused on assessing the sensitivity of the locomotor bioassay (Thackray et al. 2016). For these experiments, this group used full-length and cytosolic ovine PrP–VRQ. These flies were exposed to a serial dilution of brain scrapie PG127 from 10^{-2} to 10^{-14}. The fly bioassay showed sensitivity to detect a reduction in locomotor performance from 10^{-2} to 10^{-10} (Thackray et al. 2016). This assay was then used to assess the sensitivity of prions in plasma, which is traditionally a more challenging extract in mouse bioassays. The fly locomotor assay was sensitive to prions in plasma diluted 10^{-1}. These studies were expanded in a follow-up publication that examined the amplification of transmissible PrP in flies as they aged (Thackray et al. 2018). In this case, they mainly used PMCA to detect the presence of PK-resistant PrP in flies expressing ovine PrP–VRQ exposed to scrapie PG127 brain extracts. Flies were incubated for 5–40 days and head homogenates were subjected to PMCA. Younger samples incubated for 5 or 10 days were negative for PMCA amplification (Thackray et al. 2018). However, flies aged for 20, 30, or 40 days were positive with increasing concentrations of PK-resistant PrP with age. Overall, these studies in flies established flies expressing ovine PrP as sensitive models for the replication and detection of scrapie prions in a behavioral assay and in PMCA. These models have the advantage over the standard mouse bioassays that they are faster, economical, and reduce the number of vertebrate animals used in the laboratory.

An additional key property of prions is the preservation of strain properties over serial passages. To examine this property in the prions detected in flies expressing ovine PrP, brain extracts from fly heads were inoculated into mouse mice expressing ovine PrP (tg338). The result was that 6/6 (100%) of the mice inoculated developed disease (Thackray et al. 2016). These mice showed accumulation of PK-resistant PrP and biochemical characteristics that suggested the preservation of the original properties of the sheep prion inoculated into flies. In a follow-up, the authors examined the ability of fly extracts from flies aged 5–40 days to infect mice (Thackray et al. 2018). tg338 mice inoculated with extracts from prion-exposed flies aged for 5 or 10 days showed no infection. Mice exposed to fly extracts from flies aged for 20 days showed partial attack rates (4/6, 66%), whereas extracts from flies aged for 30 or 40 days produced 100% attack rates (6/6 each) (Thackray et al. 2018). Following a second fly-to-fly passage, inoculation of 30-day-old fly extracts into tg338 mice still resulted in 100% attack rate, with the presence of PK-resistance with the correct electrophoretic mobility, supporting the preservation of strain properties. Furthermore, inoculation of three different ovine strains generated in tg338 mice (PG127, Pa59, and Apl338) into VRQ or ARQ flies were transmitted back into tg338. These experiments continued to show preservation of the molecular profiles of the original strains along with expected neurotropism in the brain lesions (Thackray et al. 2018). In all, the transmission back to mice expressing ovine PrP showed development of prion disease and the preservation of strain properties, indicating that passage through flies had no deleterious effect on the behavior of mammalian prions.

Transmission into Flies Expressing Rodent PrP

Following on the success with the flies expressing ovine PrP, the Bujdoso team attempted the transmission of inherited prion diseases using the rodent PrP lines described above (Thackray et al. 2017). For these experiments, they used flies expressing either mouse or hamster PrP WT, D178N, or E200K (human numbering) as from KI mice carrying the mutations (Thackray et al. 2017) as host for mouse prions. The mouse prions came from knock-in mice expressing murine PrP carrying D178N or E200K. These mice develop a disease similar to the genetic prion diseases that result in the de novo production of prions induced by the point mutations (Friedman-Levi et al. 2011; Bouybayoune et al. 2015; Dossena et al. 2008). Flies expressing mouse PrP-WT were exposed to media containing brain extracts from mice expressing D178N or E200K along with WT and null controls. The locomotor bioassay showed decreased performance in flies exposed to the two mutant strains, with a larger effect by E200K consistent with the stronger phenotype in the original mouse strain. Then, head homogenates from flies expressing mouse or hamster PrP-WT were exposed to head extracts from flies expressing mouse or hamster PrP harboring D178N or E200K mutations that developed spontaneous neurotoxicity. The flies exposed to these extracts developed a mild decline in locomotor performance only in flies in which the mutation in the inoculum and the host matched. This is suggestive of the transmission of a neurotoxic agent that is specific for each mutation. However, this study did not further characterize the biochemical properties of PrP in the host, and thus, it is unclear whether the increased neurotoxicity is due to the transmission of a de novo generated prion by mutant PrP. Alternatively,

this experiment could be interpreted as the transmission of misfolded conformations, not necessarily PrP^{Sc} capable of seeding endogenous PrP an accelerating neurotoxicity.

Transmission into Flies Expressing Bovine PrP

The most recent of the series of papers trying to demonstrate the utility of *Drosophila* models of PrP shifted to the generation of transgenic flies expressing bovine PrP-WT (Thackray et al. 2021). It is likely that the Bujdoso lab considered alternative models expressing ovine and rodent PrP for some time due to the increased safety when expressing PrP from animals with a species barrier with humans. Since the major concern for zoonotic prion transmission from the food chain comes from beef, the Bujdoso team created new flies expressing bovine PrP to apply their previous expertise with this useful model. Flies expressing bovine PrP were exposed to classical and atypical BSE inoculums, incubated the adult flies for 5–40 day post-eclosion, homogenized the heads at each timepoint, and conducted PMCA to detect the amplification of PrP. They found, as they did before with ovine PrP, that only the older flies (20–40 days) amplified PrP in PMCA (Thackray et al. 2021). Serial passage from fly-to-fly also showed that only inoculum from older flies resulted in amplification by PMCA. These infected flies showed loss of locomotor activity and decreased survival that were concentration-dependent, and the limit of detection of BSE inoculum was around 10^{-12} dilution (Thackray et al. 2021). Direct comparison of transmission of the same infectious material into flies and mice indicated that the *Drosophila* model is faster, and more sensitive and economic, suggesting a potential application of fly models to reduce the use of rodents for surveillance purposes.

Identification of Novel Genetic Pathways Mediating PrP Toxicity

A complementary approach to gain information about how a particular gene or process interact with normal cell and tissue physiology is to examine the disruption of gene networks through transcriptomics. In this case, the Bujdoso lab analyzed the differential gene expression (DEG) in flies exposed to mammalian prions with the goal of identifying candidate pathways mediating this specific neurotoxic condition. For this study, they used models expressing ovine PrP-WT and its cytosolic version lacking the GPI anchor exposed to sheep prions in the growth media, as described above (Thackray et al. 2012b, 2014b). Then, they performed RNAseq from fly heads at 5 and 40 days followed by analysis of DEG against control flies not expressing PrP. This work identified more DEG in flies expressing PrP-WT compared to the cytosolic form (Thackray et al. 2020). The early changes identified in these flies were up-regulation of cell cycle and DNA damage repair networks. Later changes showed repression of protein synthesis and the mTOR-signaling pathway, two key survival pathways. Moreover, several down-regulated genes indicated that loss of mitochondrial homeostasis was driver of neurotoxicity in this model (Thackray et al. 2020). Overall, these results are consistent with known processes mediating prion disease. Altered protein expression has been identified in previous publications as a key modulator of prion toxicity, particularly through phosphorylation of eIF2a following the activation of the UPR (Myers et al. 2022; Moreno et al. 2012; Hughes and Mallucci 2019; Hetz and Soto 2006). On the other hand, disruption on mitochondria homeostasis is a known mediator of degenerative processes in general.

17.4 Concluding Remarks

Fruit flies will never be a substitute for rodent-based experiments in the pursuit of understanding and treating human diseases. Yet, *Drosophila* provides a complementary experimental system with its own strengths, including the faster and economic development of transgenic animals carrying constructs to address specific questions. Over the last 10+ years, existing fly models have been utilized in novel experimental approaches and many new models have been generated to ask new questions. The overall outlook is that *Drosophila* models are advancing the knowledge base toward unraveling the rules governing the conformational dynamics and toxicity of PrP, identifying the genetics mechanisms modulating neurotoxicity, and developing alternative bioassays that will reduce the use of mice in the surveillance for prions in farm and wild animals.

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Part VI
Human Prion Diseases and Other Pathologies

Chapter 18
Human Sporadic Prion Diseases

Pierluigi Gambetti and Ignazio Cali

Abstract Sporadic or idiopathic prion diseases account for over 90% of all human prion diseases, and sporadic Creutzfeldt–Jakob disease (sCJD) is by far the most common. The heterogeneity of the sCJD clinical features, which was noted soon after a significant number of cases became available, led to the introduction of increasing number of "forms" or phenotypes under descriptive labels, such as myoclonic, ataxic, and amaurotic. In the 90s, Gambetti, Parchi and colleagues proposed a molecular mechanism based on the pairing of the prion protein (PrP) genotype at the methionine (M)/valine (V) polymorphic codon 129, and the type 1 or 2, of the disease-associated PrP (PrP^D). This mechanism led to a rational and robust classification of sporadic prion diseases that, with some adjustments to the increasing complexities of the sporadic prion diseases, is currently in use worldwide, and has been the subject of several reviews. Recent data, however, have highlighted an additional mechanism of phenotypic heterogeneity that pertains to the sCJD subtypes heterozygous at codon 129 denoted as MV2C, MV2K, and MV1, and have further characterized the sCJDVV1 subtype as well as sporadic fatal insomnia and variably protease-sensitive prionopathy, the two prion diseases recently set apart from sCJD. This review focuses on these new data that further support and expand the molecular mechanism of phenotypic heterogeneity originally proposed. We also review a novel application of magnetic resonance imaging to identify in vivo the brain region initially impacted (epicenter) and the subsequent propagation pathway of the disease process in the major subtypes of sCJD. It is hoped that a better understanding of phenotypic heterogeneity and strain determination coupled with technologies leading to early and accurate diagnosis of sCJD subtype in vivo will lead to early and targeted therapeutics.

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18.1 Introduction

Creutzfeldt–Jakob (or Jakob–Creutzfeldt) disease (CJD), first tentatively identified by Creutzfeldt in 1920 and definitively established in 1921 by Jakob, acquired wider recognition following the early reviews by Alemà and Bignami (1959), and Kirschbaum (1968). The broad clinical and histopathological spectrum of human prion diseases was further underscored shortly after (Brownell and Oppenheimer 1965; Gomori et al. 1973).

In the late 1990s, the phenotypic heterogeneity of sporadic human prion diseases entered the molecular era. Six studies proposed a correlation between disease heterogeneity and specific molecular features (Collinge et al. 1996; Gambetti et al. 2003; Parchi et al. 1996, 1999, 2000; Puoti et al. 1999). Subsequently, new technologies became accessible that were applicable to the study of phenotypic heterogeneity of human prion diseases. They include: (i) modeling in genetically modified (humanized) mice expressing the human normal or cellular prion protein (PrP^{C}), (ii) prion conformational assays, and (iii) diagnostic tests based on the amplification of the disease-related prion protein (PrP^{D}). These new approaches have basically confirmed and refined the model proposed by Gambetti, Parchi and colleagues adding novel subtypes and leading to the application of the strain concept to the subtypes of human prion diseases (Table 18.1). These advances have been the topic of several recent reviews (Baiardi et al. 2019; Puoti et al. 2012; Ritchie and Ironside 2017; Gambetti et al. 2013; Zerr et al. 2018). However, further developments encourage to revisit this issue; they are highlighted by (i) a novel mechanism of sporadic CJD (sCJD) phenotype determination in the 129MV heterozygous group encompassing the MV2C, MV2K, and MV1 subtypes; (ii) the first brain mapping identifying the locale of first disease detection or epicenter and the ensuing propagation pathway in sCJD subtypes; (iii) the fundamental diversity of the proteinase K (PK) resistant PrP^{D} ($resPrP^{D}$) aggregate formation in the variably protease-sensitive prionopathy (VPSPr) when compared with that of Gerstmann–Sträussler–Scheinker disease (GSS), two conditions often considered to be related (Cracco et al. 2019; Nemani et al. 2020; Pascuzzo et al. 2020). New data also deal with the characteristics of $resPrP^{D}$ type 1 in sCJDVV1 and of $resPrP^{D}$ type 1 and 2 co-existence in the sCJDVV1–2 subtype (Table 18.1) (Cali et al. 2021; Nemani et al. 2020).

This chapter is a current update on basic aspects of sporadic human prion diseases with emphasis on the recent developments and previous relevant findings that may have been overlooked.

Table 18.1 Classification of human sporadic prion diseases

Disease and Subtype clusters	Prevalence[a] (%)	Subtype	Prevalence[b] (%)	Age at onset (y)/Dis. durat. (m) (Mean)[b]	Dominant early clinical and histopathological features
sCJD "pure" Well established	74	MM(MV)1	63	67/3	Dementia (70%) and typical EEG (83%). Spongiform degeneration (SD) with small, non-confluent vacuoles mostly in cerebral neocortex; punctate PrP immunostaining pattern (IP). Over 90% sensitivity of diagnostic (Dx)
		VV1	2	32/11	Dementia (96%) with rare typical EEG. SD with intermediate size, non-confluent vacuoles mostly in anterior neocortex and neostriatum with gliosis and balloon cells; weak punctate IP; cerebellum (Crbll) spared. 80% and 92% sensitivity of dx tests
		MM2	6	66/14	Dementia (100%) and rare typical EEG. Large and confluent vacuoles in CC; granular PrP IP; Crbll. Spared. High sensitivity of CSF tests and MRI
		VV2	18	68/6	Ataxia; rarely typical EEG. Laminar SD with medium size vacuoles and plaque-like deposit especially in the Crbll granule cell layer with atrophy; perineuronal IP. Dx tests highly sensitive
		MV2K	10[c]	65/16	As VV2 except for prominent kuru plaques in Crbll without detectable atrophy. Dx tests highly sensitive
		MV2C	1[c]	68/25	Phenocopy of MM2 subtype

(continued)

Table 18.1 (continued)

Disease and Subtype clusters	Prevalence[a] (%)	Subtype	Prevalence[b] (%)	Age at onset (y)/Dis. durat. (m) (Mean)[b]	Dominant early clinical and histopathological features
sCJD PrP^D type 1 and 2 mixed with shared phenotypes	23	MM1-2	39	61/6	Dementia (90%); SD of both subtypes with typical PrP IP maintained and distributing with the respective PrP^D types 1 and 2
		MV1-2	35	66/14	As MM1–2 with type 2 phenotype generally under-represented
		VV1-2	23	65/8	Mixed clinical and histotype of both subtypes in measure directly related to the relative amount of the matching PrP^D type
sCJD Phenotype mixed with shared type 2	–	MV2C-K	2[c]	66/14	Ataxia and/or dementia; histotypes including PrP IP of each pure subtype variably mixed as for representation and topographic distribution
sCJD very rare	<1	MM-p^{WM}	<1	65/20	Dementia. Widespread cortical and white matter (WM) subcortical atrophy. Kuru plaques in subcortical WM and WM component of subcortical nuclei, brain stem and cerebellum with WM degeneration.
sFI	1	MM2	–	39/17	Dementia and sleep disorder. Severe neuronal loss and astrogliosis of thalamic nuclei with minimal or no SD. SD presence in cerebral cortex related to disease duration, when present, similar to MM2 SD. Variable atrophy of inf. olives; minimal PrP IP. MRI generally negative; positive PET scanning

(continued)

Table 18.1 (continued)

Disease and Subtype clusters	Prevalence[a] (%)	Subtype	Prevalence[b] (%)	Age at onset (y)/Dis. durat. (m) (Mean)[b]	Dominant early clinical and histopathological features
VPSPr	2	MM	19	77/32	Psychiatric signs, ataxia, parkinsonism frontal dementia in various mixes. SD with vacuoles predominantly small but including larger sizes with MM1-like distribution. Mini amyloid plaques in Crbll molecular layer. IP: granules of different sizes sometimes clustered in a tigroid or target-like pattern
		MV	11	76/30	
		VV	70	66/16	

[a]Data based on 2319 cases provided by the NPDPSC (Cleveland, OH, USA)
[b]Data on the "pure" cluster, MV1-2, sFI and VPSPr provided by the NPDPSC; data on other sCJD clusters obtained from references (Berghoff et al. 2015; Cali et al. 2009, 2020; Gelpi et al. 2013; Kobayashi et al. 2013; Parchi and Saverioni 2012; Rossi et al. 2017)
[c]Estimated prevalence values assessed from the 12% prevalence of all MV2 subtypes combined provided by the NPDPSC. Data on age and duration of MM(MV)1, MM2, MV1-2, sFI and VPSPr provided by the NPDPSC

18.2 Individual Types and Subtypes

sCJD: the established subtypes. Demographics, clinical, and histopathological characteristics of the most common subtypes of sCJD are well known and have been recently reviewed (Baiardi et al. 2019; Polymenidou et al. 2005; Zanusso et al. 2007). Table 18.1 summarizes these features related to all the types and subtypes of human sporadic prion diseases discussed in this chapter, which include not only the "pure" and most common sCJD subtypes, but also the two groups of "mixed" subtypes: (i) those where resPrP^{D} types 1 and 2 and their respective phenotypes (sCJDMM/MV1-2 and VV1-2) coexist in various ratios and (ii) those where two phenotypes co-exist in association with the resPrP^{D} type 2 variants (MV2C-K). As for the clinical features, among the established subtypes of notice is the early onset of the VV1 subtype, while disease duration distinguishes a cluster of cases with short duration (~ 4–7 months), which includes MM/MV1 and VV2 subtypes, from a second cluster with longer disease duration (~15–17 months) encompassing the other three subtypes (MM2, MV2, and VV1). Similarly, the subtypes may be grouped according to the dominant clinical sign at presentation: cognitive decline or ataxia (or other cerebellar signs) (Baiardi et al. 2019; Puoti et al. 2012; Zerr and Parchi 2018). However, each subtype can also be clearly identified and diagnosed according to the topography and type of the lesions, especially the characteristics of the vacuoles populating the spongiform degeneration (SD) and of the PrP immunostaining patterns (Fig. 18.2).

sCJD Associated with Coexisting PrP^D Types 1 and 2 First observed in 1999 (Parchi et al. 1999; Puoti et al. 1999), the coexistence of both PrP^D types 1 and 2 (1–2) was initially reported in all SCJD-affected brains (Polymenidou et al. 2005) raising questions about the validity of the sCJD classifications based on $resPrP^D$ typing. However, several detailed studies did not confirm the ubiquity of $resPrP^D$ types 1–2 coexistence in sCJD, which was attributed to the lack of complete PK-digestion and adequate buffer capacity required for accurate $resPrP^D$ tying (Cali et al. 2009; Notari et al. 2004, 2007). Nonetheless, when cases with only traces of one of the two types are included in the sCJDVV1–2 subtype, the type mixed cohort amounts to up to 57% of the total sCJDVV population (Cali et al. 2020).

sCJD-Mixed Subtypes MV2C and MV2K Showing Coexisting Clinical and Histopathological Phenotypes but Sharing Type 2 $resPrP^D$ When they harbor mixed phenotypes as in MV2C-K, these two subtypes essentially follow the same rules governing phenotype and $resPrP^D$ representation as the PrP^D 1–2 subtypes but through a different mechanism (see the following section) (Nemani et al. 2020).

Very Rare Subtypes

(a) With atypical glycotype (AG): $sCJDMV^{AG}$: The most striking molecular characteristic of the first of the two cases independently reported is the electrophoretic profile of $resPrP^D$, where the diglycosylated and one of the two monoglycosylated $resPrP^D$ isoforms are lacking, while the unglycosylated is over-represented (Table 18.1 and Fig. 18.1) (Tanev and Yilma 2009; Zanusso et al. 2007). A ~ 19 kDa, fragment consistent with $resPrP^D$ type 2, has been detected only in the pellet-enriched fraction or following mild denaturation with GdnHCl (Galeno et al. 2017). Of note, mass spectrometry identified $resPrP^D$ N-termini of Q67, G71, and G90 which do not seem to be consistent with either $resPrP^D$ type 1 (G82) or type 2 (S97) (Parchi et al. 2000; Zanusso et al. 2007). $sCJDMV^{AG}$ could be transmitted only to humanized mouse line expressing PrP^C-129VV (not the lines expressing PrP^C 129MM and -MV) and to bank voles. The 129VV mice faithfully reproduced electrophoretic profile of the original $resPrP^D$, but did not show histotypic changes, such as an immune-detectable PrP deposition pattern (Galeno et al. 2017). Transmission to bank voles was complete and generated distinct features. The second independently reported case also lacks the $resPrP^D$ diglycosylated isoform (Tanev and Yilma 2009), but differs from the first case in several features: (i) the unglycosylated $resPrP^D$ isoform is not overrepresented; (ii) the genotype at codon 129 is VV not MV; (iii) the disease onset is ~30 years earlier; and (iv) the histotype mimics that of sCJDVV1 and lacks the intracellular PrP^D deposition of the first case (Tanev and Yilma 2009).

(b) With white matter PrP plaques: $sCJDMM\text{-}p^{WM}$: Seven of the nine reported cases (Berghoff et al. 2015; Gelpi et al. 2013; Kobayashi et al. 2008; Rossi et al. 2017) that were fully analyzed harbored $resPrP^D$ type 1 (Kobayashi et al. 2008; Rossi et al. 2017), while types 1 and 2 coexisted in equal amount in two cases

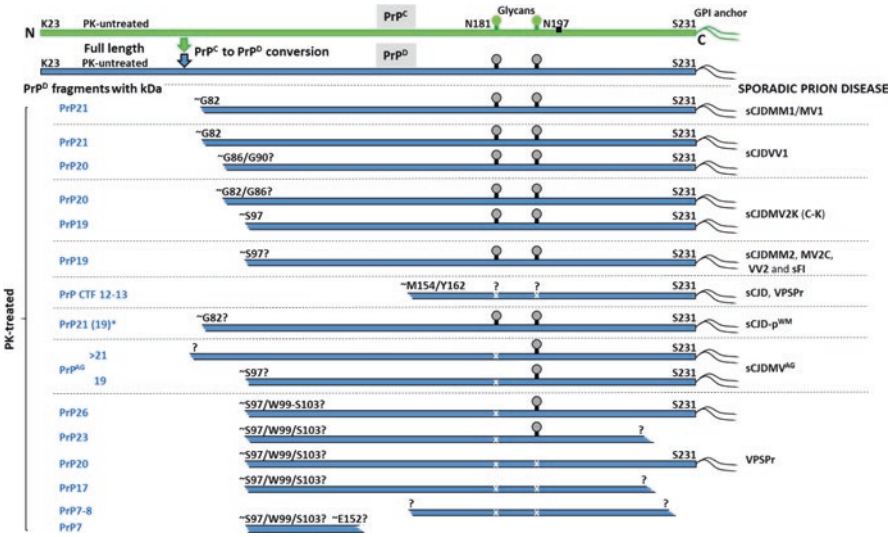

Fig. 18.1 Schematic representation of prion protein (PrP) fragments associated with sporadic prion diseases including sporadic Creutzfeldt–Jakob disease (sCJD) subtypes, sporadic fatal insomnia (sFI), and variably protease-sensitive prionopathy (VPSPr). PrP^C, PrP^D, and PK refer to normal or cellular, abnormal or disease-related prion protein, and to proteinase K, respectively. Lack of one or both glycans is indicated by the x embedded in the blue bars. Letters and numbers atop each bar specify symbol and number in the PrP sequence of one or more amino acids identified at the N- and C-termini (N and C); the lack or presence of the question mark indicates terminus identification by sequencing or epitope mapping and other indirect approaches, respectively. CTF: C-terminus fragment; sCJDMM, -MV, -VV followed by 1 or 2 refer to the allotype at the MV polymorphic codon 129 and to the type 1 or 2 of the associated PK-resistant PrP^D that characterize most sCJD subtypes; K and C: presence of kuru plaques or cerebral cortical spongiform degeneration; sCJD-p^{WM} and –MV^{AG} denote kuru plaques impacting the white matter and atypical glycoform PrP^D profile. *One case was also associated with the PrP^D type 2 (19 kDa) fragment. References used to determine the N- and C-termini of all fragments: sCJD major subtypes (Nemani et al. 2020; Parchi et al. 2000); -MV^{AG} (Galeno et al. 2017; Zanusso et al. 2007); -p^{WM} subtype (Rossi et al. 2017); VPSPr (Notari et al. 2018; Pirisinu et al. 2013; Zhang et al. 2021)

(Berghoff et al. 2015; Rossi et al. 2017). The resPrP^D type 1 associated with MM-p^{WM} mimics gel mobility, glycoform ratios, and conformational characteristics of the MM1 subtype. However, the MM-p^{WM} clinical course of 25 months is significantly longer than that of MM1 (Table 18.1). sCJDMM-p^{WM} transmissibility has been demonstrated in bank voles.

The extreme rarity of sCJDMV^{AG} and the heterogeneity of the only two cases reported necessitate further analyses or detection of additional cases before -MV^{AG} can be considered a bona fide subtype of sCJD. Similarly, MM-p^{WM} needs analyses aimed at the PrP^D species harbored in the white matter or associated with the kuru plaques, as well as a better understanding of its relationship to MV2K; transmission properties should also be assessed in humanized mice with different codon 129 allotypes.

Sporadic Fatal Insomnia (sFI) is commonly viewed as the phenocopy of fatal familial insomnia, a genetic prion disease linked to the D178N PrP gene mutation and coupled with the 129 allotypes MM or MV (but not VV) (Cracco et al. 2017; Goldfarb et al. 1992; Medori et al. 1992). sFI shares PrP^{D} type 2 with MM2, but differs from the latter for the pronounced neuronal loss and gliosis without SD impacting the medial thalamic nuclei. However, there are unquestionable affinities between the two diseases. For example, although the presence of SD is not required for the diagnosis of sFI, when it is present, as is in sFI subjects with long disease duration, it mimics the SD associated with MM2 (Cracco et al. 2018).

VPSPr First reported in 2008 and more comprehensively in 2010, VPSPr clearly differs from sCJD due to the generally longer course, clinical features (more consistent with non-prion neurodegenerative diseases), distinct histopathology, and, more strikingly, for the electrophoretic profile (see the next section) (Table 18.1 and Figs. 18.1 and 18.2) (Gambetti et al. 2008; Notari et al. 2018; Zou et al. 2010). VPSPr also shows additional intriguing features: as sCJD, it comprises variants

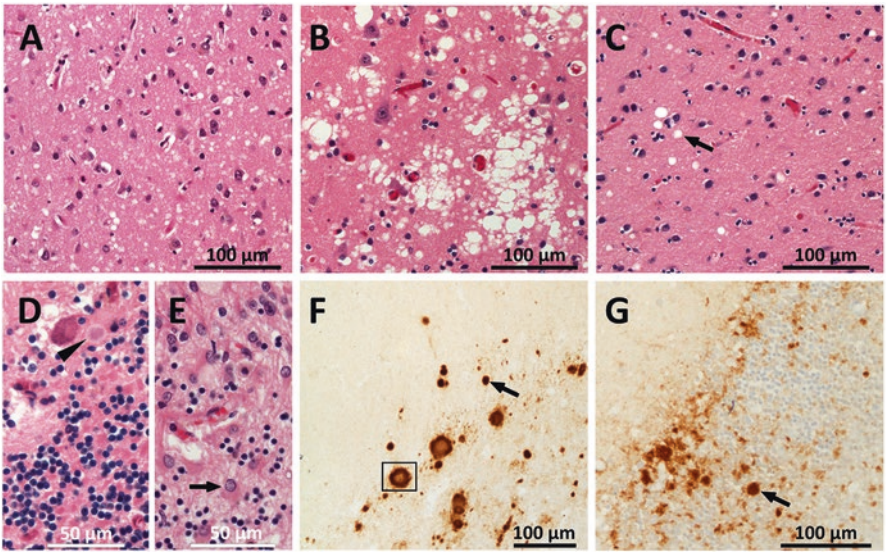

Fig. 18.2 Histopathology in subtypes of sporadic Creutzfeldt–Jakob disease (sCJD) and in variably protease-sensitive prionopathy (VPSPr). (**a**–**c**) Types of spongiform degeneration (SD) in MM1 and MM2 subtypes, and in VPSPr, respectively; (**d**–**g**) cerebellar histopathology and PrP immunostaining pattern in MV2K (**d**, **f**) and in VV2 (**e**, **g**). Note the distinct patterns of SD in the cerebral cortex: fine vacuoles in MM1 (**a**); large, clustered and often confluent vacuoles in MM2 (**b**); intermediate-size vacuoles in VPSPr (**c**). (**d**, **e**) Compare the apparently good preservation of cerebellar granule cells in MV2K (arrowhead: Kuru plaque) (**d**) with the granule cell depopulation and reactive astrogliosis (arrow) in VV2 (**e**). (**f**, **g**) Kuru plaques with core (square) and coreless plaque-like PrP aggregates (arrow) in MV2K (**f**) compared with predominantly an apparently coreless plaque-like profiles in VV2 an apparently (arrow) (**g**). (**a**–**e**) Hematoxylin–eosin. (**f**, **g**) PrP immunostaining (3F4 antibody)

associated with each of the three 129 allotypes (MM, MV, and VV), but with an individual prevalence almost opposite to that of sCJD (11, 24, and 65 VPSPr vs. 71, 12, and 17 sCJD), which suggests that the 129MV polymorphism plays a different role in the two diseases. Additionally, there is a relatively high frequency of familial history of dementia, and finally, it shares the resPrP^{D} electrophoretic profile with the genetic prion disease bearing the V180I mutation (Notari et al. 2018; Xiao et al. 2013).

18.3 Mechanisms of Sporadic Prion Disease Heterogeneity: Old and New

sCJD Major Subtypes The classification of the most common subtypes of sCJD proposed by Gambetti, Parchi, and coworkers (Parchi et al. 1996, 1999) resolved the stumbling block of the phenotypic heterogeneity in sCJD by pairing the patient's PrP^{C} genotype at the MV polymorphic codon 129 with type, 1 or 2, of the resPrP^{D}. The resPrP^{D} was designated type 1 or 2 according to its electrophoretic mobility either to 21 (type 1) or 19 kDa (type 2), which is referred for convenience to the mobilities of the respective unglycosylated isoform. The distinct electrophoretic mobilities of resPrP^{D} types 1 and 2 relate to the different lengths of their respective protease-resistant cores, which are revealed by PK-digestion. These data have been confirmed by amino acid sequencing that has identified the major N-termini of resPrP^{D} types 1 and 2 at glycine 82 (G82) and asparagine 97 (S97), respectively (Fig. 18.1) (Parchi et al. 2000). It has also been reported that PrP^{D} type 1 is paired with the 129MM allotype in over 90% of the patients, while type 2 associates with the 129MV and -VV allotypes in ~80% of the cases, which suggests that PrP^{D} typing is at least partially controlled by the genotype at codon 129 (Gambetti et al. 2003).

The pairing of the 129 PrP genotype and PrP^{D} type (Parchi et al. 1996, 1999) currently provides a straightforward classification of the "pure" 129 homozygous subtypes MM1, MM2, and VV2 (Table 18.1). The 1-2 type mixed subtypes comprising MM1-2 and VV1-2 also fit the Gambetti, Parchi, and coworkers classification, because the type-specific phenotype directly correlates with the dosage of the respective resPrP^{D} type when the amount of the corresponding resPrP^{D} exceeds the threshold of the ~20% ratio (Cali et al. 2009, 2020). Furthermore, a distinct mechanism of phenotypic determination has been recently identified in the 129 heterozygous subtypes MV2C and MV2K as well as in MV1, which applies to both "pure" and phenotype-mixed forms. Finally, recent studies have added complexity to the type 1 resPrP^{D} associated with the VV1 and VV1–2 subtypes that are noteworthy (Cali et al. 2020, 2021).

The two sCJD subtypes, MV2C and MV2K, seemingly challenge the basic tenet of phenotypic determination in sCJD, because they share the 129 allotype (MV) and the resPrP^{D} type 2 that are the two major phenotypic determinants, yet they feature

distinct phenotypes: SD prominently impacts the cerebral cortex (C) in MV2C and Kuru plaques (K) set MV2K apart (Table 18.1, Fig. 18.2) (Gambetti et al. 2003). We recently described an additional mechanism of phenotypic heterogeneity and mixing in 129MV sCJD, whereby both respective 129 M and -V PrP^C variants are expressed and converted to $resPrP^D$, and the relative abundance of each of 129 M and -V $resPrP^D$ correlates with the dosage of the MV2C and MV2K phenotypes (Nemani et al. 2020). More specifically, "pure" MV2C and MV2K phenotypes are associated with $resPrP^D$-129 M and -V, respectively, in the same reciprocal ratio of ~80:20%, while closer ratios result in the co-existence of both phenotypes in ratios that mirror those of the respective $resPrP^D$ 129 M and -V species (Nemani et al. 2020). The electrophoretic profile of phenotypically pure MV2K showed a 19 kDa $resPrP^D$ fragment apparently identical to the 19 kDa harbored by VV2 as they shared also the S97 N-terminus. This component, however, co-existed with a 20 kDa fragment that immunoreacted as $resPrP^D$ type 1 but featured two N-termini at G82 and G86 which differed slightly from the canonical G82 major and G78 minor cleavage sites of MM1 (Fig. 18.1) (Kobayashi et al. 2013; Nemani et al. 2020; Parchi et al. 2000). Despite these discrepancies of the electrophoretic profiles, conformational testing revealed no difference between the 19 and 20 kDa components as well as between these two components (individually or combined) and the 19 kDa $resPrP^D$ associated with VV2. These results suggest that the conformational properties of MV2K- and VV2-associated PrP^D are homogeneous (Nemani et al. 2020). The $resPrP^D$ profile of MV2C, as a whole, appears to mimic that of the MM2 subtype (Kobayashi et al. 2013; Nemani et al. 2020). In summary, this new mechanism of phenotypic heterogeneity is again consistent, also at allotypic level, with the original hypothesis of Parchi, Gambetti, and colleagues (Parchi et al. 1996, 1999) that the genotype at PrP codon 129 is a determinant of the PrP^D characteristics, which, in turn, affects the phenotype (Gambetti et al. 2003).

The determination of the resPrP-129 M and -V relative amounts in the MV1 subtype, which is widely considered to be a phenocopy of the MM1 subtype, was expected to yield an 80:20 ratio in favor of $resPrP^D$-129 M, mimicking the ratio of $resPrP^D$ type 2 in "pure" MV2C (Table 18.1). This ratio would seem to be the most economical way to express the MM1 "pure" phenotype in a 129MV background. Surprisingly, we observed a 50:50 ratio of 129 M to 129 V $resPrP^D$ type 1. The explanation as of how a phenotype matching that of MM1 can be achieved with an equal representation of $resPrP^D$-129 M and -129 V remains speculative at this time (see also the next section).

Finally, a recent study adds complexity to the characteristics of the sCJDVV1 subtype as it reports that VV1 $resPrP^D$ comprises two fragments of 20 and 21 kDa which feature N-termini at the likely residues G86 and G82, respectively (Fig. 18.1) (Cali et al. 2020, 2021; Parchi et al. 2000). The 20 and 21 kDa fragments may coexist as a doublet in variable ratios or individually. Conformational tests have highlighted significant differences between these two fragments. Furthermore, although the 20 and 21 kDa fragments transmitted with similar metrics in humanized mice expressing PrP^C-129 V, contrary to the 20 kDa, the 21 kDa failed to replicate in similar mice expressing human PrP^C-129 M further supporting the heterogeneity of

the two fragments. Remarkably, no significant variations of the histopathological features were detected between cases associated with each of the three electrophoretic profiles (Cali et al. 2021).

sFI poses a challenge to the Parchi, Gambetti, and coworkers' (Parchi et al. 1996, 1999) mechanism of phenotypic heterogeneity, given that both sFI and the sCJDMM2 subtypes are associated with electrophoretically indistinguishable resPrP^{D} type 2, even though the two conditions are phenotypically distinct (Table 18.1) (Cracco et al. 2017). However, significant conformational difference between the resPrP^{D} type 2 species associated with these prion diseases has been reported (Cracco et al. 2017). Furthermore, quantitative variations in PK-sensitive PrP^{D}, the other components of total PrP^{D}, were also observed that clearly distinguished the two diseases. They included: glycoform ratios, one- and two-dimensional electrophoretic profiles and aggregate distribution following equilibrium and velocity sucrose gradients (Cracco et al. 2017). Finally, distinct characteristics of transmission have been uncovered following inoculation of sFI and sCJDMM2 brain isolates to humanized mice (Moda et al. 2012).

The VPSPr electrophoretic profile is challenging due to the number and diversity of the resPrP^{D} fragments that are "variably" resistant to PK digestion and can be fully demonstrated only with selected antibodies (Abs) to the PrP^{C} N- and C-terminal regions (Fig. 18.1) (Notari et al. 2018; Zhang et al. 2021; Zou et al. 2010). Another striking feature of this family of resPrP^{D} fragments is the lack of the resPrP^{D} isoforms monoglycosylated at residue N181 and diglycosylated, in spite of the presence of these glycoforms in the PrP^{C} isolates (Xiao et al. 2013). Nonetheless, the resPrP^{D} electrophoretic pattern of VPSPr remains complex (Fig. 18.1). Altogether, the resPrP^{D} population includes seven fragments comprising the mono- and unglycosylated isoforms; five of which have a molecular mass of ~26, ~23, ~20, ~17, and ~7, and are preferentially detected by the Abs to the PrP^{C} N-terminus that have also affinity for the resPrP^{D} type 2 fragment (Notari et al. 2018); two fragments of 12/13 and 7–8 kDa immunoreact with Abs to the PrP^{C} C-terminus (Fig. 18.1) (Notari et al. 2018). The 26 and 20 kDa fragments have been tentatively identified as the monoglycosylated and unglycosylated resPrP^{D} isoforms, respectively, both bearing the intact C-terminus including the glycophosphatidylinositol (GPI) anchor. Conversely, the resPrP^{D} 23 and 17 kDa fragments would derive from the 26 and 20 kDa listed above following cleavage of the GPI anchor (Notari et al. 2018). Finally, the 7 and 7–8 kDa are distinct internal fragments: the N-terminal 7 kDa might result from cleavages at residues ~S97–W99–S103 and ~ E152, while the cleavages for the distal C-terminal 7–8 internal fragment are currently undetermined (Notari et al. 2018). The 12/13 fragment matches the CTF (C-terminal fragment) of 12 and 13 kDa originally described in sCJD by Zou and co-workers (Notari et al. 2018; Pirisinu et al. 2013; Zou et al. 2003). Following deglycosylation, three N-terminus-truncated fragments of 20, 17, and 7 kDa were detectable by Abs to the N-terminus, while C-terminus Abs revealed the CTF-12/13 and 7–8 kDa fragments with unchanged electrophoretic mobility (Notari et al. 2018). The PK-resistance varied in part according to the 129 allotype and the band size. The four fragments

with 26–17 kDa molecular weight range showed the lowest PK-resistance in VPSPr-VV compared to the -MM and -MV allotypes (Gambetti et al. 2008; Zou et al. 2010). By contrast, the 7 kDa fragment was invariably resistant in all three allotypes (Zou et al. 2010). Finally, the presence of the three-band resPrP^{D} typically associated with sCJD subtypes has been occasionally mentioned in the original and subsequent reports (Gambetti et al. 2008; Peden et al. 2014; Rodríguez-Martínez et al. 2012).

18.4 Phenotypic Heterogeneity and Prion Strains

Recently, prion strains have been defined as "heritable disease phenotypes that are encoded by specific conformations of PrP^{D}" (Bartz 2021). The "quasi-species" concept dictates that strains are initially generated as a spectrum of conformers that undergo a Darwinian selection. Thus, the "quasi-species" concept provides some guidelines on the early stages of prion strain formation (Bartz 2021; Cracco et al. 2017; Gambetti and Notari 2013; Weissmann et al. 2011). Indeed, the co-existence of distinct PrP^{D} conformers as is posited by the "quasi-species" hypothesis fits well with the tendency of individual sporadic prion diseases to co-occur with various ratios of distinct PrP^{D} conformers each expressing a distinct phenotype (Table 18.1). The rare occurrence of certain types of sporadic prion diseases such as sCJDVV1, sFI, and VPSPr also fits with the strain selection hypothesis that may result in a hierarchy of strain frequency with some strain being rarely selected. Finally, the wide variety of the subtypes in sporadic prion diseases should not be so surprising given the complexity of the primary, secondary, and tertiary structures of the human PrP^{C}, which provides the substrate for the spontaneous conversion to PrP^{D} (Gill and Castle 2018). The PrP^{C} primary structure includes two polymorphisms, MV at codon 129 and EK at codon 219 resulting in nine possible primary structure variants. The N-terminal region is considered intrinsically disordered and is followed by a sequence of ~20 hydrophobic amino acids, while two β-strands and three α-helices populate the C-terminal region. Tertiary structures include a disulfide bond and two sites of non-obligatory N-glycosylation generating di-, mono-, and unglycosylated variants. Furthermore, a GPI anchor is attached to the C-terminus. This complexity at multiple translational levels fosters the generation of a highly heterogeneous population of pathogenic variants upon conformational conversion. This in turn results in the generation of multiple prion strains that are associated with distinct disease subtypes, even though they all derive from a single protein (Gill and Castle 2018). Nonetheless, the staggering diversity of phenotypes and of PrP^{D} structural characteristics exhibited by the sporadic prion diseases' subtypes requires caution and humility in accepting or rejecting the associated PrP^{D} variants as a distinct strain.

The PrP^{D} isolates associated with sCJDMM/MV1, MM2, VV1, and VV2 along with their resPrP^{D} type-mixed variants are currently accepted as established strains along with the PrP^{D} isolate associated with sFI, while VPSPr has been put on hold

(Baiardi et al. 2019). The recent data reported in this review partially support this view, but raise several relevant issues that may change the current perspectives on sporadic human prion strains. The new data on the MV2K, MV1, and VV1 and the VPSPr re-evaluation are emblematic.

sCJDMV2K is commonly believed to share the PrP^D strain 19 kDa with -VV2 based on the finding that conformational tests as well as transmission characteristics following inoculations to humanized mice reveal no difference between the two $resPrP^D$ isolates (Baiardi et al. 2019; Bishop et al. 2010; Zerr and Parchi 2018). However, as described above, the electrophoretic profile of $resPrP^D$ differs in the two subtypes, given that in VV2, it consists exclusively of type 2 (19 kDa), while MV2K also comprises a $resPrP^D$ type 1 variant of 20 kDa that originates from the 129 M allotype (Kobayashi et al. 2013; Nemani et al. 2020; Parchi et al. 2009). Despite this diversity, no significant difference in conformation was detected between the 20 and 19 kDa components of MV2K $resPrP^D$ either individually or combined, as well as between the $resPrP^D$ components of MV2K and the 19 kDa of the VV2 subtype (Nemani et al. 2020). On the other hand, several observations support the uniqueness of the MV2K subtype and the associated $resPrP^D$. Transmission of "pure" MV2K to humanized mouse lines expressing each of the 129 allotypes (MM, MV, and VV) has shown that only the MV allotype is competent to reproduce the 20 and 19 kDa $resPrP^D$ doublet, while the 20 and 19 kDa variants were reproduced separately in the 129MM and -VV background, respectively (Kobayashi et al. 2013). Moreover, MV2K and VV2 subtypes show relevant clinical and histopathological differences: disease duration is about three times longer in MV2K (17 vs. 6 months), and PrP^D predominantly forms amyloid plaques in the cerebellum with apparently no cytotoxic effects on adjacent tissue, while non-amyloid deposits associated with granule cell depopulation predominate in VV2 (Fig. 18.2) (Cali et al. 2020) (Cali I., unpublished data). Although detailed studies of this issue are needed, these observations suggest that PrP^D amyloid fibrils are formed in MV2K and not, or in lower quantities, in VV2, where the deposits are less or not fibrillar and, contrary to those of MV2K, appear to be cytotoxic (Sevillano et al. 2020). Although detailed studies of the quaternary structure associated with the PrP^D populating the two types of deposits are needed, these observations raise the possibility of major differences in the PrP^D aggregation pattern between MV2K and VV2. Since these differences, that could be regional, may not be detectable in the conformational studies conducted to date, it seems prudent to reserve judgement on the nature of these two strains.

The VV1 subtype has been for a long time associated with a $resPrP^D$ electrophoretic profile essentially indistinguishable from that of MM/MV1, which has been regarded as a separate strain in consideration of its conformation and experimental transmission characteristics (Baiardi et al. 2019). The recent finding proposing the existence two $resPrP^D$ electrophoretic profiles of 20 and 21 kDa, which may coexist or occur separately, along with the heterogeneity of their conformational and transmission characteristics raises new issues concerning the role of these three sets of $resPrP^D$ variants as prion strains (see also the previous section) (Cali et al. 2020, 2021). The issue is confusing, because despite this heterogeneity, the human disease

associated with each of these resPrP^{D} variants did not show distinguishing clinical or histotypic features co-distributing with each of the three variants (20, 21, and 20–21 kDa). The histotypes of the humanized mouse models were also similar. The conclusion suggested by these findings, that conformationally distinct prion strains might be individually incompetent to determine a distinct phenotype, needs further investigation. Also, the very early onset of 32 ± 5 years reported in the five cases of the "pure" VV1 subtype available is unique among sCJD subtypes and difficult to explain (Table 18.1) (Appleby et al. 2021; Cali et al. 2021). A possible mechanism can only be surmised based on the age-related etiologic model common to all sporadic conformational proteinopathies. According to this model, the proteostasis system, which ensures that abnormal proteins are degraded to prevent the accumulation of unwanted products, is impaired in aging resulting in the increases of misfolded proteins some of which are pathogenic and trigger prion and other conformational diseases (Hou et al. 2019; Morimoto 2019; Vecchi et al. 2020). Based on this scenario, it can be hypothesized that because of its particular conformation, the VV1 "prion strain" can elude even a "young" proteostasis system triggering the disease at an early age. Other mechanisms may include the identification of novel risk variants outside the PrP gene influencing PrP^{C} to PrP^{D} conversion early during life (Jones et al. 2020; Mead 2021).

Since the start of the sCJD molecular era, the MV1 variant has always been considered not only to be a phenocopy of MM1, but also expected to share with it PrP^{D} and strain characteristics. This conclusion was based on the virtually identical conformation and transmissibility metrics of the two conditions (Parchi et al. 1999). The finding that in MV1, both 129 M and -V allotypes equally contribute to the resPrP^{D} population must be adjusted to this expectation. The possibility that in MV1, both PrP^{C}-129 M and -V are equally templated to generate a copy of the MM1 strain would satisfy this requirement, and is supported by the virtually identical conformational metrics of the two 129 M and 129 V allotypes (Nemani et al. 2020). If proven, this scenario would also show that PrP^{C}-129 V can be converted to PrP^{D} type 1 of the MM1 variety. In this setting, it would also be interesting to determine the allelic origin—either from PrP^{C}129M or -129 V—of the MM2-like resPrP^{D} harbored in the MV1-2 subtype (Baiardi et al. 2019; Parchi and Saverioni 2012). The finding that in MV1-2, M1-like PrP^{D} is generated by the 129 V allele would show the remarkable capability of this allele to replicate strains until now thought to be generated exclusively in a 129MM background. It seems obvious that the strain issue should be deferred until the MV1 PrP^{D} characteristics in MV1, and possibly MV1-2, are clarified.

VPSPr is associated with a resPrP^{D} population that can be defined unique among sporadic prion diseases not only based on the electrophoretic profile (see the above section) but also according to several conformational parameters (Gambetti et al. 2008; Notari et al. 2018; Pirisinu et al. 2013; Saverioni et al. 2013). Of notice, it is the significantly lower resistance to PK-digestion than the sCJD subtypes. Similarly, the aggregate distribution profile of the total PrP (including PrP^{C} and PrP^{D}) following sucrose gradient sedimentation, was also significantly different pointing to a distinct quaternary structure of PrP^{D} in VPSPr compared to that of sCJD (Gambetti

et al. 2008; Saverioni et al. 2013). Conformational tests clearly show a difference also between the VPSPr-VV and the other two -MV and -MM allotypes consistent with a PrP^D conformational heterogeneity also at the subtype level (Pirisinu et al. 2013; Zou et al. 2010). Furthermore, VPSPr has been proven to be transmissible to humanized mice and bank voles (Diack et al. 2014a; Nonno et al. 2019; Notari et al. 2014). Transmission, however, was invariably difficult requiring on average 666 day post-inoculation (dpi) and yielding a 54% attack rate compared to a 183–609 dpi range (336 average) and 100% attack rate for the five major sCJD subtypes combined (Notari et al. 2014). Transmission appeared to be 129 allotype sensitive, since the -MV allotype failed to transmit to syngeneic humanized mice (Diack et al. 2014b). Mostly, due to the scarcity of $resPrP^D$, following VPSPr-VV inoculation, the accurate replication of the $resPrP^D$ profile could be demonstrated in only 34% of the humanized mice overexpressing PrP-129VV at eight times normal (Notari et al. 2014). The histopathology of the VPSPr challenged mice was restricted consisting of focal PrP plaque formation and SD in the hippocampal and sub-ventricular regions (Diack et al. 2014b; Notari et al. 2014). Second passage failed, possibly because of the very limited PrP^D deposition and the scarcity of the $resPrP^D$ recovered from the affected mice at first passage (Notari et al. 2014). Challenge to bank vole was similarly difficult and $resPrP^D$ replication was less accurate. However, all three allotypes were transmitted, and the second passage was successful with ~50% reduction of the transmission barrier encountered at the first passage. Moreover, the real-time quaking-induced conversion assay has shown a distinctive lower seeding activity of PrP^D associated with the three VPSPr allotypes than those of sCJD subtypes (Zhang et al. 2021). Finally, based mostly on the similarity of the $resPrP^D$ electrophoretic profile, VPSPr has been occasionally compared to classical GSS, where, in addition to an internal fragment of 7 or 8 kDa, there is a similar "ladder" of bands spanning a wide range of molecular weights. This similarity has led to the hypothesis that VPSPr might be the sporadic form of GSS (Rossi et al. 2019; Zou et al. 2010). However, the recent demonstration that in GSS, the gel "ladder" is populated by multimers of the 7 or 8 kDa fragment that has definitively ruled out this hypothesis (Cracco et al. 2019). Altogether, (i) distinct phenotype, (ii) unique $resPrP^D$ profile, and (iii) transmission and seeding characteristics strongly support the association of VPSPr with a distinct prion strain. Whether each VPSPr variant also is associated with a distinct strain requires further studies.

Recently, a new dynamic approach has been introduced which applies event-based technology to establish the time-sequential order by which DWI abnormalities are acquired with magnetic resonance imaging (MRI) of affected individuals (Pascuzzo et al. 2020). This study identified sites of first detection, or epicenters, and subsequent propagation pathways of the disease process in sCJD, showing that they are subtype-specific. In particular, the data clearly indicate that the epicenter is distinct and the propagation takes place essentially in opposite directions, in MM1 and VV2. In MM1, the epicenter was identified in the precuneus; the abnormal signal then propagated in the anterior–posterior direction within the cerebral cortex, and through subcortical formations terminally affecting thalamus and cerebellum. The most likely epicenter in VV2 was the cerebellum, while the propagation ended

in the posterior cortex. Remarkably, the subtypes affecting primarily the cerebral neocortex (MM1, MM2, VV1, and MV2C) seemed to share the epicenter in the precuneus. Moreover, MV2K and VV2, although similar, were not identical showing a likely later participation of the cerebellum in MV2K. Finally, sCJD MV1 appeared to differ from MM1 as for the epicenter (cingulate gyrus/insula vs. precuneus) and for a more convoluted propagation pathway, although terminally, many common anatomic regions were impacted (Pascuzzo et al. 2020).

Overall, these data confirm, and add fine granularity to, the current classification of sCJD subtypes. More importantly, they demonstrate the predictability of both epicenter and propagation trajectory in the individual subtypes. This approach is likely to impact early diagnosis and might facilitate better targeted brain therapeutics should they become available.

18.5 Concluding Remarks

1. This chapter updates the pathogenetic and molecular mechanisms that lay the foundation for understanding phenotypic heterogeneity and, hopefully, strains in sporadic human prion diseases.
2. Overall, the recent findings confirm the original ordering of sCJD-established subtypes, but they also afford a sharper mechanistic insight into the phenotypic and strain determination in 129MV heterozygous subtypes of sCJD, VV1, and VPSPr.
   - In MV2C and MV2K, mass spectrometric analyses revealed that both 129 M and -V PrP^C allotypes are converted to their respective $resPrP^D$ with reciprocal ratios of approximately 20:80 for the phenotypically "pure" forms. In the mixed MV2C-K (C: dominance of cerebral SD; K: presence of kuru plaque), the extent and locales of the two histotypes directly mirror the ratios of $resPrP^D$-129 M (C) and -129 V (K), respectively. This complexity as well as a longer disease duration and the presence of amyloid assemblies point to the presence in MV2K of a strain distinct from that of the VV2 subtype.
   - The MV1 variant, ever considered an MM1 phenocopy because of the phenotype and $resPrP^D$ characteristics, showed a 50:50 ratio of $resPrP^D$-129 M and -V, while the 129 V PrP^C allotype was previously ignored or thought to be incompetent to undergo templated conversion. A plausible scenario proposing the conversion of both -129 M and -129 V PrP^C allotypes to the same MM type 1 strain is discussed.
   - VV1, the rarest of the well-established sCJD subtypes, has been shown to be associated with a novel set of two $resPrP^D$ isolates that occur together or separately. Even though they show distinct conformational properties, they are associated with the same phenotype, an unprecedented condition in sCJD.
   - VPSPr has recently been further characterized, and its unique $resPrP^D$ population, transmissibility and seeding characteristics indicate that it is associated with a distinct prion strain.

Finally, the current molecular classification has also been strengthened and further defined by a recent MRI study aimed at identifying in the sCJD individual subtypes the first brain anatomic site of disease detection or epicenter and the subsequent propagation pathway of the lesions in vivo. It is hoped that this and other similar studies will foster early and accurate diagnoses when the disease process is still confined, maximizing successful treatment of prion and other conformational proteinopathies.

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Chapter 19
Genetics of Prion Disease

James A. Mastrianni

Abstract The Prion Diseases (PrDs) are rare transmissible neurodegenerative diseases that result from the accumulation of a misfolded isoform (PrP^{Sc}) of the normal cellular form of the prion protein (PrP^{C}), a naturally occurring surface membrane glycoprotein highly concentrated in neurons. Although the vast majority of PrDs are sporadic in nature, roughly 15 % are attributed to an autosomal dominant mutation of the prion protein gene (*PRNP*). More than 50 *PRNP* variants that result in single amino acid substitutions, variably lengthed insertions, deletions, or truncations at multiple positions within the prion protein (PrP), have been reported. Genotype-phenotype correlations are based primarily on the underlying histopathology that classify the disease as Creutzfeldt-Jakob disease (CJD), Gerstmann Sträussler Scheinker disease (GSS), or fatal familial insomnia (FFI). Some, but not all have demonstrated transmissibility to susceptible experimental hosts. Whereas some variants, such as the common polymorphic codon 129, affect risk to sporadic PrD, most are causal. Variable penetrance among some *PRNP* variants is notable, leading to define the variant as risk-associated or causal. This chapter reviews these issues that define the current state of understanding of the complex genetics of PrD, with special focus on the most common *PRNP* variants linked to genetic PrD.

Keywords Genetic CJD · GSS · FFI · *PRNP* variants

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19.1 Introduction

Prion diseases (PrDs) are rare neurodegenerative disorders that result from the accumulation of a misfolded conformer (PrP^{Sc}) of the cellular prion protein (PrP^{C}), a surface membrane protein highly expressed in neurons. Common features of PrD include progressive dementia, ataxia, myoclonus, weakness, and rigidity, in addition to visual and behavioral disturbances. The clinical diagnosis of these diseases can be difficult, but in recent years, it has been markedly enhanced by the application of diffusion-weighted (DWI) magnetic resonance imaging (MRI) that reveals hyperintense signal within the gray matter cortical ribbon and/or deep nuclei, especially those of the basal ganglia, and the highly specific real-time quaking-induced conversion assay (RT-QuIC) that detects low levels of PrP^{Sc} within cerebrospinal fluid (CSF). Pathological confirmation of disease is based on the presence of protease-resistance of PrP^{Sc} and the presence of one or more pathognomic histopathologic findings of either spongiform degeneration or extracellular amyloid plaque deposits composed of PrP. Based on a combination of clinicopathologic features and the western blot profile of protease-resistant PrP^{Sc} (PrP^{res}), several distinct subtypes of PrD have been recognized, including Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal insomnia (FI), variant CJD (vCJD), iatrogenic CJD (iCJD), and variably protease-sensitive prionopathy (VPSPr). These differing phenotypes are remarkably linked to the conformational variations acquired by PrP^{Sc} (Telling et al. 1996; Rossi et al. 2019). Although the vast majority of PrDs are sporadic, roughly 10–15% are attributed to a sequence alteration in the PrP gene (*PRNP*). Sequence variations of *PRNP* that induce PrD are predicted to generate instability in PrP, leading to its misfolding with some degree of conformational selectivity.

19.2 Prion Protein (PrP)

In humans, the PrP gene (*PRNP*) is positioned on the short arm of chromosome 20 (Liao et al. 1986; Sparkes et al. 1986). The entire open-reading frame is contained within the second of two exons and it does not undergo differential splicing. During translation and translocation into the ER, a 22 amino acid signal peptide is cleaved from PrP as is a 23-residue signal sequence from the carboxy terminus prior to addition of a glycoinositol phospholipid (GPI) anchor through which PrP attaches to the outer leaflet of the plasma membrane (Stahl et al. 1987). The amino-terminal of mature PrP is an unstructured flexible segment extending from residues 23 to 124 and contains a highly conserved octarepeat segment between residues 51 and 91. This includes a series of five repeating elements of Pro-(His/Gln)-Gly-Gly-Gly-(-/Gly)-Trp-Gly-Gln. The first of five segments includes nine amino acid residues, while the four subsequent repeats are eight residues each. The C-terminal segment (aa 125–228) is a globular structure with significant secondary structure that

includes three a-helical segments (aa 144–154, 173–194, and 200–228), and two short β-strands (aa 128–131 and 161–164). Two asparagine-linked glycosylation sites at positions 181 and 197 lie within a loop region of the protein created by a single disulfide bond (Fig. 19.1). A hydrophobic domain that appears to play an important role in PrD promotion or protection extends from residues 112 to 133. PrP is regulated during development and is constitutively expressed in the adult.

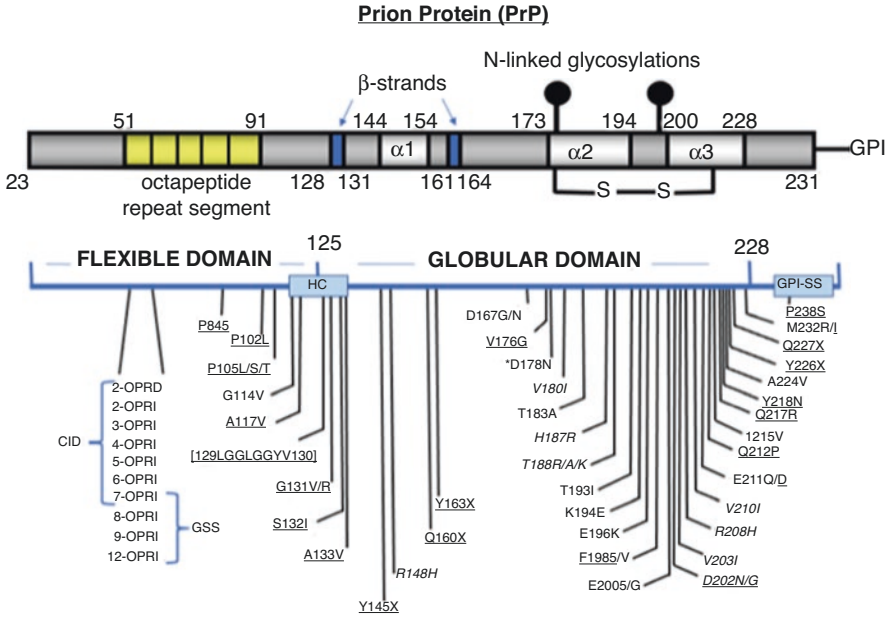

Fig. 19.1 Prion Protein (PrP^C) schematic and associated variants. Mature PrP^C after post-translational processing. The first 22 amino acids that represent the ER entry signal sequence are removed and the GPI anchor is attached at residue 231. N-linked glycosylation sites at positions 181 and 197 and a loop created by a disulfide bond between cysteines at positions 179 and 214. Positions of the three α-helical segments are indicated, as are the two β-strands. The flexible and globular domain boundaries are represented, as is the hydrophobic core (HC) (aa. 112–133) and the GPI anchor signal sequence (aa. 231–254), for reference. Disease-associated variants are displayed at their relative positions within PrP. The letter preceding the residue number is the normal amino acid at that position and the letter following the residue number is the disease-associated substitution. Variants include missense mutations, a 2-octapeptide repeat deletion(OPRD), 2–9 octapeptide repeat insertions (OPRI), a unique duplication insertion at codon 129 (in brackets), and nonsense mutations that introduce a stop codon, designated by X. Underlined variants are those characterized as GSS, based primarily on histopathology, if available, and CJD-associated variants are in bold. OPRIs are labeled as GSS vs CJD, based on variability in pathology, especially 7-OPRI. Missense mutations with low or intermediate penetrance are italicized. The remainder of the variants have either not been well characterized because of too few examples, or do not have histopathology, so are not designated as CJD or GSS. The key polymorphisms (G127V, M129V, E219K, and 1-OPRD) are not included but are discussed in the text. *D178N promotes CJD when 129V is on the same allele (i.e., D178N-129V) and FFI when D178N is paired with 129M. *A* alanine, *D* aspartate, *E* glutamate, *F* phenylalanine, *G* glycine, *H* histidine, *I* isoleucine, *K* lysine, *L* leucine, *M* methionine, *N* asparagine, *P* proline, *Q* glutamine, *R* arginine, *S* serine, *T* threonine, *V* valine, *Y* tyrosine

The levels of mRNA do not appear to change during disease development in animals (Oesch et al. 1985; Jendroska et al. 1991). The highest levels of expression are within neurons (Kretzschmar et al. 1986), but it is also expressed in other cells within the central nervous system (CNS), including astrocytes (Lima et al. 2007), oligodendrocytes (Moser et al. 1995), and microglia (Adle-Biassette et al. 2006), in addition to peripheral tissues, including lung, heart, kidney, pancreas, testis, white blood cells (Bendheim et al. 1992), and platelets (Perini et al. 1996), albeit at significantly lower levels than in CNS. Several functions of PrP have been described, including synapse formation (Collinge et al. 1994), signaling (Spielhaupter and Schatzl 2001; Solforosi et al. 2004), neuroprotection (Roucou and LeBlanc 2005), copper binding and delivery (Brown et al. 1997; Stöckel et al. 1998), and as a receptor for Aβ and other neurodegenerative proteins (Lauren et al. 2009; Corbett et al. 2020) (Table 19.1).

19.3 Molecular Genetics of Prion Disease

The worldwide incidence of PrD is roughly 1–2 cases per 10^6 population per year for sporadic PrD and 1–2 cases per 10^8 per year for genetic PrD. A gender bias does not appear to be present (Brown et al. 1987). The median age for sporadic PrD is ~67 years, although the range is broad, from teenagers to nonagenarians, depending on the subtype of disease. The clinical course is more often rapidly progressive, with death occurring in under a year, whereas genetic PrD generally manifests at a younger age and has a more protracted course overall, although some genetic forms present with striking similarity to sCJD, such as the E200K and V210I variants. Disease-causing *PRNP* variants display autosomal dominant inheritance and expression, although the estimate of ~10% occurrence of all cases may be spuriously low because of the variable and often late age of disease onset in some forms of genetic PrD (Spudich et al. 1995). This is also affected by the variability in penetrance among *PRNP* variants. Somatic mutations of *PRNP* have been hypothesized as a potential cause of PrD, although evidence for this is generally lacking. However, in a study using the Cancer Genome Atlas (TCGA) database of 10,967 cancer patients, 48 somatic mutations of *PRNP* were identified, 8 of which were previously determined to be pathogenic, supporting the potential for the development of somatic mutations (Kim et al. 2020). A unique patient diagnosed with CJD was found to have somatic mosaicism, carrying the normal sequence in addition to a D178N/129M and D178N/129V variant, while both parents had a normal *PRNP* gene (Alzualde et al. 2010a). Whereas environmental factors are associated with a number of acquired forms of CJD, including iCJD that results from exposure to prion-contaminated biologicals such as growth hormone and dura mater grafts (Brown et al. 2012), vCJD caused by ingestion of food contaminated with bovine spongiform encephalopathy (BSE) (Diack et al. 2014), and kuru that resulted from human prion ingestion during cannibalistic rituals among the Fore people of New Guinea (Gajdusek and Zigas 1957), there has been no environmental factor linked

Table 19.1 Genetic PrD compared with sporadic Creutzfeldt–Jakob disease

	Sporadic Creutzfeldt–Jakob disease (sCJD)	Creutzfeldt–Jakob disease (CJD)	Gerstmann–Sträussler–Scheinker disease (GSS)	Fatal Familial Insomnia (FFI)
Typical presentation	Progressive cognitive decline, ataxia, and myoclonus.	Progressive cognitive decline, ataxia, and myoclonus.	Progressive ataxia of gait, pyramidal and extrapyramidal features, dysarthria, and late dementia	Progressive sleep disturbance, dysautonomia, later ataxia and cognitive decline.
Additional features	Visual disturbances, seizures, hallucinations, behavioral changes (depression, aggression), hemi-neglect, alien limb, delusions, weakness, tremors, rigidity, insomnia, chorea, etc.	As in sCJD.	Lower extremity areflexia (P102L), dementia onset (A117V, G131V, H187R), spastic paraparesis (P105L), aphasia and frontal lobe syndrome (P105S)	Visual disturbances, spatial disorientation, hallucinations, dysarthria, bulbar symptoms, weight loss.
Age at onset (range)	68 years (17–91)	Avg. 55–60 years (20–90)	Median 45 years (24–71)	50–60 years (18–74)
Disease course (range)	4–6 months (up to 15 months rarely)	Avg. 15 months (12–60)	Median 8 years (1–11, rarely to 20)	Mean 16 months (8–72)
Histopathological features	Spongiform degeneration within cortical and deep grey nuclei	As in sCJD	Extracellular PrP amyloid plaques, usually in the presence of minimal spongiform degeneration, although mixtures of both occur in many cases.	Neuronal loss with gliosis within the anterior nucleus (AN) and dorsomedial nucleus (DM), and olivary nucleus

(continued)

Table 19.1 (continued)

	Sporadic Creutzfeldt–Jakob disease (sCJD)	Creutzfeldt–Jakob disease (CJD)	Gerstmann–Sträussler–Scheinker disease (GSS)	Fatal Familial Insomnia (FFI)
EEG (PSWCs)	Positive in majority (~67%)	As in sCJD (~67%), especially with cases with rapid progression	Typically negative, slow wave discharges are common	Typically negative, but may display slow wave discharges
MRI (DWI hyperintensities)	Positive in more than 90% of all sCJD, but in some rare subtypes it may be negative.	Positive in ~90% of E200K and V180I, M232R, but in less than 50% for other variants.	Typically negative, except rare cases of P102L and P105L	Negative with rare exceptions
CSF testing (14-3-3, t-tau, RT-QuIC)	All positive in vast majority	All show reduced positivity than sCJD, dependent on variant. Faster progression variants (E200K, V210I) more likely positive.[a]	14-3-3 and tau are mostly negative, with rare exceptions (e.g., P102L). RT-QuIC variable, but 90% positive in one series of P102L cases.[b]	Negative 14-3-3, but positive RT-QuIC in 83% in one series[c]
Most common variants	129M/V	E200K[c], V210I, V180I, D178N-129V, M232R. Less common – P105T, R208H, E211Q, T188R.	P102L[c], P105L, A117V	D178N-129M

[a] Sano et al. (2013)
[b] Franceschini et al. (2017)
[c] Most common variant within each group

to the onset of genetic PrD. Because of the aggressive nature of some variants, families will often ascribe a preceding physical or emotional event as the possible trigger for disease. It is curious, however, that there is wide variation in disease onset reported for most genetic forms of PrD, even in monozygotic twins with a *PRNP* variant (Webb et al. 2009; Hamasaki et al. 1998), suggesting a potential environmental influence on genetic PrD. In addition to external factors, within the *PRNP* gene are well-characterized polymorphisms, especially at codon 129, which can impart a profound effect on disease susceptibility and/or phenotype of sporadic, acquired, and genetic PrD, which will be discussed below.

In 1989, Hsiao et al. (1989a) identified a C-to-T transition within codon 102 of the *PRNP* gene, resulting in a leucine substitution of proline at residue 102 (P102L) in affected members of two unrelated families with GSS. In that same year,

Goldgaber et al. (1989) identified the same mutation in three affected members of a family with GSS. These early findings established the *PRNP* gene as central to inherited PrD. Since then, well-over 50 variants of *PRNP* have been identified (Table 19.2). The most common pathogenic variants worldwide are the E200K, P102L, D178N, V210I, and V180I (Minikel et al. 2016).

19.4 Penetrance

Not all *PRNP* variants display complete penetrance. Roughly 47% of individuals identified with a *PRNP* variant in a large European survey did not report a family history of PrD (Goldman et al. 2004; Kovacs et al. 2005). However, a more detailed review of the family history in these cases found occasional misdiagnosed dementias or other neuropsychiatric illness (Goldman et al. 2004; Kovacs et al. 2005). With the discovery of the *PRNP* gene, early efforts to link variant *PRNP* sequences to families with possible genetic PrD benefitted from the study of large pedigrees from which carriers and non-carriers could confirm the co-segregation of the variant with disease. These early studies led to linkage of the highly penetrant P102L, D178N, E200K, and 6-OPRI variants to heritable PrD. With expanded access to genetic testing, many *PRNP* variants have been discovered in subjects with less clear evidence for full penetrance. Problems that contribute to this include an unrecorded or unknown family history, the early unrelated death of family members, and the variability in age at onset that sometimes suggests an age-dependent increase in penetrance (Spudich et al. 1995) and may lead to a missed occurrence due to death of the carrier in early life. For some variants, there are clear questions of causality. In a recent study that took advantage of a large scale publicly derived genetic database and ~16,000 cases of genetic PrD collected from several prion research centers, Minikel et al. (2016) predicted "lifetime risk" estimates of penetrance for several *PRNP* variants. Although that survey confirmed the nearly 100% penetrance of the most common variants of P102L, A117V, D178N, and E200K, some previously reported variants were found at higher prevalence in the general population than expected, suggesting reduced penetrance and raising questions as to their pathogenicity. In particular, the M232R, V180I, and V210I variants were estimated to carry lifetime risks for PrD of 0.02%, 1.0%, and 10%, respectively. Octapeptide repeat insertions were not studied, based on the inability of the sequencing method used in the publicly derived database to detect insert segments. Thus, whereas many PrDs have clear evidence for their heritable nature, many do not. As such, the general term "genetic PrD" has replaced "familial PrD", although even that terminology may not be entirely appropriate for a number of *PRNP* variants that are limited by a single or low occurrence and which lack sufficient evidence for causality. In such cases, the variant in question might simply be a non-pathogenic sequence change coincidently identified in a patient with sporadic PrD.

Table 19.2 Missense mutations of *PRNP*

Codon	Sequence change	Amino Acid change[a]	Codon 129	Pathologic phenotype	References
17	GGG -> GAG	Ser (S) -> Gly (G)	Met		Zhang et al. (2016)
84		Pro (P) -> Ser (S)	ND	GSS	Jones et al. (2014)
97	AGT -> AAT	Ser (S) -> Asn (N)	Met	N/A	Zheng et al. (2008)
102	CCG -> CTG	Pro (P) -> Leu (L)	Met	GSS	Hsiao et al. (1989a)
102	CTG	Leu (L)	Val	GSS	Young et al. (1997)
105	CCA -> CTA	Pro (P) -> Leu (L)	Val	GSS	Kitamoto et al. (1993b) and Mano et al. (2016)
105	ACA	Thr (T)	Val or Met	GSS	Kim et al. (2018) and Rogaeva et al. (2006)
105	TCA	Ser (S)	Val	Atypical GSS	Tunnell et al. (2008)
114	GGT -> GTT	Gly (G) -> Val (V)	Met	CJD	Rodriguez et al. (2005)
117	GCA -> GTG	Ala (A) -> Val (V)	Val	GSS	Doh-ura et al. (1989), Hsiao et al. (1991c) and Mastrianni et al. (1995)
127	GCG -> AGC	Gly (G) -> Ser (S)		Protective	Ch'ng et al. (2015)
129	ATG or GTG	Met (M) or Val (V)		[c]	Owen et al. (1990) and Hsiao et al. (1989b)
131	GGA -> GTA	Gly (G) -> Val (V)	Met	Atypical GSS	Panegyres et al. (2001)
131	GGA -> AGA	Gly (G) -> Arg (R)	Val	N/A	Alshaikh et al. (2020)
132	AGT -> ATT	Ser (S) -> Ile (I)	Met	GSS	Hilton et al. (2009)
133	GCA -> GTG	Ala (A) -> Val (V)	Met	Atypical GSS	Rowe et al. (2007)
136	AGG -> AGT	Arg (R) -> Ser (S)	Met	GSS	Ximelis et al. (2021)
145	TAT -> TAG	Tyr (Y) -> Stop (-)	Met	GSS w/NFTs (PrP-CAA)	Kitamoto et al. (1993c)
148	CGT -> CAT	Arg (R) -> His (H)	Met	CJD	Pastore et al. (2005)
160		Gln (Q) -> Stop (-)	Met	GSS	Finckh et al. (2000)
160		Gln (Q) -> Stop (-)	Met	PrP with NFTs	Jayadev et al. (2011)
163		Tyr (Y) -> Stop (-)		GSS	Themistocleous et al. (2014)

(continued)

Table 19.2 (continued)

Codon	Sequence change	Amino Acid change[a]	Codon 129	Pathologic phenotype	References
167	GAT -> GGT	Asp (D) -> Gly (G)	Met	CJD	Bishop et al. (2009)
167	GAT -> AGT	Asp (D) -> Asn (N)	Met	CJD	Beck et al. (2010)
169	TAC -> TAA	Tyr (Y) -> Stop (-)	Met		Capellari et al. (2018)
171	AAC -> AGC	Asn (N) -> Ser (S)	Val	[c]	Fink et al. (1994)
176		Val (V) -> Gly (G)	Val	GSS	Kim et al. (2018)
178	GAC -> AAC	Asp (D) -> Asn (N)	Val	CJD	Goldfarb et al. (1991b)
178	AAC	Asn (N)	Met	FFI	Medori et al. (1992a, b)
180	GTC -> ATC	Val (V) -> Ile (I)	Met	CJD	Hitoshi et al. (1993)
183	ACA -> ACG	Thr (T) -> Ala (A)	Met	CJD	Nitrini et al. (1997)
187	CAC -> CGC	His (H) -> Arg (R)	Val	Atypical[b]	Hall et al. (2005) and Bütefisch et al. (2000)
188		Thr (T) -> Lys (K)	?	CJD	Finckh et al. (2000)
188	ACG -> AAG	Thr (T) -> Lys (K) homozygous	Met	CJD	Shan et al. (2022)
188	ACG -> AGG	Thr (T) -> Arg (R)	Val	CJD	Tartaglia et al. (2010)
188		Thr (T) -> Ala (A)	Met	CJD	Kim et al. (2018)
189	GTC -> ATC	(Val) V -> Ile (I)	Met	CJD	Di Fede et al. (2019)
193		Thr (T) -> Ile (I)	Met		Kim et al. (2018)
196	GAG -> AAG	Glu (E) -> Lys (K)	Met	CJD	Peoc'h et al. (2000)
196	GAG -> GCG	Glu (E) > Ala (A)	Met		Dai et al. (2019)
198	TTC -> TCC	Phe (F) -> Ser (S)	Val	GSS w/NFTs	Hsiao et al. (1990b)
198	TTC -> GTC	Phe (F) -> Val (V)	Met		Zheng et al. (2008)
200	GAG -> AAG	Glu (E) -> Lys (K)	Met/ Val	CJD	Hainfellner et al. (1999) and Goldfarb et al. (1990b)
200		Glu (E) -> Asp (D) homozygous	Met		Hassan et al. (2021)

(continued)

Table 19.2 (continued)

Codon	Sequence change	Amino Acid change[a]	Codon 129	Pathologic phenotype	References
200		Glu (E) -> Gly (G)	Val	CJD	Kim et al. (2018)
202	GAC -> AAC	Asp (D) -> Asn (N)	Val	GSS	Piccardo et al. (1998)
202		Asp (D) -> Gly (G)	Val		Kim et al. (2018)
203	GTT -> ATT	Val (V) -> Ile (I)	Met or Val	CJD	Peoc'h et al. (2000) and Jeong et al. (2010)
208	CGC -> CAC	Arg (R) -> His (H)	Met	CJD	Mastrianni et al. (1996)
208	CGC -> TGC	Arg (R) -> Cys (C)	Met		Zheng et al. (2008)
210	GTT -> ATT	Val (V) -> Ile (I)	Met	CJD	Pocchiari et al. (1993), Ripoll et al. (1993) and Mastrianni et al. (2001)
211	GAG -> CAG	Glu (E) -> Gln (Q)	Met	CJD	Peoc'h et al. (2000)
211	GAG -> GAC	Glu (E) -> Asp (D)	Val	GSS	Kim et al. (2018)
212	CAG -> CCG	Gln (Q) -> Pro (P)	Val	GSS	Piccardo et al. (1998)
212		Gln (Q) -> Pro (P) homozygous	Met	GSS	Beck et al. (2010)
215		Ile (I) -> Val (V)	Met	CJD	Kim et al. (2018)
217	CAG -> CGG	Gln (Q) -> Arg (R)	Val	GSS w/NFTs	Hsiao et al. (1992)
218	TAC -> AAC	Tyr (Y) -> Asn (N)	Val	GSS w/NFTs	Alzualde et al. (2010b)
219	GAG -> AAG	Glu (E) -> Lys (K)	Met	[c]	Furukawa et al. (1995)
224		Ala (A) -> Val (V)	Val	CJD	Kim et al. (2018)
225	TAT -> TGT	Tyr (Y) -> Cys (C)	Met	Atypical CJD	Bagyinszky et al. (2019)
226	TAC -> TAA	Tyr (Y) -> Stop (-)	Val	PrP-CAA	Jansen et al. (2010)
227	CAG -> TAG	Gln (Q) -> Stop (-)	Val	GSS	Jansen et al. (2010)
232	ATG -> AGG	Met (M) -> Arg (R)	Met	CJD	Hitoshi et al. (1993) and Hoque et al. (1996)
232		Met (M) -> Thr (T)		PrP and Aβ	Bratosiewicz et al. (2000)
238	CCA -> TCA	Pro (P) -> Ser (S)	Met	CJD	Windl et al. (1999)

(continued)

Table 19.2 (continued)

Codon	Sequence change	Amino Acid change[a]	Codon 129	Pathologic phenotype	References
Non-pathogenic variants (also present in unaffected individuals)					
39		Pro (P) > Lys (L)	Met		Oldoni et al. (2016)
54		Gly (G) -> Ser (S)	Met	PrP	Beck et al. (2010) and Forbes et al. (2014)
142 (w/ N171S)		Gly (G) -> Ser (S)		sCJD	Beck et al. (2010)
200		Glu (E) -> Asp (D)	Met		Hassan et al. (2021)
209		Val (V) -> Met (M)			Beck et al. (2010)

NFTs = neurofibrillary tangles, *CAA* = cerebral amyloid angiopathy, *ND* = not determined (patient was 129MV, but allele not known)
[a]Letters in parentheses indicate letter codes for amino acids
[b]Either early psychiatric symptoms or dementia preceding ataxia w/curly PrP deposits
[c]Polymorphisms – may modify disease phenotype

19.5 Other Genes Involved in PrD

PRNP is the only gene directly linked to PrD, and in the absence of *PRNP*, PrD cannot develop. This was initially confirmed in mice with the mouse PrP gene (*Prnd*) ablated (Bueler et al. 1993). The search for additional genes that might influence risk or phenotype of PrD in humans has focused primarily on genome-wide association studies. Such studies are inherently limited by the low incidence of PrD. One study found two potential SNPs (Rs7565981, Rs4921542) that were weakly associated with the occurrence of vCJD, but not associated with sCJD (Lloyd et al. 2013). These SNPs represent an intergenic region upstream of the neuronal PAS (per-ARNT-sim) domain-containing protein 2 gene (*NPAS2*), a transcription regulatory gene, in addition to an intronic variant in the myotubularin-related protein 7 gene (*MTMR7*) that is involved in the dephosphorylation of phosphatidylinositol 3-phosphate and inositol 1,3-biphosphate. A more recent study of over 5000 subjects with probable or definite CJD of European ancestry, 41 SNPs, at three loci was found to be associated with CJD risk, including *PRNP*, *STX6,* which encodes Syntaxin-6 that may play a role in membrane targeting of PrP at an early stage of prion formation, and *GAL3ST1*, which encodes galactose-3-O-sulfotransferase 1 that plays a role in myelination, which localizes to the Golgi network (Jones et al. 2020). Confirmation that these genes impart a significant risk to sCJD will need further study in appropriate cellular and animal models.

In recent years, it has been recognized that PrP may play a role in other neurodegenerative disease, especially by acting as a surface membrane receptor and toxic effector for oligomeric amyloid beta (Aβ) (Lauren et al. 2009; Kessels et al. 2010) and possibly other neurodegenerative disease-related proteins, including α-synuclein

and tau (Corbett et al. 2020). Whether other neurodegenerative-related proteins participate in PrD has also been explored. For instance, mice with an ablated amyloid precursor protein (APP) or interleukin-1 gene, or those that over-express superoxide dismutase 1 (SOD-1), displayed a delay in onset of PrD of up to 19% following PrP^{Sc} inoculation (Tamguney et al. 2008). Additionally, prion inoculation of Tg mice that model Alzheimer's disease (AD) developed PrD earlier than non-Tg mice (Morales et al. 2010) and a significantly earlier onset of ataxia and more intense PrD-related histopathology were observed in a double Tg mouse that expressed genes associated with GSS and AD, compared with the parental TgGSS mice (Qin et al. 2019). Not all studies suggest an influence of other neurodegenerative-related proteins. For instance, inoculation of mouse PrP^{Sc} to mice lacking tau or α-synuclein did not show differences in incubation periods to control mice (Lawson et al. 2011; Asuni et al. 2010). This is an important field of study, as other genetic or epigenetic influences are thought to explain the clinical and histopathologic variability associated with genetic forms of PrD.

19.6 Genetic Prion Disease Subtypes

19.6.1 Creutzfeldt–Jakob Disease (CJD)

The onset of cognitive dysfunction with a rapidly evolving dementia, followed by ataxia of gait and limbs, myoclonus, and an eventual akinetic mute state, is the typical clinical picture observed for CJD. A variety of additional neurologic symptoms, most commonly including extrapyramidal and pyramidal features, but also visual distortions or hallucinations, weakness, rigidity, bradykinesia, tremor, chorea, alien limb syndrome, psychiatric symptoms of mood and behavioral changes, sleep dysregulation, other vegetative symptoms, and sensory disturbances (Brown and Mastrianni 2010). As many as 25% of sCJD patients display ataxia at onset (Gomori et al. 1973), with a much smaller percentage beginning with cortical blindness, a phenotype which has been named the Heidenhain variant. Comparison of gCJD and sCJD suggests a significant overlap in the clinical phenotype. Typical onset for sCJD is 68 with a mean duration of 4–6 months, with only a small percentage surviving slightly beyond a year. Genetic forms of CJD generally present earlier than sCJD. In one European study, the mean age of onset was 60 years, albeit with significant variability ranging from 20 to 90 years of age (Kovacs et al. 2005). Despite an earlier mean age of onset, observational data suggest that disease duration is similar between gCJD and sCJD (Kovacs et al. 2005; Brown et al. 1986; Cali et al. 2006; Kim et al. 2018), although this too is highly variable in that 75% of patients exhibit a 20 month disease course, whereas a disease course of 8 years or more have been described (Kovacs et al. 2002). Nearly, 98% of gCJD cases report progressive dementia, 70% display cerebellar ataxia, 60–70% exhibit myoclonus, and roughly 50% display extrapyramidal features (Kovacs et al. 2002; Takada et al. 2018; Meiner

et al. 1997). It should be noted that these estimates may be biased due to the predominance of the E200K variant, the most common genetic variant associated with CJD and PrD.

Helpful diagnostic studies include EEG, MRI, and CSF testing. EEG typically shows bilateral periodic sharp wave complexes (PSWCs) at a frequency of 0.5–2 s; when observed in the presence of rapidly progressive dementia, myoclonus, and ataxia, diagnostic certainty of CJD is ~90% (Brown et al. 1984, 1986; Chiafalo et al. 1980). However, the EEG is negative in a third of sCJD patients and the yield is generally lower for gCJD (Figgie Jr. and Appleby 2021). The MRI, particularly DWI sequences and less so, fluid-attenuated inversion recovery (FLAIR) sequences, shows hyperintense signal within the cortical ribbon or deep grey nuclei in ~95% of sCJD cases (Vitali et al. 2011) (Fig. 19.2). In general, the yield for a positive MRI is dependent on the *PRNP* variant in that some, such as the E200K and V210I variant, show high rates of typical MRI findings for CJD, although some do not. Deep gray nuclei involvement may be more common than cortical ribboning according to some studies (Breithaupt et al. 2013; Takada et al. 2017). CSF testing for tau, 14-3-3, and RT-QuIC are now considered routine in the diagnostic work up due to their collective sensitivities and specificities for the diagnosis of PrD, particularly CJD. The presence of 14-3-3 within the CSF has shown a high sensitivity of 90%, but specificity varies from 40% to 100%, depending on the study (Sanchez-Juan et al. 2006; Geschwind et al. 2003), whereas elevated tau levels beyond 1150 pg/mL have a slightly lower sensitivity but more consistently higher specificity of between 87% and 95% (Figgie Jr. and Appleby 2021; Sanchez-Juan et al. 2006). The highest specificity CSF test is the RT-QuIC which approaches 100% and a sensitivity between

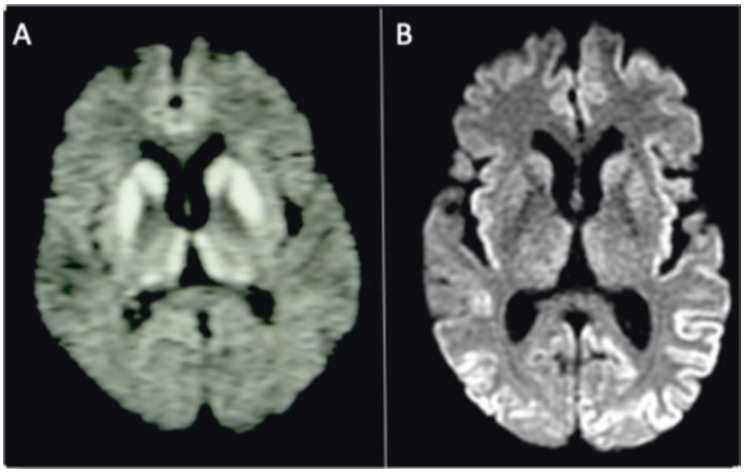

Fig. 19.2 Diffusion-weighted imaging (DWI) MRI sequences. Typical deep nuclei basal ganglia (caudate and putamen) hyperintensities (**A**) and cortical ribbon hyperintensities (**B**) seen in patients with CJD. Such DWI positive findings are very common in sCJD but less common in gCJD, with the exception of the E200K, V210I, and M232R variants. DWI signal changes are typically absent in GSS and FFI

90% and 97% (Atarashi et al. 2011; McGuire et al. 2016). These numbers reflect the findings within the best-studied group of sCJD, but in some cases of gCJD, the sensitivity of CSF total tau may be higher than that of 14-3-3 (Kovacs et al. 2005, 2011; Breithaupt et al. 2013; Sano et al. 2013; Krasnianski et al. 2016) and RT-QuIC sensitivity may be reduced, depending on the particular *PRNP* variant. In one study of a series of Japanese patients with the E200K variant, the phenotype of which most closely approximates that of sCJD, the sensitivity of RT-QuIC was 83% (Sano et al. 2013; Higuma et al. 2013), somewhat lower than that in sCJD. Since, in many cases of gPrD, diagnostic studies may be less likely to show positive results, the detection of a PRNP variant may help to confirm a diagnosis.

Pathological analysis of CJD reveals vacuolization, more commonly termed spongiform degeneration, that involves grey matter distributed within the neocortex, subiculum of hippocampus, basal ganglia (caudate and putamen), thalamus, and molecular layer of the cerebellar cortex, typically accompanied by reactive gliosis (DeArmond and Prusiner 1997) (Fig. 19.3). The vacuoles are evident on light microscopy and are macroscopic representations of focal swellings of axonal and dendritic processes of neurons. Additional findings on electron microscopy include loss of synaptic organelles and accumulation of abnormal membranes (Beck et al. 1982; Chou et al. 1980; Lampert et al. 1972). The relative intensity, distribution, and size of the vacuoles may differ among the different phenotypes of sCJD and within and among the different variants associated with genetic CJD. The most commonly reported missense variants associated with CJD, in order of incidence include E200K, V210I, V180I, D178N-129V, and M232R, whereas R208H, P105T, T188R, and E211Q are much less common with roughly a dozen cases reported for each, and the remaining known variants have been reported with even less incidence, sometimes one or two cases (Takada et al. 2017).

19.6.2 Gerstmann–Sträussler–Scheinker Disease (GSS)

First described by Gerstmann (Gerstmann et al. 1936; Gerstmann 1928), this disease classically presents with progressive gait ataxia and/or speech dysarthria, followed by variable pyramidal and extrapyramidal features, and delayed development of dementia. Age at onset is generally earlier than that of CJD, with a median age of 45 years, and a duration between 2 and 11 years, although there are some reports of considerably longer duration. Several phenotypes associated with GSS pathology have been described, including the onset of a movement disorder, prominent behavioral features mimicking a frontal lobe syndrome, a progressive cognitive disorder, spastic paraparesis, neuropathy, and autonomic dysfunction with severe diarrhea as a feature, among others. This variation in phenotype combined with the generally slower rate of progression compared with CJD can often result in a delay in diagnosis or misdiagnosis for another neurological disease, such as AD, Parkinson's disease, amyotrophic lateral sclerosis, etc. (Kong et al. 2004). The EEG typically does not show PSWCs, although slow wave activity may be present. The MRI is also less

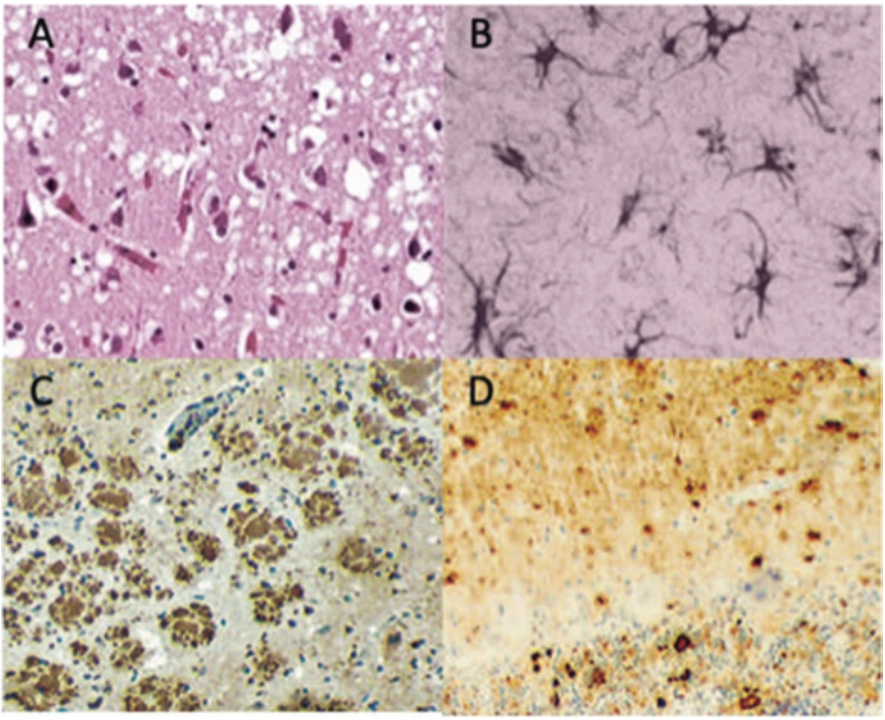

Fig. 19.3 Major histopathologic features of gPrD. (**A**) Light microscopy of a case of gCJD due to E200K demonstrates typical spongiform degeneration of the neuropil. The vacuoles of spongiform degeneration represent axonal and dendritic swelling associated with the loss of synaptic organelles and the accumulation of abnormal membranes. Their size varies from 5 to 25 μm, and may be most evident in cerebral cortex, deep cortical nuclei, and the molecular layer of the cerebellar cortex. (**B**) Reactive gliosis following GFAP staining is consistently associated with spongiform degeneration. In FFI, gliosis and neuronal dropout within the anterior and dorsomedial nucleus of the thalamus may be the only histopathologic features present, although longer duration cases may also display limited spongiform degeneration. (**C**) GSS multicentric amyloid plaques are the pathognomic feature of GSS. Multicentric plaques typically display a central core of amyloid that is surrounded by multiple smaller amyloid satellites of varying size. This section is from the hippocampus of a 31-year-old woman who carried the P105S variant of GSS and who displayed a presentation suggestive of frontotemporal dementia (Tunnell et al. 2008). (**D**) Unicentric PrP plaques, as in this section of cerebellum, are also common in GSS

helpful than it is for CJD, as DWI abnormalities are typically not present (Vitali et al. 2011). CSF biomarker proteins, such as 14-3-3, total tau, and even RT-QuIC, are more often negative (Kovacs et al. 2005; Jones et al. 2014; Rhoads et al. 2020), although a positive RT-QuIC has been reported in some cases of the P102L variant (Sano et al. 2013; Franceschini et al. 2017).

The characteristic histopathology of GSS is the presence of extracellular PrP amyloid plaque deposits with gliosis and less prominent spongiform degeneration than in CJD. However, as will be discussed, some GSS-associated variants may

display rather prominent spongiform degeneration. Plaques are most often described as multicentric, typified as a collection of multiple plaques of varying sizes, sometimes with a dense central core surrounded by smaller satellites; however, unicentric plaques may also be prominent in some forms of GSS (DeArmond and Prusiner 1997) (Fig. 19.3). A variety of *PRNP* variants have been described in association with a clinical or pathological phenotype of GSS, the most common of which is the P102L variant, which was retrospectively traced back to the original family described by Gerstmann (Hainfellner et al. 1995). Several other GSS-related variants are recognized (Fig. 19.1 and Table 19.2). In contrast to CJD, which can occur sporadically and on a genetic basis, there are no sporadic cases of GSS, although some consider VPSPr a potential sporadic form of GSS.

19.6.3 Familial Fatal Insomnia (FFI)

First described by Lugaresi et al. (Lugaresi et al. 1986) as a familial thalamic-type dementia heralded by progressive and recalcitrant insomnia, dysautonomia, and motor features with brain histopathology revealing only neuronal dropout and gliosis primarily within the anterior and dorsomedial nuclei of the thalamus, with some involvement of the inferior olivary nucleus, this disease was subsequently linked to the D178N variant of the *PRNP* gene (Medori et al. 1992a). However, the D178N variant was also found in families with CJD. Goldfarb et al. (1992a) determined that the polymorphic codon 129 allelic to the D178N mutation defined whether *Familial FI* (FFI) or CJD would develop in the carrier. Thus, FFI results with the *PRNP*-D178N/129M genotype, whereas the D178N/129V genotype predicts CJD. Over 70 kindreds carrying the *PRNP*-D178N/129M variant have been reported as of this writing. Disease onset is in mid-life, but varies broadly from age 18 to 74, with a mean range of 50–60 years and an average duration of 16 ± 2.2 months (range 8–72 months) (Cracco et al. 2018).

As with most other genetic PrDs, phenotypic heterogeneity exists with FFI, among and within families (McLean et al. 1997). Although sleep disruption is the most characteristic clinical feature, this may not be the most prominent feature at presentation, sometimes requiring a polysomnogram to detect a reduction in total sleep time that usually first affects the deeper slow wave sleep phase (Montagna et al. 2003). The dysautonomia that occurs may include hypertension, tachycardia, diaphoresis, excessive lacrimation or salivation, or impotence (Krasnianski et al. 2014; Gambetti et al. 2003a). Neurological features are typically delayed by about 5 months from onset and include primarily gait ataxia, dysarthria, cognitive dysfunction, visual symptoms, hallucinations, psychiatric features, and seizures, which may be more delayed. The cognitive disorder associated with FFI is one that primarily affects attention and vigilance (Gallassi et al. 1996). Diagnostic criteria for FFI have been proposed (Krasnianski et al. 2014), although the presence of the *PRNP* D178N-129M genotype provides diagnostic confirmation in a symptomatic patient. As in other examples of genetic PrD, codon 129 genotype has been shown to

modify the disease presentation (Krasnianski et al. 2014). For instance, homozygosity (i.e., 129MM) promotes a faster progression and earlier onset of disease compared with heterozygosity (Krasnianski et al. 2014; Gambetti et al. 2003b). Diagnostic testing is limited, as MRI and CSF testing for 14-3-3 are typically negative, with rare exceptions (Takada et al. 2017; Sano et al. 2013; Chen et al. 2013, 2018). The RT-QuIC may be more helpful, as one study found 10 of 12 patients with FFI had a positive RT-QuIC test compared with 14-3-3, in which only one of 12 were positive (Sano et al. 2013). A polysomnogram can document the reduction in total sleep time and loss of sleep-related features, such as K-complexes and sleep spindles (Montagna et al. 2003). A brain fluorodeoxyglucose (FDG) positron emission tomography (PET) scan is often quite helpful, as it typically reveals thalamic hypometabolism even at an early stage of disease (Cortelli et al. 1997; Mastrianni et al. 1999; Perani et al. 1993).

19.7 Genotype–Phenotype Correlations

Following the discovery of the *PRNP* gene, initial studies focused on linking familial occurrences of PrD to specific *PRNP* variants. Genotype–phenotype correlations were proposed to conveniently categorize each newly recognized variant within one of the three major PrD phenotypes of CJD, GSS, or FFI. However, as more cases of each variant continued to be identified, wide variations in the clinical phenotype have emerged among individuals and even within the same pedigree. Although the clinical phenotype varies significantly, the histopathological findings of disease that determine GSS, CJD, and FFI appear to be more tightly linked to the causal *PRNP* variant. However, variation in the distribution, or relative predominance, of the defining pathology and even mixed pathologies of spongiform degeneration and PrP plaques within the brain can vary among carriers. In general, the presence of prominent spongiform degeneration and gliosis in the absence of other pathologies typically defines CJD (Gambetti et al. 2003a), whereas the presence of extracellular PrP plaques, especially multicentric type, with less prominent or absent spongiform degeneration supports a GSS classification (Ghetti et al. 2003). The FFI phenotype is defined by the presence of thalamic gliosis and neuronal dropout, often with limited spongiform degeneration (Gambetti et al. 1995) (Fig. 19.3). With some exceptions that are highlighted within the descriptions of selected variants, particularly some OPRI variants that may display mixed pathologies, and the D178N-129M genotype linked to the FFI phenotype, all other variants are classified as either CJD or GSS, based on the predominant histopathology. Additional histopathological findings, especially neurofibrillary tangles (NFTs) and cerebrovascular amyloid angiopathy (CAA) resulting from PrP amyloid accumulation within blood vessels, are also observed in some *PRNP* variants, particularly P105L, Y145X, F198S, and Q217R variants.

Subtype classification of gPrD is also supported by differences in the western blot pattern and migration rate of PrP^{res}. The rate of migration of PrP^{res} reflects the

site of N-terminal cleavage after incubation with Proteinase-K. Whereas cleavage normally occurs around residue 90, different conformations of PrP^{Sc} expose more or less of the protein in that region, leading to slightly altered sites of cleavage and larger or smaller PrP^{res} fragments. Three principal patterns of PrP^{res} correspond to CJD, GSS, and FFI, although several variations are observed (Fig. 19.4). In general, PrP^{res} from CJD displays all three glycoforms (un-, mono-, and di-glycosylated PrP) with some exceptions (i.e., the T183A and V180I variants display only unglycosylated and monoglycosylated PrP^{res}) (Chasseigneaux et al. 2006; Grasbon-Frodl et al. 2004a). Type 1 PrP^{res} has a 21 kDa unglycosylated fragment and Type 2 carries a 19 kDa fragment. PrP^{res} from FFI also displays all three glycoforms, but only with a Type 2 pattern. GSS typically displays a smaller unglycosylated fragment of 7, 8, or 14 kDa, depending on the variant, without the mono- and di-glycosylated fractions, as a result of cleavage at amino- and carboxy-terminal ends (Parchi et al. 1998;

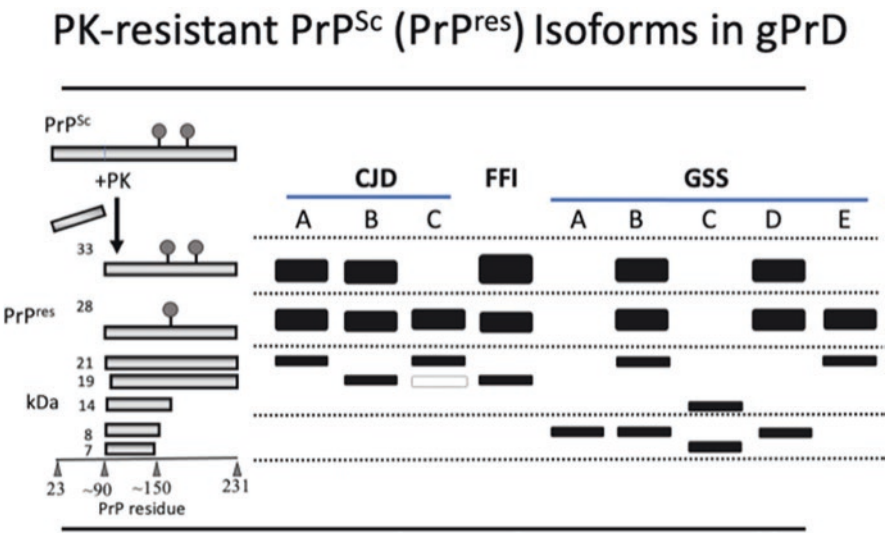

Fig. 19.4 Proteinase-K-resistant isoforms of PrP^{Sc} (PrP^{res}). CJD(A) = Type 1, with a 21 kDa unglycosylated band and mono- and di-glycoforms. This is observed in several variants. CJD(B) = Type 2, with all glycoforms and a 19 kDa unglycosylated band. This is also observed in many variants. CJD(C) = Atypical pattern, lacking the diglycosylated PrP^{res} fraction and the monogycosylated fraction may be either 21 or 19 kDa. Examples are the V180I (closed box) and T183A (open box). FFI is difficult to distinguish from Type 2 PrP^{res}. GSS patterns are more varied and generally demonstrate a smaller PrP^{res} fragment of between 7 and 14 kDa. In many cases the higher molecular weight mono- and di-glycosylated fragments are absent, as in GSS (A and C), whereas other variants may display either a poorly defined or well-defined pattern of higher molecular weight bands that represent all glycoforms. The latter situation typically occurs with variants that variably display spongiform degeneration along with GSS plaques, as with P102L. Examples of variants: GSS(A) = P102L lacking spongiform degeneration, GSS(B) = P102L with spongiform degeneration. In both cases, a small ~8 kDa band is also present. GSS(C) = A117V variant that displays a ~7 and/or 14 kDa PrP^{res} band. GSS(D) = G131V, F198S, D202N, Q217R, and Q212P. GSS(E) = P105S, which looks similar to CJD due to T183A and V180I

Tagliavini et al. 2001). In some cases of GSS that also display spongiform degeneration, a mix of PrP^{res} patterns exists that includes an 8 kD band and the typical three bands associated with a CJD phenotype (Parchi et al. 1998; Piccardo et al. 1998). In the case of the P105S variant, which also associated with a mixed pathology, a ~21 and 26 kD fragment is observed (Tunnell et al. 2008).

19.8 Transmissibility of Genetic PrD

Historically, passage of PrD from an affected host to an experimental host has been the defining proof for the presence of prions (PrP^{Sc}). Non-genetic and genetic forms of PrD have been studied extensively in non-human primates (Brown et al. 1994) and in transgenic (Tg) mice that express either a chimeric mouse–human PrP transgene or a transgene encoding human PrP (Telling et al. 1994; Marín-Moreno et al. 2020). Whereas nearly all forms of sCJD have proven to be efficiently transmissible to experimental hosts, with rare exception (Cali et al. 2018), genetic forms of PrD have not displayed uniform transmissibility. Although most cases of genetic CJD (gCJD) tested for transmissibility using Tg mice have been successful, the majority of GSS cases have not proven to be transmissible. In 1981, Masters et al. (Baker et al. 1990) first reported transmission of the GSS-P102L variant to some recipient non-human primates, although spongiform degeneration was the predominant pathology. Brown et al. (1994) performed a series of transmissions of PrD to non-human primates and found the transmission rate of GSS to be only 40% compared with 85% transmission for sporadic and genetic CJD. However, the most common GSS cases in that series came from subjects with the P102L variant, which can display spongiform degeneration and protease-resistant PrP^{Sc} (i.e., PrP^{res}) with the 21 kDa band in some instances, which was later shown to predict transmissibility (Parchi et al. 1998). Tg knock-in mice expressing mouse PrP-P101L, the homolog of human PrP-P102L, developed disease when inoculated with GSS-P102L with the 21 kDa PrP^{res} (Piccardo et al. 2007), although GSS-P102L with the 8 kDa PrP^{res} induced PrP plaque deposition, but not clinical disease (Piccardo et al. 2007; Asante et al. 2009). The GSS-A117V variant was also found to be transmissible with low efficiency and very prolonged incubation period when inoculated into Tg mice expressing human PrP-A117V (Asante et al. 2013). Such studies that involve inoculation of human cases of genetic PrD into Tg mice that carry matching sequences of the variant can be argued as demonstration of facilitation or promotion of disease rather than transmission, as GSS cases displaying only the 8 kDa fragment have not been transmitted to Tg mice carrying normal sequence human PrP. Transmission of the Y226X variant into Tg mice that express human PrP 8–16-fold higher than physiological levels induced PrP^{Sc} deposition within the brain that was detectable by immunohistochemistry, immunoblotting, and RT-QuIC after extended incubation periods of over 560 days in 50% of mice, documenting the first and only truncation mutation to be transmitted, However, the same investigators found that transmission of the G131V variant resulted in positive PrP^{Sc}

immunohistochemistry, but negative immunoblotting for PrP^{res} or RT-QuIC amplification (Race et al. 2018). In recent years, the bank vole has proven to be permissive to human prions and even GSS, at least from *PRNP* variants P102L, A117V, and F198S, despite the presence of the 7 or 8 kDa or the 21 kDa PrP^{res} fragment, with evidence of both spongiform degeneration and PrP plaque histopathology in affected voles (Pirisinu et al. 2016; Nonno et al. 2006). As proof that infectious prions were generated, the brains from these animals were efficiently transmitted to healthy bank voles, providing support for the neurodegenerative and transmissible features of prions. Transmission studies of genetic PrD due to OPRIs have been incompletely studied, but those that support a CJD phenotype are more likely to be transmissible.

19.9 *PRNP* Polymorphisms

Non-pathogenic *PRNP* variants include single amino acid substitutions, a single octapeptide repeat deletion (OPRD), a single OPRI (Beck et al. 2010), and several "silent" base-pair alterations that do not induce a sequence change within PrP. However, there are important polymorphisms that do not cause disease, but may have a significant impact on the risk to sporadic and acquired forms of disease, the clinical phenotype, rate of disease progression, histopathological features, and physicochemical properties of the PrP^{Sc} molecule in non-genetic and genetic forms of PrD. The most important of these is the polymorphic codon 129, which can encode either methionine (Met) or valine (Val) and which plays a profound role in PrD. The best studied polymorphisms are discussed below.

19.9.1 Polymorphisms with a Well-Documented Effect on PrD

Codon 129 Codon 129 is either ATG, which encodes Met (M), or GTG, which encodes Val (V). Initial estimates of allelic frequency in the Caucasian population were 0.66 for M and 0.34 for V, resulting in a genotype distribution of 37% MM, 51% MV, and 12% VV (Owen et al. 1990). However, in contrast to the predicted 49% homozygosity (i.e., 129MM or 129VV) in the general population, 80–90% of CJD patients sampled from several countries were found to carry a homozygous state at 129 (Owen et al. 1990; Palmer et al. 1991; Laplanche et al. 1994; Salvatore et al. 1994). The exception to this is Japan, where the allelic frequencies of codon 129 are 0.96 for 129M and 0.04 for 129V, obscuring the risk of homozygosity in that population (Doh-ura et al. 1991). A recent meta-analysis of 13 appropriately designed case–control studies on the role of codon 129 in the risk of sCJD estimated the current genotype frequencies within the general European population as 55% MM, 36% MV, and 9% VV, and confirmed that homozygosity, in particular 129MM,

is a potent risk factor for sCJD, with a calculated odds ratio of 4.9611 (Kim and Jeong 2021). Homozygosity at codon 129 is also over-represented in iCJD resulting from exposure to biologicals such as growth hormone and dura mater grafts contaminated with human prions (Brown et al. 2012). The most profound association with PrD risk and codon 129 is observed with vCJD, an acquired PrD that results from ingestion of beef contaminated with BSE, in which the 129MM genotype is observed in nearly 100% of cases (Diack et al. 2014; Collee et al. 2006), although there is evidence for asymptomatic prion infection in 129MV and 129VV carriers, based on detection of prions in peripheral lymphoid organs of spleen and appendix (Peden et al. 2004, 2010), and there is a case of clinical and pathologically likely, but not molecularly confirmed, vCJD reported in a 36-year-old with the 129MV genotype (Mok et al. 2017).

Prion transmission studies using Tg mice expressing human PrP genes carrying either 129M, 129V, or 129MV have supported the protective nature of the heterozygous state by the extension of the incubation period when the 129 residue of the recipient host animal differs from that of the source prion inoculum and the facilitation of disease transmission when the 129 residues match (Wadsworth et al. 2004; Mallik et al. 2010; Korth et al. 2003; Bishop et al. 2006). Live cell FRET studies also revealed tighter interaction between PrP molecules homozygous at residue 129 compared with heterozygous molecules, thereby supporting the facilitation of the templated conversion process that occurs between PrP^{Sc} and PrP^{C} when codon 129 sequences match (Mallik et al. 2010).

The genotype at codon 129 also features in determining the phenotype of sCJD. In general, the 129MM genotype is associated with a dementia onset and rapid progression, whereas a slower progression and ataxic at onset is more common with the 129MV or 129VV genotype (Parchi et al. 1996). However, several subtypes of sCJD that depend on the codon 129 genotype and the conformation of PrP^{Sc} have been defined. Whereas the 129MM genotype is most often associated with Type 1 PrP^{res} (~21 kDa), and the 129 VV genotype typically results in Type 2 PrP^{res} (~19 kDa) (Parchi et al. 1996, 1997), these are not invariant. Thus, six primary subtypes of sCJD are defined, based on the combination of 129 genotype, PrP^{Sc} typing, and neuropathologic features (i.e., MM/MV 1, VV2, MV 2K, MM/MV 2C, MM 2T, VV1, and mixed phenotypes of MM/MV 1 + 2C), with C, K, and T representing histopathologic features denoting spongiform degeneration within cortex (C) or thalamus (T), and the presence of kuru plaques (K) (Parchi et al. 2012).

In some gPrDs codon 129 profoundly affects the molecular and clinical phenotype of disease, as in the case of the D178N variant, which can result in FFI when 129M is in *cis* with the variant allele or CJD when D178N is in *cis* with 129V. These two subtypes differ not only in clinicopathologic phenotypes, but in the migration rate of PrP^{res}, which is 19 kDa for CJD (D178N-129V) and 21 kDa for FFI (D178N-129M) (Monari et al. 1994). Although age at onset of PrD is generally difficult to predict because of its broad variation among and within variants, some associations with individual variants have been proposed. For instance, carriers of the F198S or 6-OPRI variants who are homozygous at codon 129 generally display

an earlier age at onset and shorter disease duration. Also, as noted earlier, 129 genotype influences the course of disease in FFI; a recent assessment of ~130 cases that found 129M homozygosity was associated with a significantly faster rate of progression (11.13 ± 5.92 months) compared with 129MV (26.79 ± 13.62 months) (Zhang et al. 2022).

E219K A substitution of lysine (K) for glutamate (E) at codon 219 occurs in ~6% of the Japanese population (Furukawa et al. 1995), although it is not seen in any cases of sCJD, suggesting a protective effect (Shibuya et al. 1998). This polymorphism was also detected on the same allele as the dominant P102L variant in a Japanese family in which dementia rather than ataxia was prominent and cerebellar plaque pathology was less prominent compared with *PRNP* P102L variants that lack the E219K polymorphism (Tanaka et al. 1997). However, similar variability in the presentation of GSS has been observed in other families with the *PRNP*-P102L variant, limiting the conclusions. The E219K polymorphism was also reported in two cases of vCJD (Lukic et al. 2010) and studies in knock-in Tg mice suggest that it is not resistant to conversion. However, whereas knock-in Tg mice expressing only human PrP^{219K} were susceptible to both sCJD and vCJD, a significant delay in disease onset was observed in those expressing human PrP^{219E} when challenged with vCJD, suggesting that PrP^{219K} mice confer enhanced susceptibility of PrP to adopt the conformation of PrP^{Sc} associated with vCJD (Hizume et al. 2009). Further, a dominant-negative inhibitor effect was observed when PrP^{219E} and PrP^{219K} were co-expressed (Hizume et al. 2009; Perrier et al. 2002), which may be the basis for the observed "protective" effect of the E219K polymorphism.

G127V This polymorphism was initially identified in healthy individuals in Papau, New Guinea, a population at risk for exposure to kuru that was endemic to that region (Mead et al. 2009). It is always allelic with 129M and has not been reported in other populations. It is predicted that this polymorphism has undergone population selection because of its protective effect against kuru within this high-exposure population. Tg mice that express only human PrP^{127V} were, in fact, resistant to kuru, vCJD, and sCJD challenge and Tg mice co-expressing normal human PrP^{127G} and varying gene doses of PrP^{127V} showed a dose-dependent dominant-negative inhibition to prion challenge, confirming the protective potential of this polymorphism (Asante et al. 2015).

19.9.2 Polymorphisms with Unclear or No Effect on Disease

24-base Pair (bp) Deletion or Insertion These result in the loss or gain of eight amino acids within the octapeptide repeat segment of PrP. 1-OPRD was initially detected in three healthy members of a Moroccan family (Laplanche et al. 1990) and then incidentally in a cosmid library construct derived from the HeLa human cell line (Puckett et al. 1991). It is found in ~3% of the normal population (Laplanche et al. 1990; Vnencak-Jones and Phillips 1992; Palmer et al. 1993) and has not been

reported in association with genetic PrD, although it has been incidentally detected in subjects with sCJD (Areškevičiūtė et al. 2021) and in the presence of other *PRNP* variants (Bosque et al. 1992; Alshaikh et al. 2020). 1-OPRI is also considered non-pathogenic, as it can be found in the unaffected population (Beck et al. 2010).

N171S The N171S polymorphism was incidentally noted in a 69-year-old healthy control subject (Fink et al. 1994), but when identified in a family with psychiatric disease, it was suggested to be potentially linked to schizophrenia. However, one healthy member of this family also carried the polymorphism (Samaia et al. 1997) and a follow-up study was unable to demonstrate the N171S polymorphism in a schizophrenia population (Tsai et al. 2001), suggesting that it plays no role in disease.

19.10 Disease-Associated *PRNP* Variants

The vast majority of *PRNP* variants are due to single base-pair substitutions within the highly structured globular domain. These result primarily in missense mutations that do not alter the length of PrP but act to destabilize its structure to facilitate its conversion to PrP^{Sc}; however, at least six of these result in nonsense mutations that introduce an early stop sequence that result in variably lengthed C-terminally truncated PrP molecules. An additional nonsense mutation results from a frame-shift mutation that introduces a stop sequence. Other mutations result in a lengthening of PrP. These include a number of insertions within the octapeptide repeat segment between codons 51 and 90, and a single small non-octapeptide repeat duplication insert just downstream from this region. A selection of *PRNP* varia are described below. These are selected based on their frequency of occurrence in one or more populations, or their unique property that contributes to the understanding of genetic PrD. All known reported variants are listed separately in Table 19.2.

A. **Missense Mutations:**

CJD-Associated:

PRNP-**E200K** This is the most common *PRNP* variant worldwide. It was first detected in rural Slovakia (Goldfarb et al. 1990a) and then in a Libyan Jewish family (Hsiao et al. 1991a), both of which were allelic with 129M coding. Based on additional reports of this mutation in a Japanese family (Inoue et al. 1994) and its identification in association with Val coding at codon 129 (Hainfellner et al. 1999), at least two additional founders are predicted, and further suggest that this mutation arose spontaneously in several populations from the deamination of a methylated CpG in a germline *PRNP* gene. Clusters of this mutation are seen in populations from Israel, Chile, and Eastern Europe. Surveillance studies from France and England have detected the E200K mutation in patients without a clear family history, supporting its variable age at onset (Laplanche et al. 1994; Windl et al. 1996).

The clinical phenotype of this variant is quite comparable to sCJD. The *PRNP*-E200K variant is allelic with 129M in all but one reported case (Hainfellner et al. 1999). The homozygous 129MM state appears to reduce the duration of disease by 50% in one survey (Mitrová and Belay 2002). Age at onset is rather broad, extending from the early 30s to mid-80s and time to death is typically between 5 and 10 months (Kovacs et al. 2005; Krasnianski et al. 2004). Penetrance estimates of this mutation vary from 60% in Slovakian Jews (Mitrová and Belay 2002) to nearly 100% age-dependent penetrance in Sephardic Jews (Spudich et al. 1995). Symptoms include dementia, ataxia, myoclonus, pyramidal signs, hallucinations, delusions, aggression, depression, insomnia, and less commonly extrapyramidal signs, including chorea or dystonia. Many patients show visual/oculomotor disturbances and ataxia in early stages of disease (Krasnianski et al. 2016). Diagnostic studies that are positive in sCJD are generally positive with the E200K variant. PSWCs on the EEG are seen in roughly 55% of cases, slightly less than the estimated 65% observed in sCJD (Kovacs et al. 2005; Krasnianski et al. 2016; Breithaupt et al. 2012). As with sCJD, deep nuclei and cortical ribbon hyperintensities on MRI–DWI and FLAIR sequences are observed in the majority (~80%) of cases (Breithaupt et al. 2012), as are CSF-14-3-3, tau, and RT-QuIC (Franceschini et al. 2017). Pathology is typified by widespread spongiform degeneration of cortex and deep nuclei, with more severe pathology favoring the neocortex, basal ganglia, and thalamus characteristic of that seen with sCJD 129MM Type 1 subtype (Kovacs et al. 2005, 2011). Gliosis parallels the spongiform degeneration. PrP plaques are absent, while PrP^{res} is Type 1 (21 kDa) in all cases except for the E200K-129V case, in which plaque-like PrP deposits were found in the cerebellum and the PrP^{res} displayed a Type 2 pattern with a 19 kD unglycosylated fragment (Hainfellner et al. 1999).

PRNP-**D178N** This variant is linked to two classical phenotypes, CJD and FFI, which are largely determined by the 129 polymorphism that is allelic with the mutation. Goldfarb et al. (1992a) first recognized that the FFI phenotype of insomnia, autonomic dysregulation, and late neurological symptoms was strongly associated with the D178N-129M genotype, whereas the D178N-129V genotype was associated with a CJD-like presentation of dementia and earlier neurological signs and symptoms. Since these initial striking correlations, it has been recognized that the distinction between these two extreme phenotypes may be less clear, as several reports of the D178N-129M genotype describe a CJD phenotype (Chen et al. 2018; Bosque et al. 1992; Taniwaki et al. 2000; Zarranz et al. 2005), and a case of D178N-129V with insomnia as a presenting feature (Appleby et al. 2010), supports the notion that there exists a continuous spectrum of disease that depends on the propensity of conformational selection driven by each of the two PrP sequences. However, the D178N-129V genotype most often presents with memory impairment, followed by cerebellar ataxia and myoclonus, with varying degrees of visual disturbance, reduced speech output, extrapyramidal, and pyramidal tract features. Less than 10% develop seizures. Data on the diagnostic utility of MRI, CSF 14-3-3, tau, and RT-QuIC for the D178N-129V variant are insufficient (Franceschini et al. 2017). The EEG may lack PSWCs. Brain pathology associated with the D178N-129V

genotype includes diffuse spongiform degeneration, gliosis, and neuronal loss within the frontotemporal cerebral cortex, caudate, and basal ganglia, with relative sparing of deep thalamic nuclei and the cerebellum, while PrP^{res} is Type 1 (Gambetti et al. 1995; Brown et al. 1992). This contrasts with the focal thalamic pathology with minimal spongiform degeneration and Type 2 PrP^{res} observed with FFI (Cracco et al. 2018). Transmissibility to receptive experimental models for each variant has been documented. Comparison of the transmissibility of PrP^{Sc} from D178N-129V and D178-129M to "humanized" Tg mice revealed that the distinct conformations of PrP^{Sc} from each were stably transmitted, supporting a "conformational templating" process that transfers the PrP^{Sc} conformation onto PrP^{C} (Telling et al. 1996).

PRNP-**V210I** This variant was initially reported in Italy (Pocchiari et al. 1993) and France (Ripoll et al. 1993), although additional cases have since been reported in several countries, including the United States (Mastrianni et al. 2001), Japan (Furukawa et al. 1996), and France (Mouillet-Richard et al. 1999). It is much more prevalent in Europe than the United States and represents 42% of all variants in Italy (Ladogana et al. 2005). It is allelic with 129 M in cases. As with the E200K variant, the presentation is variable and includes a cerebellar syndrome, myoclonus, dysarthria, stroke-like features, in addition to hemisensory loss, hemiparesis, sudden onset of visual disturbances, or the onset of behavioral changes, all beginning at a mean age of ~60 years (range ~40–80) and running a rapid course that averages ~5 months (range 2–20) (Kovacs et al. 2005; Krasnianski et al. 2016). PSWCs are evident on the EEG in ~69%, comparable to sCJD (Krasnianski et al. 2016). MRI is positive in roughly 20% of cases, whereas 14-3-3, elevated tau, and RT-QuIC are positive in nearly 100% of cases (Franceschini et al. 2017; Ladogana and Kovacs 2018). PrP^{res} is similar to that of sCJD and the pathological features are comparable to sCJD(129MM1), with spongiform changes in cerebral cortex and cerebellum (Pocchiari et al. 1993; Mastrianni et al. 2001). Because healthy carriers of the V210I mutation aged 67–82 years have been identified in a population-based genetic survey (Minikel et al. 2016) and some patients lack a clear family history, penetrance of this variant is estimated at 10%, compared with other *PRNP* mutations, an important consideration in family discussions regarding carrier risk to disease.

PRNP-**V180I** This variant is rarely seen in the Caucasian population, although it is the most common variant in Japan, accounting for ~42% of all gCJD cases (Yamada et al. 2009; Nozaki et al. 2010). It results from a G to A change at the first nucleotide of codon 180 (GTC to ATC) and is allelic with 129M in all but a few rare cases that are allelic with 129 V (Qina et al. 2014). It was initially reported in two unrelated Japanese families with a presentation consistent with CJD (Kitamoto et al. 1993a). In those families, as with most cases of V180I in a large series of 84 collected in Japan (Yamada et al. 2009), a family history is generally lacking and may be present in only 2% of patients (Nozaki et al. 2010), supporting the low penetrance that has been estimated to be 0.1–1%, based on its prevalence in the healthy population (Minikel et al. 2016). Interestingly, the mean age at onset with this variant is 77.6 years (Qina et al. 2014), considerably delayed when compared with sCJD and other genetic PrDs. Clinical features include a relatively slowly progressive course

over 1–4 years that is dominated by cognitive impairment and less commonly ataxia, myoclonus, psychiatric, pyramidal tract, and visual disturbances (Nozaki et al. 2010; Qina et al. 2014). PSWCs are seen in ~10%, whereas MRI–DWI signal changes in the cerebral cortex or basal ganglia are present in 99% of cases. The CSF RT-QuIC was positive in only 39% in one series (Higuma et al. 2013) and as high as 68% in another (Qina et al. 2014), whereas both 14-3-3 and elevated tau levels were detected in 70–90%, depending on the series (Higuma et al. 2013; Qina et al. 2014). Histopathological findings include diffuse spongiform degeneration, neuronal loss, and gliosis within the cerebral cortex, although kuru-types plaques were also observed in at least one example (Iwaski et al. 1999, 2011). The western blot pattern of PrP^{res} is reported as either Type 1 or Type 2, although it lacks the higher diglycosylated band (Fig. 19.4), despite the fact that when expressed in cultured CHO cells, PrP^{180I} appears fully glycosylated (Chasseigneaux et al. 2006; Iwasaki et al. 2011), suggesting that the substitution does not interfere with glycosylation, but does impact the conversion of the diglycosylated fraction of PrP^{180I}. The differences in clinical features, diagnostic testing, and histopathological profile compared with typical sCJD suggest an influence of the V180I substitution on PrD, but its low penetrance suggests that it may function primarily as a risk factor rather than a pathogenic mutation.

PRNP-**T183A** Although uncommon, this is discussed, because of the effect, this mutation has on the normal post-translational processing of PrP and the resultant PrP^{Sc}. The substitution of threonine (T) with alanine (A) at codon 183 in combination with 129M coding was first reported in a Brazilian family with nine affected members who presented with a syndrome of progressive dementia and behavioral features that suggested possible frontotemporal dementia (FTD), with aggressiveness, hyperorality, and stereotypic speech at the onset, beginning in the 40s and progressing over 2–9 years (average 4 years) (Nitrini et al. 1997). It has since been reported in Germany (Grasbon-Frodl et al. 2004a) and China (Shi et al. 2015), also with dementia as the primary feature. Brain MRI is negative for typical CJD findings, as is the presence of 14-3-3 in CSF. RT-QuIC is not reported. Pathology in the Brazilian family revealed intense spongiform degeneration primarily within frontotemporal regions and putamen, in addition to some plaque-like deposits in cerebellum (Grasbon-Frodl et al. 2004a; Nitrini et al. 1997). PrP^{res} is Type 2, although the diglycosylated band is poorly visualized, based on the proximity of the residue to the second N-linked glycosylation site (Fig. 19.4). In contrast to the findings of the V180I variant, when expressed in cultured cells, PrP^{183A} is poorly diglycosylated and has impaired trafficking through the secretory pathway (Grasbon-Frodl et al. 2004a).

PRNP-**M232R** This variant is one of the most common in Asians, having been reported in Japan (Yamada et al. 2009; Nozaki et al. 2010; Koh et al. 2015), China (Zheng et al. 2008), and South Korea (Choi et al. 2009), yet not in Caucasians. It is allelic with 129M in all cases. However, because this variant was shown to be present in the general population at a rather high level (Minikel et al. 2016), its penetrance has been estimated to be only 0.1%, suggesting it may be an incidental

polymorphism found in subjects with sCJD, or perhaps it functions as a modifier of disease, although a distinct effect on phenotype has not been confirmed. In one series in Japan, as many as 15% of CJD cases carried this variant. Interestingly, two Japanese patients were reported to carry the M232R variant with the V180I, although rather than developing disease at an early age, as might be expected with a double mutation, the affected individuals were 74 and 84 years at onset (Koh et al. 2015; Hitoshi et al. 1993). Typical disease onset is 64 years ± 12 (range 15–81) with an average duration of 8 months, although a duration of up to 32 months has been reported (Nozaki et al. 2010). EEG is positive in 63% and MRI is positive in 84% of cases, while 78% have a positive 14-3-3 CSF test. The histopathology associated with disease is characteristic of typical CJD, with generalized spongiform degeneration and an absence of PrP plaques (Shiga et al. 2007).

GSS Associated:

PRNP-**P102L** The substitution of proline for leucine at codon 102 is the most common GSS-related mutation worldwide and the first mutation of *PRNP* linked to PrD (Hsiao et al. 1989b). It has been recognized in multiple families from at least nine countries, suggesting it is the result of deamination of methylated CpG in the germline of multiple founders (Goldgaber et al. 1989; Doh-ura et al. 1989; Kretzschmar et al. 1992; Goldhammer et al. 1993; Young et al. 1995). Most reported mutations are allelic with 129M. The E219K polymorphism was also reported allelic with the P102L mutation in a Japanese family (Tanaka et al. 1997). The *PRNP*-P102L variant has an LOD score of greater than 4.5 and demonstrates complete penetrance (Hsiao et al. 1989a; Speer et al. 1991). Median age at onset is 52 (range 25–70). The clinical phenotype associated with this mutation is characteristic of the classic phenotype of GSS, with progressive cerebellar ataxia, often with accompanying weakness and/or rigidity of muscles that develops over ~4 years (range 5 months to 17 years) (Mead 2006), although variation in the presentation is common among and within families (Hainfellner et al. 1995; Kretzschmar et al. 1992; Young et al. 1995). Some patients experience areflexia with a suggestion of lower motor neuron signs or a myopathic process. Cognitive problems typically relate to frontal-executive dysfunction that may be more prominent in later stages of disease. The EEG may show diffuse slowing, but typically lacks PSWCs. Pathology includes multicentric, primitive, and/or dense core plaques that are observed throughout the cerebrum, but more prominently observed within the molecular layer of the cerebellum (Hainfellner et al. 1995). Whereas plaques are a constant feature and spongiform degeneration is often limited, the latter may be conspicuously present in a subset of patients. Interestingly, those patients with prominent spongiform degeneration mixed with GSS plaques typically display PrP^{res} that is more characteristic of that observed in CJD, such that all glycoforms are present and the unglycosylated fraction is ~21 kD, alone or in combination with the small ~8 kDa fragment common to patients with exclusively plaque pathology (Parchi et al. 1998; Piccardo et al. 1998) (Fig. 19.4). This variant was the first genetic PrD to be modeled in Tg mice, which provided key evidence that mutations within *PRNP* are disease-associated (Hsiao et al. 1990a). Tg (PrP-P101L) mice over-express mouse PrP with the mouse

homolog of the P102L mutation and develop progressive gait ataxia at ~150 days of age, spongiform degeneration, and a low level of PrP plaques (Hsiao et al. 1991b).

PRNP-**P105L/T/S** Three variants of *PRNP* have been reported at codon 105. The P105L variant results from a cytosine to thymine transition at the second nucleotide of codon 105 (CCA to CTA), leading to a leucine (Leu) substitution of proline (Pro). In all cases, this variant is allelic with 129V. The clinical syndrome is characterized by spastic paraparesis with weakness, hyperreflexia, and extensor plantar responses prior to or coincident with the development of cognitive decline. Age at onset ranges from 38 to 48 years. Over a 7–12-year course, the affected individual progresses to tetraparesis (Kitamoto et al. 1993b; Yamada et al. 1993; Amano et al. 1992). In some subjects, a more typical presentation of GSS, with progressive cerebellar ataxia, develops. Myoclonus is not reported. EEG typically lacks PSWCs. The MRI is typically negative. CSF data are not available. Pathology consists of diffuse-type PrP plaques distributed throughout the cerebral cortex, especially within frontal motor cortex and deep gray nuclei of the basal ganglia and thalamus. These same areas also display severe neuronal loss and gliosis but lack spongiform change.

The P105T variant (CCA to ACA), which is allelic with 129M, was first reported in a 13-year-old of East Indian descent who developed anxiety followed by ataxia, dysarthria, spasticity, and dystonia, with late dementia, whereas the other five affected family members presented between 33 and 41 years of age with rapidly progressive dementia, ataxia, spasticity, and in some, extrapyramidal features. Disease duration was 2–3 years. MRI was positive in ~25%. CSF data are unavailable, as is histopathological and biochemical data regarding PrP^{Sc} (Rogaeva et al. 2006). A subsequent family was reported with an onset in the late 30s with cognitive decline followed by a progressive cerebellar syndrome and eventual mutism (Polymenidou et al. 2011). Histopathology showed limited unicentric PrP plaques and spongiform degeneration, but typical PrP^{res} was lacking, although a small 6 kDa band was detected using a novel method to isolate PrP aggregates (Polymenidou et al. 2011).

The P105S variant (CCA to TCA) was found allelic with 129V in a 31-year-old woman who developed a frontal lobe syndrome and cognitive decline without ataxia, which initially suggested a diagnosis of FTD. The pathology associated with that case included intense focal vacuolation of the basal ganglia and a heavy burden of PrP multicentric plaques within the cerebellum and hippocampus (Tunnell et al. 2008). The PrP^{res} pattern was atypical, with only unglycosylated and monoglycosylated fractions present (Fig. 19.4). The prominent plaque pathology in P105L and P105S compared with P105T may relate to the fact that the former are allelic with 129V coding, and the latter is allelic with 129M.

PRNP-**A117V** First identified in a French–Alsatian family (Doh-ura et al. 1989; Hsiao et al. 1991c) and subsequently in two American families of German descent (Hsiao et al. 1991c; Mastrianni et al. 1995), this variant has also been reported in Hungary, the United Kingdom, Ireland, and Argentina (Heldt et al. 1998; Mallucci et al. 1999; Kovacs et al. 2001; Saenz-Farret et al. 2016). Case segregation studies

support it as having high penetrance (Minikel et al. 2016). This variant is consistently allelic with 129V. Age at onset is from the third to fifth decade and the average disease duration is 3 years. Two major presentations were initially thought to be associated with this variant; a typical GSS phenotype that includes ataxia with pyramidal and extrapyramidal features and dementia emerging late in the disease (Mastrianni et al. 1995) and a "telencephalic" variant in which the onset of disease is heralded by progressive dementia with variable features of pyramidal, extrapyramidal, and cerebellar ataxia features (Doh-ura et al. 1989; Hsiao et al. 1991c). However, subsequent reports of the original family (Tateishi et al. 1990) and other families (Mallucci et al. 1999; Eraña et al. 2022) have revealed clinical variability that ranges from behavioral and psychiatric onset, cognitive decline, or a cerebellar syndrome, even within members of the same family. However, the pathology in most cases displays extracellular PrP plaques. As is common with GSS, EEG typically lacks PSWCs, and MRI is negative. CSF studies of 14-3-3, tau, and RT-QuIC are negative or unavailable. Interestingly, prior to the use of specific antibodies to confirm PrP plaque pathology, this disease was initially mislabeled as a familial AD, based on the presence of thioflavin S positive amyloid plaques distributed throughout the cerebral cortex and not within the cerebellum (Heston et al. 1966). PrP^{res} may be difficult to detect, although fragments of 7 and 14 kD can be observed (Ghetti et al. 2003; Eraña et al. 2022). A Tg mouse expressing the mouse homolog of human PrP-A117V with the 129V polymorphism (i.e., TgPrP-A116V/128V) at ~4× the normal level of wt PrP develops a stereotypical presentation beginning with ataxia at ~120–130 days that progresses to death ~30 days later (Yang et al. 2009). The mice display PrP plaques predominately within cerebellum and hippocampus in addition to mild spongiform degeneration, confirming the pathogenicity of this variant.

PRNP-**F198S** This variant was Initially identified in 67 affected individuals spanning 6 generations of a 1200-member kindred in Indiana, providing an LOD score of 6.37. It is allelic with 129V (Dlouhy et al. 1992). Onset of disease occurs at a median age of 52 (range 30s–60s) and duration of disease averages 6 years (range 2–12 years) (Farlow et al. 1989). Individuals with 129VV homozygosity may develop disease up to a decade earlier than 129VM carriers (Dlouhy et al. 1992). Affected individuals typically develop progressive gait ataxia, dysarthria, and impaired cognitive function, often with extrapyramidal features of bradykinesia and rigidity, in addition to oculomotor abnormalities. Myoclonus is present in some, but not all. PSWCs are not present, although the EEG is typically slow. Diffusely distributed PrP plaques throughout the cerebral and cerebellar cortex, with minimal spongiform degeneration, is the typical histopathology (Ghetti et al. 1989). NFTs, comprised of tau protein within the frontal, parahippocampal, and insular cortex, in addition to cingulate gyrus, are also present in many cases (Ghetti et al. 1992, 1995).

B. **Nonsense Mutations:**

All nonsense mutations of *PRNP* that result in a truncated molecule are associated with a GSS phenotype, as a result of the lack of the GPI-anchor that normally anchors PrP to the plasma membrane, thereby resulting in its secretion and

extracellular deposition as PrP amyloid plaques. This was demonstrated best by Tg mice constructed to express PrP lacking the GPI anchor, which displayed prominent PrP plaque deposits (Chesebro et al. 2005). The first truncation mutation was identified at position 145 in a Japanese individual with a 20-year course of dementia, leading to death at age 59 (Kitamoto et al. 1993c; Ghetti et al. 1996). EEG did not show PSWCs and the presence of PrP plaque deposits in small and medium blood vessels have led to the labeling of this as a cerebral amyloid antipathy (CAA) due to PrP (i.e., PrP-CAA) (Ghetti et al. 2003). Five additional truncation mutations, including Q160X (Finckh et al. 2000), Y162X, Y163X (Mead et al. 2013), Y226X (Jansen et al. 2010), and Q227X (Jansen et al. 2010), have also been reported. A younger age of onset, ranging from the 20s to the 50s, and a more protracted course of 2 to 6 or more years is generally associated with each of these variants. Cognitive decline, aphasia, and hallucinations were reported with the Y226X variant, whereas a hypokinetic rigid gait with parkinsonism and cognitive decline was reported for the Q227X variant, and autonomic disturbances, peripheral neuropathy, and an FTD-like orbitofrontal syndrome was described for the Q160X variant (Fong et al. 2017). The Y163X variant is linked to a debilitating dysautonomia with associated diarrhea, autonomic failure, and length-dependent axonal sensory neuropathy (Mead et al. 2013). PrP plaques are present in all, with PrP-CAA observed in Q160X, Y163X, and Y226X variants, but not in the Q227X variant. NFTs are also found throughout the cerebral cortex of the Q160X, Y163X, and Q227X variants, but not in the Y226X variant. NFTs in the Q160X variant are composed of paired helical filaments and straight filaments of 3-repeat and 4-repeat tau isoforms, similar to the profile observed in typical AD (Hallinan et al. 2021). The Y163X variant is associated with peripheral deposition of PrP amyloid within several peripheral organs, especially bowel and peripheral nerves, presumably accounting for the severe autonomic failure in those patients (Mead et al. 2013).

An interesting nonsense mutation at codon 195 was identified in a Japanese family that developed early cognitive decline with a subsequent pan-autonomic-sensory neuropathy that manifested with severe orthostatic hypotension, syncope, vomiting, and diarrhea (Matsuzono et al. 2013). In contrast to all other early stop sequence variants, this variant results from a frame shift as a result of a 2-base pair deletion at codon 178. As with other truncation mutations, affected family members were young, with symptom onset at age 31 in the proband, 47 in her mother, and 52 in her grandfather. Pathology of the brain was not available, although it was noted that PrP accumulations were found within myelin of a sural nerve biopsy.

19.11 Octapeptide Repeat Insertions (OPRIs) and Deletions (OPRD)

The octapeptide segment of PrP consists of an initial 9-peptide repeat segment, followed by four 8-peptide repeats with minor variations. The repeat sequence with possible variations is represented as P(H/Q)GGG(−/G)YGQ. “R” is used to

designate repeat units, with the normal sequence represented by R1-R2-R2-R3-R4. R2, R3, and R4 are each distinguished from R1 by non-coding nucleotide differences. Additional repeats of between 1–9 and 12, designated as X-OPRI, with X indicating the number of extra inserts (see Fig. 19.1 and Table 19.2). These variants are proposed to result from unequal crossover and recombination (Goldfarb et al. 1991a). There is no obvious anticipation with these repeat inserts and the length of the repeat segment is stable across generations. There is remarkable clinicopathologic heterogeneity among this group of variants, although broad associations that relate to insert length have been recognized. Age at onset generally correlates inversely with the length of the OPRI, whereas disease duration is generally proportional to repeat length, at least in cases with 7 or less octarepeats (Goldfarb et al. 1996; Capellari et al. 1997; Croes et al. 2004). Individuals with up to four additional OPRIs develop disease between the sixth and seventh decade that runs a course as short as 5 months, whereas those with higher numbers of OPRIs may develop symptoms as early as the third or fourth decade with a course that runs beyond 10 years (Goldfarb et al. 1996; Croes et al. 2004; Dermaut et al. 2000). The clinical phenotype in the majority of OPRIs that result in CJD type histopathology and PrP^{res} profile is often described with atypical features compared with sCJD, including aphasia, apraxia, and a personality disorder, in addition to dementia, cerebellar ataxia, and extrapyramidal features. Myoclonus occurs in less than 50% and PSWCs on EEG are observed in less than 30% (Goldfarb et al. 1991a; van Gool et al. 1995; Duchen et al. 1993). Although the 1-OPRD is a well-documented polymorphism (see above), a 2-OPRD has been reported in two patients with CJD, one diagnosed on a clinical basis (Beck et al. 2001) and the other pathologically confirmed (Capellari et al. 2002). Although a family history was not evident in either case, the absence of this variant in a large population survey of ~3000 subjects supports its pathogenicity.

The histopathologic findings also generally vary with the length of the insert in that CJD-type spongiform degeneration predominates in cases with up to four extra OPRIs, whereas GSS-type histopathology with PrP plaque deposits are more often observed in those with eight or more OPRIs. Those with 5–7 OPRIs have greater variability of the pathology, not only among different families but even within the same family, with a mixture of spongiform degeneration and GSS-type pathology. A dose-dependent effect on penetrance (Goldfarb et al. 1991a) and the rate of PrP^{res} production (Moore et al. 2006) have also been suggested to correlate with increasing expansion of the OPRI segment. Although these generalizations are helpful to provide a framework for genetic counseling of family members of OPRI variants, the extreme variation in presentation with many of these variants should always be emphasized during counseling.

1- to 4-OPRI As a group, these variants generally have reduced penetrance, based on the finding that a strong family history is typically lacking. A single report of 1-OPRI (Beck et al. 2010), four cases of 2-OPRI (Croes et al. 2004; Goldfarb et al. 1993; van Harten et al. 2000), three reports of 3-OPRI (Yu et al. 2004; Grasbon-Frodl et al. 2004b; Nishida et al. 2004), and 11 cases of 4-OPRI (Laplanche et al.

1995; Kaski et al. 2011; Sánchez-Valle et al. 2012) are known. The 1-OPRI variant is considered non-pathogenic, as it has been detected in healthy individuals (Beck et al. 2010), whereas the 2-OPRI, associated with either Met or Val at 129, has been found in four patients aged between 58 and 75 that displayed varying disease phenotypes of slowly progressive dementia over 13 years (Goldfarb et al. 1993), a 3-month course of rapidly progressive dementia at age 58, a 10 year slowly progressive dementia in a 39-year-old (Croes et al. 2004), and a 7-year course of progressive ataxia and dementia in a 61-year-old (van Harten et al. 2000). Only one case had confirmation of CJD pathology (Goldfarb et al. 1993). The 3-OPRI was also found in a healthy 11-year-old and her mother who was in her late 30s (Yu et al. 2004), suggesting it may be a risk variant rather than directly pathogenic (Yu et al. 2004). However, progressive dementia and ataxia were observed in the two other cases that began later in life. One case carried the 129V polymorphism allelic to the 3-OPRI (Grasbon-Frodl et al. 2004b) and the other carried 129M in combination with the E219K polymorphism (Nishida et al. 2004). Generally, age at onset is older in this group and the course of disease is more progressive than cases of longer OPRIs (Croes et al. 2004), although there are clear exceptions of long duration up to 13 years (Goldfarb et al. 1993) and earlier onset with ages ranging from 39 to 85 (Kaski et al. 2011). Cognitive dysfunction appears to dominate the clinical picture, especially with the 4-OPRI, although aphasia with mutism was reported in a case of 2-OPRI (Croes et al. 2004) and ataxia and myoclonus were present in the 3-OPRI cases. In the case of the 4-OPRI variant, up to 50% experience cerebellar ataxia and 80% experience myoclonus (Kaski et al. 2011). Diagnostic testing availability is somewhat limited, although one of five individuals from a 4-OPRI family had a DWI hyperintensities within the right caudate head and temporal lobe on MRI (Kaski et al. 2011). Only two of nine had PSWCs, and four of four had a positive CSF 14-3-3. RT-QuIC data are not available. Histopathology of the 4-OPRI is most consistent with CJD, although quite variable in the degree and region of spongiform degeneration. In addition, there are conspicuous elongated patches of PrP deposits within the molecular layer of the cerebellum that are oriented perpendicular to the surface and described as a "tigroid" pattern in some (Kaski et al. 2011) but not all (Sánchez-Valle et al. 2012) cases, perhaps explained by differences in the 129 coding, which was 129M in the former case and 129V in the latter. These were not described in the one 3-OPRI case with histopathology, which was found to display prominent spongiform degeneration with small punctate PrP plaques within the molecular layer of the cerebellum (Grasbon-Frodl et al. 2004b).

5- to 7-OPRI This group displays the greatest variability in phenotype. Several reports of 5-OPRI cases from the United States, Japan, Germany, South Africa, and the U.K. have been described (Goldfarb et al. 1991a; Cochran et al. 1996; Skworc et al. 1999; Beck et al. 2005; Mead et al. 2007). They are allelic with 129 M coding. The age at presentation is quite varied, but average age is 44 (range 26–63) and disease duration can be less than 1 year to more than 19 years (Takada et al. 2017; Beck et al. 2005). Clinical features include a premorbid change in personality followed by a cognitive presentation, often with ataxia and speech decline, leading to

akinetic mutism. One case presented with posterior cortical atrophy syndrome with predominate impairment of visual processing (Depaz et al. 2012). Clinical studies are limited, with MRI typically lacking DWI hyperintensities, although cerebral and cerebellar atrophy is generally present. PSWCs have been reported in only a few cases (Beck et al. 2005). Histopathology includes primarily spongiform degeneration, especially within frontal, temporal, and parietal lobes, in addition to the perpendicularly oriented tigroid pattern of PrP deposition within the molecular layer of the cerebellum, as seen in other OPRIs. Detection of a clear signal of PrP^{res} was unsuccessful (Mead et al. 2007).

The 6-OPRI variant was the first OPRI variant reported. It has been extensively studied in a large kindred from the U.K. (Owen et al. 1989; Collinge et al. 1990, 1992; Poulter et al. 1992). Disease onset is early, with a mean age of ~35 years (range 20–53) and a duration of 1–7 years, with the longest duration cases occurring in those presenting at a younger age (Mead et al. 2006). This variant is allelic with 129M and in those with the 129MM genotype, disease onset occurred 10.3 years earlier (31.4 ± 5.7 years, $n = 30$) than 129MV heterozygotes (41.7 ± 5.3 years, $n = 10$) (Mead et al. 2006), suggesting codon 129 heterozygosity is protective. The clinical phenotype, which is quite variable among affected individuals, is generally characterized by long-standing behavioral symptoms and personality disorders, including aggressive or apathetic features suggestive of frontal lobe dysfunction, prior to the development of progressive dementia. Cerebellar ataxia, myoclonus, dysarthria, pyramidal, and extrapyramidal signs are present in many. PSWCs are lacking and MRI shows diffuse atrophy, and in those in whom DWI sequences were completed, typical hyperintensities seen in CJD were not present (Takada et al. 2017; Mead et al. 2007). CSF 14-3-3 and RT-QuIC studies are not available. The pathology varies from severe spongiform degeneration to no obvious pathology, in addition to the tigroid pattern of PrP accumulations within the molecular layer of the cerebellum, in addition to occasional small cerebellar PrP plaque-like deposits (Mead et al. 2006). PrP^{res} that displays all three glycoforms, comparable to that seen in sCJD, is present and transmission of disease from brain samples to receptive Tg mice expressing human PrP^{C} (Mead et al. 2006). Other cases of 6-OPRI generally compare with the clinicopathologic phenotype of the well-studied UK kindred (Takada et al. 2017; Capellari et al. 1997; Kovács et al. 2007).

Whereas the clinicopathologic features of the 5- and 6-OPRI cases predominantly display features and histopathology consistent with a CJD phenotype, the 7-OPRI appears to promote either CJD or GSS-type pathology. Initially reported in a Dutch family, the histopathologic features were characteristic of GSS, with uni- and multi-centric PrP plaques diffusely distributed throughout the cerebrum and cerebellum (Jansen et al. 2011). In addition, PrP^{res} revealed an 8 kDa fragment from the cerebellum of one patient, characteristic of GSS. The clinical picture includes cognitive decline, psychiatric symptoms, extrapyramidal features, and apraxia that begins in the fifth to sixth decade and progress over a few years. In the two genetically tested affected members of the Dutch family, 129V was allelic with the 7-OPRI. In a family from Belgium with the 7-OPRI, but with 129M allelic to the mutation, PrP plaques were absent and the tigroid pattern of PrP deposits was

present within the cerebellum, as is observed in other CJD-like OPRI cases (Dermaut et al. 2000). The clinical phenotype also differed from the Dutch family, as it is generally described as a slowly progressive dementia beginning at a younger age (mean of ~29 years) and with an extended duration of ~10 years (Dermaut et al. 2000). A recently described de novo case of 7-OPRI allelic with 129M revealed similar histopathology as the Belgium family (Cali et al. 2020), suggesting that, as with other genetic PrD, codon 129 genotype may have an influence on the disease phenotype. Western blotting from this case revealed a PrP^{res} pattern consistent with Type 1 sCJD in most brain regions except the cerebellum, where a mixture of Type 1 and 2 were observed (Cali et al. 2020).

8- to 12-OPRI This group more consistently displays a GSS histopathologic subtype with varied clinical presentations, but often including psychiatric symptoms of mania and/or aggression followed by progressive dementia (van Gool et al. 1995; Goldfarb et al. 1992b; Laplanche et al. 1999). The 8-OPRI variant occurs with either 129M or 129V on the same allele, although a major clinical phenotype difference has not been reported. A French–Breton family reported by Goldfarb et al. (Goldfarb et al. 1992b) displayed varied clinical symptoms including cognitive decline, psychiatric and personality changes, gait ataxic, extrapyramidal symptoms, and myoclonus beginning in the fourth and fifth decade and progressing at a generally slow pace, although disease duration between 3 months and 13 years has been reported (Goldfarb et al. 1992b). In the M–E family of Laplanche et al. (1999), the mean age was 28, and disease duration was generally prolonged at 10 or more years, with a few exceptions of short duration lasting only a few months. Histopathology revealed characteristic multicentric PrP plaques in cerebellum and cerebrum, classifying this as GSS, although restricted spongiform degeneration, especially within the thalamus, was also reported (Laplanche et al. 1999). Another 8-OPRI family with early onset (mean age 37 years) and long duration (mean of 12 years) disease was initially reported as a phenocopy of Huntington's disease (Xiang et al. 1998), but later found to harbor the 8-OPRI variant (Moore et al. 2001), further emphasizing the range of disease phenotype associated with OPRIs and PrD in general.

The 9-OPRI variant, allelic with 129M, has been reported in a few families, all of which exhibit histopathologic features that support a GSS subtype (Takada et al. 2017; Duchen et al. 1993; Owen et al. 1992; Krasemann et al. 1995). Age at onset is commonly between the fifth and sixth decade and disease progresses over a 2–3-year period with progressive dementia and gait ataxia most often along with varying degrees of behavioral changes, dysarthria, apraxia, and myoclonus. Diagnostic studies are limited, although at least one case revealed cortical ribboning on the DWI MRI and 14-3-3 was detected in the CSF (Takada et al. 2017). A Tg mouse expressing PrP carrying a 9-OPRI has been shown to develop a progressive neurological disease with ataxia, but they lack obvious spongiform degeneration and carried a low level of insoluble PrP that was not transmissible to mice (Harris et al. 2003).

The largest OPRI is a 288-base pair insertion or 12-OPRI, described initially in 2011 within a single family with members from three generations, some of which

were longitudinally followed (Kumar et al. 2011; Townley et al. 2020). Affected family members exhibited symptoms in the 40s and 50s with a protracted course of ~5–10 years. The 12-OPRI is allelic with 129M. Clinical phenotype is best described as an atypical FTD-like presentation, highlighted by executive dysfunction, language impairment, and some with child-like or other personality behaviors, with slowly progressive ataxia that becomes profound late in the course. Generalized tonic–clonic seizures were evident in some affected members and the EEG revealed spike and wave discharges but no PSWCs. FDG–PET revealed frontal lobe hypometabolism and MRI revealed frontal lobe atrophy but not DWI hyperintensities. Neuropathology includes PrP-positive multicentric plaques concentrated especially in frontal and cerebellar cortex, in addition to widespread NFTs.

19.12 Non-OPRI Insertion

A 24-nucleotide insertion between codons 129 and 130 was reported in a young male who presented with night terrors at age 26 (Hinnell et al. 2011). The insertion was determined to be derived from the duplication of a segment extending between nucleotides 361 and 420, resulting in an 8-amino acid insertion (LGGLGGYV) directly following position 129. The mutation was allelic with 129V. The duplication was not present in the parents of the patient. Other clinical features included progressive memory and executive dysfunction, dysarthria, gait impairment, and behavioral changes that progressively worsened after an episode of status epilepticus at age 34 (Hinnell et al. 2011). Myoclonus was present, but restricted diffusion on MRI was not. EEG was primarily slow and without PSWCs, but multifocal epileptiform discharges were present. Pathologic examination revealed multicentric plaques and minimal spongiform degeneration, consistent with a GSS phenotype. The CSF was 14-3-3 and tau positive.

19.13 Summary

Genetic PrD can represent a diagnostic challenge to the clinician. Compared with sCJD, apart from the E200K and V210I variants, which mimic sCJD remarkably well, most variants follow a more protracted course that, because of the rarity of these diseases, will often lead to consideration of a more common neurodegenerative disease, such as AD, Parkinson's disease, FTD, or Lewy Body Dementia. A delay in diagnosis of PrD is further contributed by the generally poor rate of positive findings on the diagnostic studies that often signal sCJD, including hyperintensities on DWI sequences of the MRI, PSWCs on EEG, and positive CSF studies of 14-3-3, tau, and RT-QuIC. Although the generally younger age of onset at presentation might raise suspicion for a genetic condition, many variants display a broad range of age at onset, as with the OPRI variants, or a late age at onset, as with the V180I

variant. This feature, coupled with the varying degrees of penetrance of the variants, facilitates missing a possible genetic cause and appropriate diagnosis. As such, one could argue that routine genetic testing for *PRNP* variants should be considered for all patients who present with a neurodegenerative disease. The widespread availability and progressively lower cost for genetic testing make this a reasonable consideration.

Genetic PrDs are rare disorders of a rare disease, but the value in recognizing and understanding this group of diseases goes beyond the efficient clinical diagnosis of these currently untreatable diseases. As therapies are developed, carriers of *PRNP* variants are likely to be the initial beneficiaries as they have the potential, through early genetic testing, to be recognized far ahead of the onset of disease, when preventative therapies would be most beneficial. Gene therapies that selectively knock down or replace the mutated allele of such individuals, leaving a single copy of normal *PRNP,* would be a highly advantageous approach. Such studies are in progress and may provide an option for families affected by these rare but devastating diseases.

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Chapter 20
Glycoform-Selective Prions in Sporadic and Genetic Variably Protease-Sensitive Prionopathies

Zerui Wang, Jue Yuan, Tricia Gilliland, Maria Gerasimenko, Syed Zahid Ali Shah, and Wen-Quan Zou

Abstract Unlike other human prion diseases, including the most common Creutzfeldt–Jakob disease (CJD), variably protease-sensitive prionopathy (VPSPr) is characterized by the deposition of a unique glycoform-selective pathogenic prion protein (PrP^{Sc}) in the brain. The proteinase K (PK) sensitivity of PrP^{Sc} from VPSPr is highly variable and appears to be mediated by PrP-129 polymorphisms. Its PK-resistant PrP^{Sc} (PrP^{res}) consists of a pathognostic ladder-like electrophoretic gel profile consisting of two sets of multiple N- and C-terminally truncated fragments. Formation of this distinctive PrP^{Sc} is glycoform-selective, by which only two out of four normal PrP glycoforms (PrP^{C}) convert to PrP^{Sc}. Strikingly, all the PrP^{Sc} features of sporadic VPSPr are shared by a genetic prion disease linked to the valine to isoleucine mutation at residue 180 of PrP (PrP^{V180I}), previously defined as atypical genetic CJD. This chapter highlights the features of VPSPr PrP^{Sc} and provides evidence supporting the hypothesis that the condition with PrP^{V180I} is the first genetic form of VPSPr.

Keywords Prions · Prion disease · Prion protein · Creutzfeldt · Jakob disease (CJD) · Variably protease-sensitive prionopathy (VPSPr) · Gerstmann · Sträussler · Scheinker (GSS) · Mutation · Proteinase K (PK) · Glycosylation · Glycoform-selective prion formation · Transmissibility · RT-QuIC · PMCA

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20.1 Introduction

Prion diseases are a group of remarkable diseases; they are highly heterogeneous with three different etiologies, including sporadic, genetic, and acquired through transmission from individuals to individuals, affecting both animals and humans. They include scrapie in sheep and goats, mad cow disease or bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer, elk, moose and reindeer, as well as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), and fatal insomnia in humans. They are all associated with the deposition and accumulation of a single infectious misfolded prion protein pathogen termed PrP^{Sc} in the brain. PrP^{Sc} is derived from its normal cellular prion protein (PrP^{C}) through a conformational transition from α-helixes to β-sheet structures.

PrP^{C} is a membrane protein expressed mainly in the central nervous system (CNS) but also to lesser extent in the peripheral tissues and organs. Different topological forms have been observed including the major one that is attached onto the cell surface through the glycosylphosphatidylinositol (GPI) anchor (Stahl et al. 1987; Lehmann and Harris 1995) and less common transmembrane forms that span the various cell membranes (Hegde et al. 1998; Hay et al. 1987; De Fea et al. 1994; Stewart and Harris 2001; Faris et al. 2017). The PrP^{C} molecule carries two N-linked glycosylation sites. Four PrP^{C} glycoforms can be discerned from the human brain samples by western blotting (WB) with anti-PrP antibodies including diglycosylated at both residue 181 (N181) and 197 (N197), monoglycosylated at residue 181 or 197, and un-glycosylated, which hereafter are termed $PrP^{N181\text{-}197}$, PrP^{N181}, PrP^{N197}, and PrP^{Un}, respectively (Fig. 20.1). In virtually all sporadic and acquired CJD and most genetic CJD, the four PrP^{C} glycoforms convert into their related PrP^{Sc} conformers. They form proteinase K (PK)-resistant PrP fragments (PrP^{res}) upon treatment of prion-infected brain homogenate with PK by removing part of N-terminus of the protein in vitro.

The PrP^{res} is the biomarker for molecular diagnosis of various human and animal prion diseases. WB analysis of PrP^{res} from brain homogenates probing with 3F4, the most widely used anti-PrP antibody, has become the conventional method of diagnosis and typing in the routine surveillance and research of human prion diseases worldwide. A definite diagnosis of CJD can be made based on the typical four PrP^{res} bands from the PK-digested four PrP^{Sc} migrating at between 19 and 30 kDa on electrophoretic gels. Moreover, two types of PrP^{Sc} have been identified in the most common sporadic CJD (sCJD) based on the N-terminal PK-cutting sites and electrophoretic gel mobilities of PrP^{Un}:PrP^{Sc} type 1 is of the major N-terminal cutting site at residue 82 with a gel mobility of PrP^{Un} at 21 kDa, while the major N-terminal cutting site of PrP^{Sc} type 2 is at residue 97 and its PrP^{Un} migrates at ~19 kDa (Parchi et al. 1996, 1999, 2000; Gambetti and Cali 2022) (Fig. 20.1). Combination of both typing of PrP^{Sc} and PrP-129 methionine (M)/valine (V)

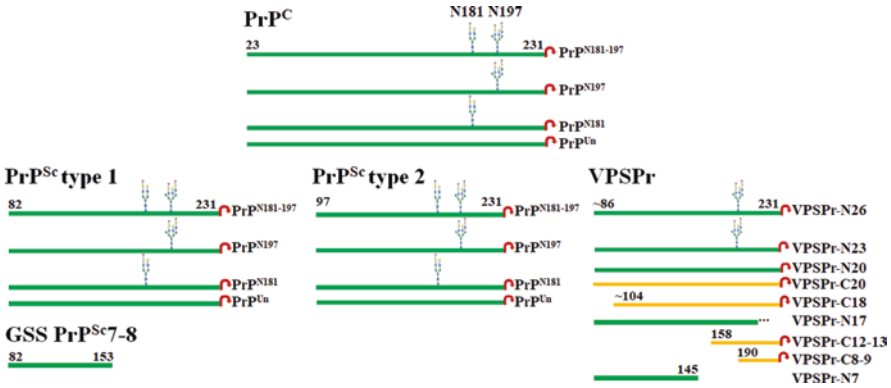

Fig. 20.1 Diagram of PrP^{C} glycoforms and various PK-resistant PrP^{Sc} fragments. The mature full-length PrP^{C} is composed of amino acids from the first N-terminal residue 23 to the last C-terminal residue 231 that links to a GPI anchor (a red arrow U-turn). There are two N-linked glycosylation sites at residues 181 (N181) and 197 (N197) carrying glycans. $PrP^{N181–197}$: PrP glycoform carrying two N-linked glycans at residues 181 and 197. PrP^{N197}: PrP glycoform carrying one of the two N-linked glycans at residue 197. PrP^{N181}: PrP glycoform carrying one of the two N-linked glycans at residue 181. PrP^{Un}: PrP carrying no N-linked glycans. PrP^{Sc} type 1: PK-resistant sCJD PrP^{Sc} type 1 with a main PK cleavage site at N-terminal residue 82, detected by N-terminal sequencing (Parchi et al. 2000), having four detectable glycoforms. PrP^{Sc} type 2: PK-resistant sCJD PrP^{Sc} type 2 with a primary PK cleavage site at N-terminal residue 97, determined by N-terminal sequencing (Parchi et al. 2000), having four detectable glycoforms. VPSPr-PrP^{Sc}: PK-resistant VPSPr PrP^{Sc} with a main PK cleavage site at N-terminal residue 86, determined by antibody epitope mapping (Yuan et al. 2008; Zhang et al. 2021; Zou et al. 2010a, 2013), linked to five PK-resistant N-terminal VPSPr PrP^{Sc} fragments including VPSPr-N26, -N23, -N20, -N17 and -N7 (green thin bars) detected by anti-PrP antibodies 1E4 and Tohoku 2 (Gambetti et al. 2008; Xiao et al. 2013; Zhang et al. 2021; Zou et al. 2010b, 2013) or linked to four PK-resistant C-terminal VPSPr PrP^{Sc} fragments including VPSPr-C20, -C18, -C12–13, and -C8-9 (yellow thin bars) detected by anti-PrP antibodies anti-C and EP1802Y (Zhang et al. 2021; Zou et al. 2010b). GSS PrP^{Sc}7–8: PK-resistant PrP^{Sc} fragment migrating at 7–8 kDa with N- and C-terminal PK cleavage sites at residues 82 and 153, respectively (Parchi et al. 1998, 2000; Piccardo et al. 1995, 1996, 2001; Tagliavini et al. 2001)

polymorphism has identified nine subtypes of sCJD (Gambetti and Cali 2022). In addition, a 7–8 kDa PrP^{res} band is the molecular signature for most of GSS with different PrP mutations (Parchi et al. 1998, 2000; Piccardo et al. 1995, 1996, 2001; Tagliavini et al. 2001) (Fig. 20.1). Moreover, the detection and characterization of the brain PrP^{Sc} are also used for screening new and atypical prion diseases. For instance, by comparing the gel profiles of the PrP^{res} glycoforms among prion-infected human, cow, and mouse models, PrP^{Sc} from mad cow disease or BSE has been confirmed to link to the variant CJD, a human prion disease derived from consumption of BSE-contaminated foods (Will et al. 1996; Bruce et al. 1997; Hill et al. 1997; Brown 2022). Through the screening work, different PrP^{Sc} molecules identified in single individual atypical cases were also reported from time to time (Chasseigneaux et al. 2006; Giaccone et al. 2007; Zanusso et al. 2007).

20.2 Identification of Sporadic Variably Protease-Sensitive Prionopathy

20.2.1 Dominant PK-Sensitive PrP^{Sc}

The identification of the variably protease-sensitive prionopathy (VPSPr) has depended on our understanding of the unique features of its pathogenic PrP^{Sc}, which was also reflected in the changes in the name of this novel disease. The first striking feature that we noticed was that no PrP^{res} was detectable in the brain by conventional WB probing with the 3F4 antibody, although minor spongiform degeneration and PrP immunostaining were observed in all 11 cases homozygous for PrP-129 VV polymorphism that were examined (Gambetti et al. 2008). The 3F4 antibody has an epitope containing residues 106–112 (Zou et al. 2010a). It detected PrP^{Sc} in these cases only after enrichment of PrP^{Sc} with gene 5 protein (g5p, a single-stranded DNA-binding protein) and sodium phosphotungstate (NaPTA) that capture misfolded PrP aggregates but not normal PrP^{C} (Gambetti et al. 2008; Zou et al. 2010a, 2004; Wadsworth et al. 2001) or by ultracentrifugation in detergent buffers (Zou et al. 2010a; Zhang et al. 2021). More than 70% of the misfolded PrP aggregates captured by g5p from these cases can be sensitive to PK-digestion, whereas only about 10% of captured PrP^{Sc} was PK-sensitive in classic sCJD (Gambetti et al. 2008). We also noticed that the amounts of PrP^{res} were variable within different brain areas in individual cases. For instance, neocortex areas exhibited the lowest or undetectable PrP^{res} by 3F4 compared to the subcortical regions, such as substantia nigra, putamen, and thalamus (Gambetti et al. 2008). As a result, we initially termed this new condition as the protease-sensitive prionopathy (PSPr) after examination of PrP^{Sc} limited to the 11 PrP–129 VV homozygotes (Gambetti et al. 2008).

20.2.2 Unique Multiple Ladder-Like Electrophoretic Gel Profile of PK-Resistant PrP^{Sc}

Meanwhile, we also identified its second striking feature. As mentioned above, the 3F4-based conventional WB reveals that almost all PrP^{Sc} in the neocortex areas of PSPr cases seems to be PK-sensitive. When we used another anti-PrP antibody termed 1E4, however, multiple intense PrP^{res} bands surprisingly come to light; these previously unrecognized hidden PrP^{res} fragments consist of five pathognostic ladder-like PrP^{res} bands migrating at ~26 kDa, ~23 kDa, ~20 kDa, ~17 kDa, and ~6–7 kDa, while this antibody detects the typical 3 PrP^{res} bands in sCJD (Gambetti et al. 2008). Of the five ladder-like electrophoretic PrP^{res} fragments, only the 26 kDa and 20 kDa fragments match the monoglycosylated and unglycosylated PrP^{res} in sCJD. The 6–7 kDa fragment is unglycosylated, reminiscent of the 7 kDa fragment of GSS. These fragments are most obvious at PK concentrations of 5–10 μg/mL and decrease upon increases in PK concentrations. The previously unknown multiple

ladder-like PrP^{res} bands detected by 1E4 were highly reproducible and were observed in all 11 PSPr cases examined. The finding with 1E4 has turned out to be the most striking feature and pathognostic for this novel disease after our subsequent full characterization of its PrP^{Sc} along with other genotypes linked to PrP-129MV and 129MM (Xiao et al. 2013; Zou et al. 2010b, 2013) (see more below).

Notably, it was also the 1E4 antibody that made the insoluble PK-resistant PrP^{C} aggregates (termed $iPrP^{C}$) in uninfected human and animal brain tissues come to light (Yuan et al. 2006). Like PrP^{res} in PSPr, the $iPrP^{C}$ molecule is mainly detectable by 1E4 but not 3F4 (Yuan et al. 2006). We also examined the PrP^{Sc} from PSPr cases with this antibody, because it happened that we were examining the epitope of 1E4 and comparing its immunoreactivity features with various human PrP molecules derived from *E. coli*, cultured cells and brain tissues with 3F4 (Yuan et al. 2008). Using epitope mapping by PrP peptide membrane arrays, we uncovered that 1E4 has an epitope between PrP residues 98 and 105 (Yuan et al. 2008). It is exactly next to the epitope of 3F4 from residues 106 to 112 (Zou et al. 2010a). Interestingly, we observed that in general, 1E4 exhibits a higher affinity for the PrP truncated at residues 86–90 than for the full-length PrP species, whereas 3F4 is opposite, with a higher affinity for the full length than for the truncated PrP species (Yuan et al. 2008). Moreover, this phenomenon can be clearly obvious when it reacts with the protein from brains and cultured cells. To compare the affinities of the two antibodies for the two protein species, we calculated the ratio of the truncated to the full-length PrP intensity on the western blots. Based on this ratio, we revealed that 1E4 exhibits 1.3-fold higher affinity than 3F4 for the truncated recombinant PrP. 1E4 has 4.1-fold higher affinity than 3F4 for the deglycosylated PrP species from human brain tissues and is 10.2-fold higher than 3F4 for the deglycosylated PrP species from cultured cells (Yuan et al. 2008). We also observed that 1E4 has higher affinity for the sCJD PrP^{Sc} type 2 and lower affinity for PrP^{Sc} type 1 compared to 3F4 (Yuan et al. 2008). Since we did not see significant effect of residues 93–96 on the 1E4 epitope PrP98-105 accessibility using the peptide membrane arrays, the weak affinity of 1E4 for PrP^{Sc} type 1 may suggest that the first 11 residues 82–92 from the N-terminal PrP^{Sc} type 1 may partially block the accessibility of 1E4 to its epitope.

20.2.3 PrP-129 Polymorphism-Dependent Variable PK-Resistant PrP^{Sc}

The birth of the current name VPSPr took place in a subsequent study in which we identified cases with other two PrP-129 genotypes and discovered that the levels of PK-resistance of PrP^{Sc} are a function of its PrP-129 polymorphism (Zou et al. 2010b). The study compared and characterized all three different PrP-129 polymorphisms, including PrP-129MM and PrP-129MV, along with additional new PrP-129VV cases (Zou et al. 2010b). We revealed that the electrophoretic gel profile of the ladder-like multiple PrP^{res} fragments detected by 1E4 in VV cases is shared by subjects carrying the 129MM and 129MV genotypes. In other words, upon

PK-treatment, they all exhibit five major PrP bands migrating at approximately 26 kDa, 23 kDa, 20 kDa, 17 kDa, and 6–7 kDa that we termed VPSPr26, VPSPr23, VPSPr20, VPSPr17, and VPSPr7, respectively (Fig. 20.1). Notably, more PK-resistant PrP^{res} was found in 129MM and 129MV than in 129VV cases. It appears that the PrP-129 M allele prefers to form the PK-resistant PrP^{Sc}, while the PrP-129 V allele favors to form the PK-sensitive PrP^{Sc}. For instance, both 1E4 and 3F4 revealed the relative highest PK resistance of PrP^{Sc} in the 129MM cases, followed by 129MV cases, and low or even undetectable in the 129VV cases. The PrP-129 polymorphism-dependent PrP^{res} is considered as the third feature of PrP^{Sc} in VPSPr. As a result, we changed the name of this novel disease from the initial PSPr (Gambetti et al. 2008) to current name VPSPr (Zou et al. 2010b) to faithfully reflect the nature of variable amounts of PrP^{Sc} for its three genotypes.

Of the five PrP^{res} fragments, the PK-resistance of the VPSPr7 fragment is highly stable and consistent. It can be detected until 100 μg/mL of PK among the three genotypes. In contrast, the other four fragments appear to follow two distinct patterns, which were similar and involved pairs of the same fragments in both 129MV and 129MM. The intensity of both VPSPr26 and VPSPr20 decreases upon increased PK concentrations. The intensity of PSPr23 and PSPr17 first enhances upon increases in PK concentrations until 25 μg/mL of PK, then peaks between 25 and 50 μg/mL of PK, and finally declines at 100 μg/mL of PK. As expected, the PK resistance of the 129VV fragments is much lower compared to 129MV and 129MM, except for VPSPr7 (Zou et al. 2010b). Based on these observations and our recent study, it is expected that VPSPr23 derives from C-terminal truncation of VPSPr26, VPSPr17 from C-terminal truncation of VPSPr20, and VPSPr7 probably from C-terminal truncation of both VPSPr20 and VPSPr17. Indeed, the intensity of VPSPr26 and VPSPr20 is decreased, while there is an increase in the intensity of VPSPr23, 17, and 7 over increasing PK concentrations until 50 μg/mL (Zhang et al. 2021). PNGase F treatment after PK-digestion shows that VPSPr20, 17, and 7 are the core PrP^{res}, which we termed VPSPr-N20, VPSPr-N17, and VPSPr-N7, respectively (Zhang et al. 2021), while VPSPr26 and VPSPr23 are the glycosylated VPSPr-N20 and VPSPr-N17, respectively (Zhang et al. 2021; Zou et al. 2010b). Moreover, the intensity of VPSPr20 decreases, while the intensity of VPSPr17 and 7 is increased over an increase in the PK concentrations until 200 μg/mL (Zhang et al. 2021). All these results indicate that the generation of the peculiar ladder-like electrophoretic profile of PrP^{res} is a PK dose-dependent two-step process, which is boosted by basic pH, such as pH 8.0 (Zhang et al. 2021), suggesting that PrP^{Sc} from VPSPr has a confirmation that has never been previously observed in any sporadic human prion diseases.

20.2.4 Two Sets of PK-Resistant PrP^{Sc} Core Fragments

Further characterization of the core fragments with the antibody anti-C, whose epitope is in C-terminal PrP 220–231, demonstrated 4 PrP bands migrating at approximately 20 kDa, 18 kDa, 12–13 kDa, and 8 kDa, which we termed VPSPr-C20,

VPSPr-C18, VPSPr-C12/13, and VPSPr-C8-9, respectively (Fig. 20.1). Of the four anti-C detected PrP^{res}, only the VPSPr-C20 kDa band matched VPSPr-N20 detected with 1E4 in three genotypes and 3F4 in PrP-129MV and PrP-129MM genotypes. This suggests that the two could be the same fragment with both epitopes. The other three fragments undetected by 1E4 and 3F4 must comprise the C-terminal region (reactive with anti-C) lacking the 97–112 sequence encompassing the 1E4 and 3F4 epitopes. Therefore, a total of six core fragments migrating between 20 kDa and 7 kDa were identified by the combination of 1E4 and anti-C. Our study also revealed considerable similarities in the electrophoretic mobilities of PrP^{res} between VPSPr and GSS linked to A117V mutation of PrP (GSS^{A117V}), especially on 1E4 and anti-C blots. The PrP^{res} fragments appear to have similar sizes but different ratios and antibody reactivities between the two conditions. Apparently, 3F4 does not show the 20 kDa band, whereas 1E4 detects an additional band migrating at 22–23 kDa in GSS^{A117V} (Zou et al. 2010b). Moreover, our recent study further identified two additional antibodies that are able to detect the two sets of PrP^{res} fragments from VPSPr. A polyclonal antibody termed Tohoku 2 (T2) with an epitope localized between PrP97-103 possesses 1E4-like affinity to recognize VPSPr-N26, -N23, -N20, -N17, and -N7, whereas a monoclonal antibody termed EP1802Y with an epitope between PrP217-226 exhibits anti-C-like ability to detect VPSPr-C20, -C18, -C12/13, and -C8 (Zhang et al. 2021).

20.2.5 Glycoform-Selective PrP^{Sc} Formation

To determine the molecular mechanism underlying formation of the unique PrP^{Sc} lacking diglycosylated PrP^{res} in VPSPr, we examined the composition of PK-sensitive and -resistant PrP^{Sc} by WB probing with antibodies directed against PrP glycoforms termed V14 and Bar209 (Xiao et al. 2013). It has been shown that both V14 and Bar209 recognize unglycosylated PrP; specifically, V14 identifies PrP with the first glycosylation site (PrP168–181, mono181), while Bar209 uncovers PrP with the second glycosylation site (PrP185–196, mono197) (Moudjou et al. 2004; Eghiaian et al. 2004; Féraudet et al. 2005). Therefore, the two glycoforms-specific antibodies helped us address the question why PrP^{Sc} from VPSPr lacks the diglycosylated form. We first isolated total PrP^{Sc} using the gene 5 protein (g5p) that is able to specifically capture both PK-sensitive and -resistant PrP^{Sc} from prion-infected brain homogenates (Gambetti et al. 2008; Zou et al. 2004; Yuan et al. 2006). The g5p-captured PrP^{Sc} was either untreated or treated with PK prior to WB for determining whether both mono181 and mono197 convert into PK-sensitive and -resistant PrP^{Sc} or not.

In samples without PK-treatment, Bar209 displays 3 PrP bands, corresponding to the full-length monoglycosylated at residue 197 (mono197) and unglycosylated PrP, as well as the endogenously N-terminally truncated PrP fragment called C2. After PK-treatment, it detects the PK-resistant mono197 and unglycosylated PrP. The findings are similar to what we observed from brain homogenates of sCJD,

suggesting that like sCJD, VPSPr has the PK-sensitive and PK-resistant mono197 and unglycosylated PrP^{Sc} (Xiao et al. 2013). When probed with the V14 antibody, the full-length monoglycosylated PrP at residue 181 (mono181) and unglycosylated PrP as well as C2 are detected in the sCJD samples without PK-treatment. The PK-resistant mono181 and unglycosylated PrP are observed in PK-treated sCJD samples as well. These results suggest that mono181 PrP can convert into PK-sensitive and -resistant PrP^{Sc} in sCJD. In contrast, mono181 is not detectable in either untreated or PK-treated samples from VPSPr with V14. Therefore, different from sCJD, neither PK-resistant nor PK-sensitive PrP^{Sc} glycosylated at residue 181 is detectable, suggesting that mono181 does not convert into PrP^{Sc} in VPSPr. Therefore, VPSPr seems to have a PrP^{Sc} formation pathway different from sCJD, by which only two out of four PrP glycoforms convert from PrP^{C} into PrP^{Sc}. Specifically, only monoglycosylated PrP at residue 197 and non-glycosylated PrP convert into PrP^{Sc}, whereas diglycosylated and monoglycosylated PrP at residue 181 do not (Xiao et al. 2013). This PrP formation in VPSPr is different from that in sCJD and most of gCJD. The latter two conditions involve conversion of all four PrP^{C} glycoforms into PrP^{Sc} (Xiao et al. 2013). The glycoform-selective PrP^{Sc} can be considered as the fourth feature of PrP^{Sc} of VPSPr.

20.3 Identification of Inherited Variably Protease-Sensitive Prionopathy

We further demonstrated that the unique PrP^{res} gel profile from sporadic VPSPr is shared by a condition previously recognized as an atypical genetic CJD linked to PrP valine-to-isoleucine mutation at residue 180 ($gCJD^{V180I}$) (Xiao et al. 2013). This mutation was first identified in a Japanese patient (Kitamoto et al. 1993). It is the most common form of genetic prion disease in Japan (Qina et al. 2014). The clinical history, pathological changes, and PrP^{Sc} features of $gCJD^{V180I}$ are different from those of sCJD and other gCJD, such as $gCJD^{E200K}$, the most common genetic form in Caucasians (Chasseigneaux et al. 2006; Kitamoto et al. 1993; Qina et al. 2014; Hitoshi et al. 1993; Kong et al. 2004; Mastrianni 2022). Hitoshi et al. first reported an atypical PrP^{res} that lacks the diglycosylated PrP^{Sc} fragment in a Japanese patient linked to two mutations of V180I and M232R of *PRNP* (Hitoshi et al. 1993). Later, Chasseigneaux et al. identified a French patient with V180 mutation alone, in which the atypical PrP^{Sc} lacking the diglycosylated PrP^{res} was also detected in different brain areas, confirming that this unique PrP^{Sc} glycosylation is most likely to be associated only with PrP^{V180I} mutation (Chasseigneaux et al. 2006).

We characterized PrP^{Sc} from autopsy brain tissues of six $gCJD^{V180I}$ cases confirmed neuropathologically, including three Caucasian and three Asian patients from the USA, France, and Japan (Xiao et al. 2013). When detected with WB probed with 3F4, PrP^{Sc} from all six $gCJD^{V180I}$ cases treated with different amounts of PK exhibited two bands corresponding to mono- and un-glycosylated PrP^{res} but no

diglycosylated PrP^{res}, the same as VPSPr-129MV and VPSPr-129MM (Xiao et al. 2013). Remarkably, like VPSPr, all cases exhibited the five bands producing a ladder-like electrophoretic profile when probed with 1E4, including a predominant 1E4-preferentially detectable 7 kDa fragment. Moreover, PrP^{Sc} from $gCJD^{V180I}$ revealed a higher affinity for 1E4 than for 3F4, characteristic of PrP^{Sc} first identified in VPSPr (Gambetti et al. 2008; Zou et al. 2010b). When the PK-treated PrP^{Sc} from the two diseases is compared side by side on the gel, they exhibit virtually identical electrophoretic gel migration patterns that are different from those of sCJD.

Using the antibodies directed against glycoforms, we revealed that cases with PrP^{V180I} mutation share the same glycoform-selective PrP^{Sc} formation with VPSPr (Xiao et al. 2013). As mentioned above, we first isolated PrP^{Sc} from the brain of $gCJD^{V180I}$ cases with g5p beads, and then, the captured PrP^{Sc} was treated with or without PK prior to WB with V14 and Bar209 (Zou et al. 2004, 2010b; Xiao et al. 2013; Yuan et al. 2006). Like VPSPr and sCJD, the Bar209 antibody is able to detect the g5p-captured PrP^{Sc} monoglycosylated at residue 197 and unglycosylated PrP before and after PK-treatment, suggesting that PrP^{Sc} from $gCJD^{V180I}$ cases derives from mono197 and unglycosylated PrP. The V14 antibody showed both mono181 and unglycosylated PrP species before and after PK-treatment of brain homogenates of sCJD cases. In contrast, it detected only unglycosylated PrP^{Sc} but not mono181 before and after PK-treatment of brain homogenates from the 180 mutation cases (Xiao et al. 2013), signifying that mono181 does not convert into PrP^{Sc} in the cases with $gCJD^{V180I}$.

The molecular event underlying the unique glycoform-selective PrP^{Sc} formation currently remains unknown. There are some notable indications that may improve our understanding of this mystery. For instance, in contrast to $gCJD^{T183A}$, both VPSPr and $gCJD^{V180I}$ have two detectable N-linked glycosylation sites prior to PK-digestion. Moreover, like wild-type PrP, PrP^{V180I} in cultured cells exhibited a typical glycoform profile and generated detectable typical PrP^{res} with diglycosylated and monoglycosylated PrP form upon PK-treatment, although they were detectable only with 1E4 but not with 3F4 (Xiao et al. 2013). PrP^{V180I} mutation itself does not eliminate any glycosylation sites. Interestingly, the N-linked glycosylation prediction algorithm revealed a decrease in the glycosylation potential value for the first glycosylation site (Xiao et al. 2013), a possible indication that PrP^{V180I} alters the composition of glycans at the first site. In addition, both diglycosylated and monoglycosylated PrP molecules carrying mono181 are not converted into PK-resistant PrP in $gCJD^{V180I}$, which is not seen in cultured cells but only in the brain, where there is an additional wild-type allele. Finally, binding of the lectin ricinus communis agglutinin I (RCA-I) to monoglycosylated PrP decreased, while its binding to diglycosylated PrP increased in VPSPr and $gCJD^{V180I}$ compared to sCJD (Xiao et al. 2013), suggesting that the two diseases have a changed composition of glycans. We have proposed that glycoform-selective PrP^{Sc} formation observed in the brain involves dominant-negative inhibition caused by the interaction between misfolded and normal PrP molecules. The changed glycan composition at the first site by the mutation may alter local conformation around residue 181. This area is close to the β-sheets 2/α-helixes 2 loop; the critical region implicated in dominant-negative

inhibition (Cong et al. 2013). Indeed, there seems to be significant differences in the effect of mutations occurring at either the first or the second N-linked glycosylation site on the conversion of PrP^{C} into PrP^{Sc}. For instance, mutations at the first site often block the conversion of PrP^{C} into PrP^{Sc}, but virtually, all mutations at the second site do not in cell and animal models (Salamat et al. 2011; Tuzi et al. 2008). Interactions between different PrP^{C} glycoforms mediate the efficiency of prion formation, involving glycan-associated steric hindrance (Nishina et al. 2006). While there are no PrP mutations that have been found in VPSPr, the possibility cannot be excluded that a similar aberrant glycosylation at N181 caused by a rare stochastic event triggers the processes as described for $gCJD^{V180I}$ (Xiao et al. 2013).

Different from cases with PrP^{V180I} mutation, VPSPr is sporadic without any identified PrP mutations. Nevertheless, it is possible that an unknown co-factor involved in altering N-linked glycosylation at N181 prevents conversion of diglycosylated PrP and mono181 into PrP^{Sc}. For instance, some of reported VPSPr cases showed a familial history of dementia (Zou et al. 2013; Jansen et al. 2010). Indeed, co-factors have also been previously proposed to play a role in the pathogenesis of prion diseases, including protein X and non-proteinaceous cofactors (Telling et al. 1995; Supattapone and Miller 2022; Ma 2022). Whether protein X that was initially suggested to directly interact with PrP^{C} is necessary for prion formation remains controversial (Supattapone and Miller 2022; Colby and Prusiner 2011). However, genes or proteins that could indirectly initiate the conversion of PrP^{C} into PrP^{Sc} may exist. The possibility cannot be ruled out that a mutation in a non-PrP gene that participates in regulating PrP glycosylation at N181 causes VPSPr. If this is the case, further investigation of VPSPr may provide an opportunity to find out the possible existence of such a co-factor.

The condition linked to this mutation has been previously considered as a genetic CJD ($gCJD^{V180I}$) (Qina et al. 2014). It has been noticed that patients with the mutation often show no family history, a late onset, and a longer disease duration, different from most of gCJD with other mutations (Qina et al. 2014; Kong et al. 2004; Mastrianni 2022). Compared to classic sCJDMM1, patients with this mutation were found to have a lower possibility of developing myoclonus, cerebellar, pyramidal signs, and visual disturbance. Patients often have cognitive impairment, which is similar to that of sporadic VPSPr (Gambetti et al. 2008; Zou et al. 2010b; Qina et al. 2014; Notari et al. 2018). Diffuse hyperintensity of the cerebral cortex in diffusion-weighted MRI is believed to be helpful for diagnosis (Qina et al. 2014). Because of similarity in PrP^{Sc} gel profile, clinical manifestations and course, lab tests and imaging study, we propose that $gCJD^{V180I}$ is the inherited form of VPSPr.

20.4 Transmissibility of Sporadic and Genetic VPSPr

Prion diseases have been widely known to be transmissible, but there are some prion diseases that may not be transmissible (Zou and Gambetti 2004; Zou 2007). As reflected in its original name, transmissible spongiform encephalopathy (TSE) is

characterized by the two major characteristics: transmissibility and spongiform degeneration in the CNS. However, inconsistency can be seen in that some prion diseases lack one or two characteristics of TSE. It has been reported that approximately 10% of sCJD and 32% of genetic prion diseases are not transmissible in nonhuman primates (Brown et al. 1994). In addition, all GSS, except for 1/3 of GSS^{P102L} cases, are difficult to transmit to experimental animals (Tateishi et al. 1996). Moreover, the spongiform degeneration is not always detectable in all GSS^{P102L}, though deposits of PrP^{Sc} plus PrP-amyloid plaques are observed in the CNS (Parchi et al. 1998). In the GSS P102L animal model inoculated with GSS free of spongiform degeneration, neither symptoms nor spongiform degeneration was detected despite the presence of PrP-amyloid (Piccardo et al. 2007).

So far, there are three published studies that have investigated the transmissibility of VPSPr using animal models (Notari et al. 2014; Diack et al. 2014; Nonno et al. 2019). In the study by Notari and co-workers, brain samples from 12 subjects covering three genotypes of VPSPr were examined by intracerebral inoculation of two lines of humanized Tg mice expressing human PrP-129MM or PrP-129VV equivalent to 1–8 fold normal human brain PrP levels (Notari et al. 2014). All mice inoculated showed no prion-related clinical signs, although 54% of them revealed neuropathologic changes and 34% were found to have abnormal PK-resistant PrP with the gel profile similar to that of VPSPr. Those neuropathological changes included clustered and poorly structured plaques observed at the border of the hippocampus that also were found in the white matter, and occasionally in periventricular regions (Notari et al. 2014). Local spongiform degeneration was detectable in the lacunosum-molecular layer of the hippocampal formation. No widespread PrP staining was detectable, while plaques and spongiform degeneration areas could react intensely with the PrP antibodies (Notari et al. 2018). WB of brain homogenates of inoculated Tg mice displayed the multiple ladder-like PK-resistant PrP^{Sc}, especially with the three core fragments similar to that found in VPSPr. Nevertheless, the Tg mice upon second passage inoculated with the mouse brain homogenates from the first passage stayed healthy for their entire lifespans without detectable neuropathological changes and PK-resistant PrP^{Sc} (Notari et al. 2014).

Diack and co-workers also examined the transmissibility of three subjects with VPSPr including two homozygous for PrP-129VV [one from the Netherlands (NL-VV) and the other from UK (UK-VV)] and a heterozygous for PrP-129MV from UK (UK-MV) with three lines of Tg mice expressing human PrP either with 129MM, 129MV, or 129VV by intracerebral inoculation (Diack et al. 2014). Similar to our study (Notari et al. 2014), they observed no clinical signs in all inoculated mice. Neither spongiform degeneration by hematoxylin and eosin staining nor PK-resistant PrP^{Sc} by WB in their mice was detectable. By immunohistochemistry, no PrP^{Sc} staining was observed in the brain of Tg mice inoculated with UK-MV. PrP^{Sc} staining was mainly detected in a small number of Tg mice expressing human PrP-129VV or -129MV inoculated with UK-VV and NL-VV. No Tg mice expressing PrP-129MM were found to have PrP^{Sc}, except for one inoculated with UKVV (Diack et al. 2014). Numerous small focal plaque-like deposits were located within the corpus callosum, the stratum oriens, stratum lacunosum moleculare of the

hippocampus, and parallel to the lateral ventricle (Diack et al. 2014). Thioflavin-S staining confirmed that they were composed of amyloid. Their study concluded that VPSPr is capable of transmission to humanized Tg mice at extremely low levels, suggesting that it is a disease that has biological properties with a limited but not negligible infectivity, distinct from those of sCJD. Moreover, based on this study, it seemed that the transmissibility of VPSPr-129VV is highest, followed by VPSPr-129MV, and VPSPr-129MM as lowest.

Nonno et al. examined transmissibility of subjects with three different genotypes of VPSPr using bank voles with both PrP-109I (Bv109I) and PrP-109 M (Bv109M) (Nonno et al. 2019), small rodents that have been shown to be highly susceptible to many prion diseases including various human prion diseases. In Bv109I, the attack rate of VPSPr-MV was 15%, slightly higher than those in VPSPr-MM (12%) and VPSPr-VV (11%), which is different from the observation found in humanized Tg mice indicated above (Diack et al. 2014). In contrast to the finding observed in inoculated humanized Tg mice with no transmission in the second passage (Notari et al. 2014), all three genotypes of VPSPr had a 100% attack rate in the second passage. In Bv109M, both VPSPr-MM and -MV had the same attack rate (~7%), lower than that in Bv190I, while VPSPr-VV was not transmissible (0/14) in the first passage. For the second passage, the attack rates of VPSPr-MM and -MV became 100%, while no attack rate data were available for VPSPr-VV. In addition, three different phenotypes of neuropathological changes and PK-resistant PrP^{Sc} profiles were identified in Bv109I inoculated with three genotypes of VPSPr inocula called T1, T2 and T3, while T1 was only detected in Bv109M animals. Notably, among Bv109I inoculated with three genotypes of VPSPr, respectively, VPSPr-MV generated T1 and T2 phenotypes, while VPSPr-MM generated T1 and T3. It seemed that two VPSPr-VV cases were examined but only one of them had the western blot result that exhibited T3 type of PrP^{Sc} (Nonno et al. 2019). The lesion profiles of spongiform degeneration severity among the three phenotypes in Bv109I were different from each other, while T1 was more like sCJDMM1 and T2-like GSS. T2 exhibited spongiform degeneration most in subcortical structures and PrP^{Sc} staining showed granular deposits with occasional mini plaque-like formation in several anatomic regions, such as neocortex, hippocampus, thalamus, and superior colliculi (Nonno et al. 2019). WB of brain homogenates revealed three types of PK-resistant PrP^{Sc}; T1 and T2 were similar to the gel mobilities of sCJD PrP^{Sc} types 1 and 2, while T3 was of GSS-like PrP^{Sc} gel mobility with an intense 7 kDa band, although no VPSPr-like PK-resistant PrP^{Sc} was duplicated in the infected bank voles (Nonno et al. 2019). This study indicated that VPSPr is a transmissible prion disease despite lower transmissibility compared to sCJD. Since it was unable to faithfully duplicate the features of the PK-resistant PrP^{Sc} of VPSPr, bank voles may not be the best animal models for VPSPr.

To date, only one published study can be found about the transmissibility of cases with PrP^{V180I} mutation (Tateishi and Kitamoto 1995). In this study, brain homogenates from three cases with PrP^{V180I} were inoculated intracerebrally into New Zealand white mice. Inoculated mice showed no prion-related clinical signs during their lifespans and mice sacrificed after more than 700 days were negative for PrP^{Sc} (Tateishi and Kitamoto 1995).

20.5 PrP^{Sc} Seeding Activity of Sporadic and Inherited VPSPr

Prion seeding activity is another feature of the infectious PrP^{Sc}, which has been widely evaluated in vitro by amplification assays, including protein misfolding cyclic amplification (PMCA)(Moda et al. 2022) and real-time quaking-induced conversion (RT-QuIC) (Orrù et al. 2022). Peden and co-workers conducted PMCA assay of brain tissues from the frontal cortex and cerebellum of two VPSPr-VV cases using either human brain or humanized Tg mouse brain homogenates as substrates (Peden et al. 2014). They revealed that the PK-resistant PrP^{Sc} bands migrating at ~19 kDa and ~ 23 kDa can be amplified slightly from the cerebellum but not the ~8 kDa band in both substrates. It is worth noting that a band migrating at ~30 kDa corresponding to the diglycosylated was generated by PMCA, although the PrP^{Sc} seeds do not contain this PrP glycoform. Using RT-QuIC assay, they observed that the PrP^{Sc} seeding activity was lower in VPSPr-VV than in sCJDVV2. Moreover, the lag phase was also longer in the former than in the latter. Interestingly, they also observed that PrP^{Sc}-seeding activity remained at similar levels from the top to the bottom fractions of a sucrose-gradient of sCJDVV2 brain homogenate. In contrast, the seeding activity of VPSPr PrP^{Sc} was lower in the first top fractions compared to that of fractions 3 to all bottom fractions (Peden et al. 2014).

Using serial PMCA (sPMCA), we also investigated brain PrP^{Sc}-seeding activity of both VPSPr and $VPSPr^{V180I}$ mutation in the presence of different PrP^{C} substrates from normal human brain homogenates with either PrP-129MM (hMM) or PrP-129VV (hVV) (Wang et al. 2019). Like sCJDMM1, PrP^{Sc} from three genotypes of VPSPr and genetic $VPSPr^{V180I}$ was all amplified in the presence of hMM, but less or no amplification was observed in the presence of hVV. However, PrP^{Sc} from sCJDVV2 or $fCJD^{T183A}$ showed higher amplification in hVV than in hMM. Compared to sCJD PrP^{Sc} whose amplification could be detected as early as the first round, no PrP^{Sc} amplification can be detected until 4–5 rounds of sPMCA in VPSPr and fCJD, suggesting a lower seeding activity in VPSPr and fCJD than in sCJD. Consistent with the observation by Peden et al. (2014), all amplified PrP^{res} had a dominant diglycosylated PrP, although the PrP^{Sc} seeds contained dramatically less or no amount of such glycoform in VPSPr, $VPSPr^{V180I}$, and $fCJD^{T183A}$ (Wang et al. 2019).

Next, we switched sPMCA from human brain substrate to humanized Tg mouse brains expressing PrP-129VV (TgVV), PrP-129MM (TgMM), PrP^{V180I} (Tg180), or in vitro mixed brain homogenate from TgMM and Tg180 mice (Tg180/TgMM) (Wang et al. 2019). Similar to sCJD PrP^{Sc}, the amplification of PrP^{Sc} of all three genotypes of VPSPr and $VPSPr^{V180I}$ was more efficient in the TgVV than in the TgMM substrate. No amplification was detected using the Tg180 brain homogenate as the substrate, although the levels of PrP^{C} in Tg180 mouse brain were similar to those in the brain of TgVV and TgMM. However, amplification was rescued in the mixed substrate of TgMM and Tg180 brain homogenates (Wang et al. 2019). As in the human PrP^{C} substrate, PrP^{Sc} from VPSPr and $VPSPr^{V180I}$ was amplified in the Tg mouse-derived human PrP^{C} substrate and the amplified PrP^{Sc} also had a dominant diglycosylated PrP. However, in contrast to the human brain PrP^{C} substrate, the TgVV substrate was more susceptible to be recruited into PrP^{Sc} than the TgMM by

sCJD, VPSPr, and VPSPr^{V180I} via sPMCA. Remarkably, while PrP^{Sc} from none of the human prion diseases examined in this study was able to seed the Tg180 substrate alone, it was amplified in the substrate of combination of TgMM and Tg180 substrates (Wang et al. 2019). Same as observed by Peden et al. (2014), no small PK-resistant PrP-7 kDa fragment was amplified from any of the VPSPr cases with any of the substrates.

Our RT-QuIC assay revealed that the PrP^{Sc}-seeding activity of VPSPr and VPSPr^{V180I} in the recombinant bank vole PrP23-231 substrate was ~10^2–10^5-fold lower than that of PrP^{Sc} from sCJDMM1 and sCJDVV2 (Wang et al. 2019). We observed that the prion seeding activity was highest in fCJD^{T183A}, followed by VPSPrVV, VPSPr^{V180I}, VPSPrMM, and VPSPrMV. When recombinant hamster PrP90-231 was used as the substrate, the following order of the prion-seeding activity was observed based on the log SD_{50} per milligram of tissues: >8.6 in sCJDMV2, >7.7 in GSS^{P102L}, >7.1 in VPSPr129VV, 7.0 in VPSPr129MM, 6.1 in VPSPr129MV, and > 5.7 in VPSPr^{V180I}. In terms of the lag time of PrP^{Sc}-seeding activity at 10^{-6} dilution, the sCJDMV2 had the shortest lag time of 4.3 h, followed by 18.0 h for VPSPr129VV, 23.1 h for VPSPr129MM, 26.0 h for GSS^{P102L}, 31.1 h for VPSPr129MV, and 36.7 h for VPSPr^{V180I} (Zhang et al. 2021). In sum, PrP^{Sc}-seeding activity was lower, and its lag time was longer in VPSPr than in sCJD and GSS, consistent with previous findings by RT-QuIC assay and animal transmission studies (Notari et al. 2014; Diack et al. 2014; Peden et al. 2014; Wang et al. 2019).

20.6 Molecular Origin of PrP^{Sc} in Sporadic and Genetic VPSPr

As mentioned above, PrP^{Sc} from sporadic and genetic VPSPr exhibits high immunoreactivity with the 1E4 antibody but poor reactivity with 3F4 (Gambetti et al. 2008; Zou et al. 2010b, 2013). The two antibodies have adjacent epitopes. The 1E4 epitope (PrP97-105) is next to the N-terminus of the 3F4 epitope (PrP106-112) (Zou et al. 2010b; Yuan et al. 2008). Recently, we revealed that a polyclonal antibody termed Tohoku 2 holds an immunoreactivity similar to 1E4 with the capability of detecting the typical 5 ladder-like PK-resistant PrP^{Sc} from VPSPr (Zhang et al. 2021). This antibody is directed against human PrP97-103, an epitope same as that of 1E4 except for missing two residues at its C-terminus. This finding further confirmed that the access of the domain starting from residue 97 is critical to detect the unique PK-resistant PrP^{Sc} from VPSPr. Given the localization of the 3F4 and 1E4 epitopes and the size of the detected PrP^{res} fragments, it is most likely that all five ladder-like PrP^{res} fragments detected by 1E4 and Tohoku 2 from the two diseases should contain the 3F4 epitope. The poor affinity of 3F4 for PrP^{Sc} from sporadic and genetic VPSPr may indicate that there might be some local structures or binding molecules that block the 3F4 epitope. We have noticed that the affinity of 3F4 for PK-resistant PrP^{Sc} from VPSPr was increased in the preparations from purification

or enrichment steps compared to that from unpurified total brain homogenates (Zou et al., unpublished data). Thus, purification or enrichment procedures may somehow remove the binding molecules or alter the local structures, which might make the 3F4 epitope exposed. It is also possible that those steps simply increase the amount of the protein, so that 3F4 is able to detect them. Nevertheless, all these findings of higher affinity for 1E4 and lower affinity for 3F4 may suggest that PrP^{Sc} from VPSPr has an origin different from PrP^{Sc} detected in other human prion diseases.

Notably, using the same 1E4 antibody, we previously identified a detergent-insoluble PK-resistant PrP species termed insoluble PrP^{C} ($iPrP^{C}$) in uninfected human brains and cultured cells (Zou et al. 2013; Yuan et al. 2006; Yuan et al. 2008; Zou 2022). The small amount of $iPrP^{C}$ in uninfected brains and cells exhibited the same peculiar immunoreactivity behavior: higher affinity for 1E4 but lower affinity for 3F4. Remarkably, the three similar PK-resistant PrP core fragments migrating at ~20 kDa, ~17 kDa, and ~ 7 kDa observed in VPSPr were detected with 1E4 in uninfected human brains (Zou et al. 2011a). The same immunoreactivity behavior of $iPrP^{C}$ in uninfected brains and the PrP^{res} in VPSPr suggests that they share a common molecular metabolic pathway, origin, or distribution and that sporadic and genetic VPSPr may result from an increase in the amount of $iPrP^{C}$ (Zou 2022; Zou et al. 2011a).

20.7 VPSPr and Other Diseases

In contrast to PrP^{V180I} mutation that generates VPSPr-like PrP^{Sc} without N-181 linked glycoforms, the T183A is the only PrP mutation identified so far that specifically eliminates the first N-linked glycosylation site at residue N181 (Nitrini et al. 1997; Grasbon-Frodl et al. 2004; Zou et al. 2011b). Nevertheless, the diglycosylated PrP^{res} is notably detectable in the brain tissues of $gCJD^{T183A}$. It is most likely derived from the wild-type allele recruited by the pathological mutant allele (Hitoshi et al. 1993; Wang et al. 2019; Grasbon-Frodl et al. 2004). If this is the case, it would be evidence that PrP^{Sc} without mono181 still can recruit diglycosylated PrP^{C} to form a regular PrP^{Sc} glycoforms. Indeed, in vitro sPMCA of PrP^{Sc} from $gCJD^{T183A}$ generated the diglycosylated PrP^{res} in the presence of brain homogenates of normal human or humanized Tg mice (Wang et al. 2019). Surprisingly, no diglycosylated PrP^{Sc} was detectable in brain homogenates of $VPSPr^{V180I}$ even in the PrP^{Sc}-enriched preparation by sodium phosphotungstate (Chasseigneaux et al. 2006; Xiao et al. 2013; Hitoshi et al. 1993), suggesting that both mutant and wild-type PrP^{C} do not convert to PrP^{res} in the case of $VPSPr^{V180I}$. Nevertheless, similar to $gCJD^{T183A}$, in vitro sPMCA of PrP^{Sc} from $VPSPr^{V180I}$ generated the diglycosylated PrP^{res} in the presence of brain homogenates of normal human or humanized Tg mice (Wang et al. 2019). In addition, PrP^{T183A} mutation does not generate VPSPr-like PrP^{Sc} (Xiao et al. 2013; Grasbon-Frodl et al. 2004). Also, other mutations involved in the

changes in glycoforms including D178N, F198S, and E200K do not generate VPSPr-like PrP^{res}.

The deposition in the brain of multiple small PrP^{res}, especially the 7–8 kDa fragment, is the molecular hallmark of GSS (Tagliavini et al. 1991), similar to VPSPr. Therefore, it is reasonable to anticipate a potential association between GSS and VPSPr. Because of the long disease duration, multiple PrP^{res} fragments, and variable amount of PrP^{res}, VPSPr was once suspected to be the sporadic form of GSS linked to PrP^{A117V} mutation (GSS^{A117}) (Zou et al. 2010b; Notari et al. 2018). However, we also noted different ratios and immunoreactivity of PrP^{Sc} between VPSPr and GSS^{A117V} in our previous studies (Zou et al. 2010b). Moreover, GSS is frequently associated with a predominant cerebellar dysfunction and characterized by the deposition of *multicentric plaques* in the cerebellum (Kong et al. 2004). In contrast, VPSPr has no typical multicentric plaques but dot-like staining or small plaque-like formations in the cerebellum (Zou et al. 2010b). It is conceivable that cells and animals expressing PrP^{A117V} will provide valid models for addressing the remaining questions.

It has been noticed that VPSPr is often comorbid with tau and amyloid-β (Aβ) pathology (Gambetti et al. 2008; Zou et al. 2010b; Notari et al. 2018). Clinically, it is frequently misdiagnosed as Alzheimer's disease, because most of VPSPr cases exhibit dementia. Tau pathology is mainly observed in their lower medial temporal cortex, entorhinal and transitional cortices, and hippocampal gyrus (Zou et al. 2010b; Notari et al. 2018). Aβ pathology was also seen in VPSPr cases (Zou et al. 2010b; Head et al. 2010; Rodríguez-Martínez et al. 2012). Comorbidity with other neurodegenerative diseases was observed, such as argyrophilic grain disease (Rodríguez-Martínez et al. 2010), Lewy body disease (Head et al. 2010; Assar et al. 2015), and amyotrophic lateral sclerosis (Cannon et al. 2014). Cases with PrP^{V180I} mutation can have Alzheimer pathology, including neurofibrillary tangles and senile plaques (Qina et al. 2014; Yoshida et al. 2010; Iwasaki et al. 2011). They could be misdiagnosed as Alzheimer's disease as well.

There was a suspicion that the unique PrP^{Sc} in VPSPr may derive from anchorless PrP^{C} (Notari et al. 2018). However, our recent characterization of PrP^{Sc} in a case with PrP^{Q227X} stop mutation does not support this hypothesis (Shen et al. 2021). It is known that the PrP^{Q227X} stop mutation eliminates the GPI anchor. Our study revealed that this mutation generates a GSS-like PrP-banding pattern but not the VPSPr-like PrP^{res} (Shen et al. 2021).

20.8 Conclusions

PrP^{Sc} found in sporadic and genetic VPSPr with PrP^{V180I} mutation is clearly different from those of classic sporadic and genetic human prion diseases. Both sporadic and genetic VPSPr share similar physicochemical and biological properties of PrP^{Sc} associated with a glycoform-selective prion formation. The PrP-129 polymorphism apparently plays a significant role in the pathogenesis of VPSPr. It not only

mediates the levels of PK-resistant and -sensitive PrP^{Sc}, a phenomenon that has never been reported in other prion diseases, but also disproportionately affects PrP-129VV homozygotes. The two diseases specifically alter glycosylation N181-linked glycoforms, which may involve a non-PrP factor that participates in regulating PrP glycosylation. Because of similar immunoreactivity and core PrP^{res} fragments, PrP^{Sc} in VPSPr may share a molecular origin with $iPrP^{C}$. The inefficient transmissibility of sporadic and genetic VPSPr may result from altered post-translational modifications, including the first N-linked glycosylation site. Further PrP sequencing study and glycan analysis of purified PrP^{res} will provide insights into these issues. Also studies with the two conditions along with cell and animal models expressing PrP^{V180I} mutation will help us understand the possible co-factors and molecular mechanisms underlying the formation of the unique PrP^{Sc}.

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Chapter 21
The Spectrum of Tau Pathology in Human Prion Disease

Gabor G. Kovacs and Herbert Budka

Abstract Intracellular deposition of hyperphosphorylated tau characterizes tauopathies: there is a spectrum from neuron-predominant through mixed neuronal and glial to glia-predominant forms. However, tau pathology appears in practically all forms of human prion disease. In addition to the rare co-occurrence of a main form of tauopathy with prion disease, tau pathology may associate with prion diseases in distinct patterns. (1) Small neuritic profiles correlating with tissue lesioning can be observed in all prion diseases. (2) Larger dystrophic neurites may be observed around PrP amyloid plaques. (3) Neurofibrillary tangles may follow the distribution described by Braak and Braak as Alzheimer-related pathology but might show atypical locations. It may be associated with prominent neuropil threads in subcortical regions in certain mutations with Creutzfeldt–Jakob disease (i.e. E200K mutation). Furthermore, widespread neurofibrillary tangles in several subcortical, allo- and neocortical regions are consistently associated with certain *PRNP* mutations in PrP cerebral amyloidoses such as Gerstmann–Sträussler–Scheinker disease or PrP cerebral amyloid angiopathy. (4) Other types of tau pathologies include the rare presence of glial tau immunoreactivity. In summary, widespread application of phospho-tau immunostaining has revealed a previously underrecognized spectrum of tau pathologies in human prion diseases. The relation between tau pathology and PrP deposition and factors influencing its appearance in prion diseases merit further studies.

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Abbreviations

AGD	argyrophilic grain disease
AD	Alzheimer's disease
ARTAG	Ageing-related tau astrogliopathy
CAA	cerebral amyloid angiopathy
CBD	corticobasal degeneration
CJD	Creutzfeldt–Jakob disease
sCJD	sporadic CJD
iCJD	iatrogenic CJD
vCJD	variant CJD
gCJD	genetic CJD
DLB	Dementia with Lewy bodies
FFI	Fatal familial insomnia
GSS	Gerstmann–Sträussler–Scheinker disease
GSK3β	glycogen synthase kinase 3β
NDD	Neurodegenerative disease
NFT	neurofibrillary tangle
PART	Primary age-related tauopathy
PD	Parkinson's disease
PK	proteinase K
PrP	prion protein
PRNP	prion protein gene
PSP	progressive supranuclear palsy

21.1 Overview of Tauopathies

Prion diseases belong to the group of neurodegenerative diseases (NDDs) that are characterized by progressive loss of neurons. A prerequisite to understanding the relevance of tau pathology in prion diseases is knowledge of the spectrum of NDDs including tauopathies.

21.1.1 Classification of Neurodegenerative Diseases

Molecular pathological classification of NDDs is based on the regional and cellular sites where the deposits composed of particular proteins are found. While immunoreactivity for amyloid-β or prion protein (PrP) is located predominantly extracellularly, major proteins that deposit intracellularly include tau, α-synuclein, TAR DNA-binding protein 43 (TDP-43) or fused in sarcoma (FUS) protein (Kovacs et al. 2010). Variability in NDDs is reflected by distinctive distributions of neurodegeneration-related proteins that can accumulate in various cell types, i.e. neurons, astrocytes and oligodendroglia, moreover in cell processes, cytoplasm or nucleus. In addition, several biochemical alterations and modifications contribute to the spectrum of phenotypes (Kovacs and Budka 2009b).

21.1.2 Tau Protein

Tau is a microtubule-associated protein encoded by a single gene (*MAPT*). *MAPT* maps to chromosome 17q21.2 (Andreadis et al. 1992; Goedert 2005). Mutations lead to hereditary diseases that associate with progressive neurodegenerative syndromes and accumulation of intracellular deposits of soluble and insoluble hyperphosphorylated tau protein (Goedert 2005; Lee et al. 2001). Genetic variability in *MAPT*, in particular a dinucleotide repeat polymorphism in intron 9 defined as H1 and H2 haplotypes, may contribute to the risk of sporadic tau diseases (Dickson et al. 2007; van Swieten and Spillantini 2007).

Alternative splicing generates six isoforms, which are present in the adult human brain. In disease, four main patterns of insoluble tau are observed on Western blotting (Lee et al. 2001). These include (I) major bands at 60, 64 and 68 kDa (e.g. in AD and primary age-related tauopathy/PART); (II) bands at 64 and 68 kDa (e.g. in corticobasal degeneration/CBD, progressive supranuclear palsy/PSP, argyrophilic grain disease/AGD and globular glial tauopathies/GGT); (III) bands at 60 and 64 kDa (e.g. in Pick's disease); and (IV) a minor band at 72 kDa that usually associates with the first pattern (Kovacs 2015; Lee et al. 2001). It is also important to distinguish different isoforms of tau in diseases. The isoforms differ by the presence or absence of a 29- or 58-amino acid insert in the amino-terminal half of the protein and by the inclusion, or not, of a 31-amino acid repeat encoded by exon 10 of tau in the carboxy-terminal half of the protein. Three isoforms with 0, 1 or 2 inserts contain three microtubule-binding repeats (R) and are designated as 3R tau; and three isoforms, also with 0, 1 or 2 inserts, containing four microtubule-binding repeats, are designated as 4R tau (Goedert et al. 2006).

There are further modifications of the tau protein that are relevant for pathogenesis:

1. The most studied is *phosphorylation*, which is the physiological way of regulating the activity of tau and the microtubule binding (Reynolds et al. 2008). Normal tau is phosphorylated on 2 or 3 residues in contrast to hyperphosphorylated tau that is phosphorylated at least on 8–12 (or more) residues (Kopke et al. 1993).
2. Further modifications are also under extensive investigations but their relevance has to be defined (reviewed in Refs. (Kovacs 2016; Kovacs et al. 2010)). These

include N- and C-terminally truncated species of tau, glycosylation, oxidative and nitrative injuries, transglutamination, deamidation and formation of tau oligomers that may be present before neurofibrillary pathology becomes evident.

21.1.3 Classification of Tau-Related Conditions

Tau-related conditions can be classified on the basis of tau isoforms, 3R, 4R or both 3R and 4R. For clinicopathological classification, the histological and cytological characterization of neuronal and glial tau immunoreactivities and their anatomical distribution is also needed.

While AD features both 3R and 4R isoforms, CBD, PSP and AGD are thought to be 4R predominant, in contrast to Pick's disease, which is a 3R isoform predominant tauopathy (Cairns et al. 2007). Tauopathies associated with mutations in the *MAPT* gene may show any of the patterns and isoform predominance. Thus, tauopathies are currently defined biochemically with a signature characterized by the pattern of insoluble tau and further by the tau isoforms (Sergeant et al. 2005). Recently, high-resolution tau filament structures have been determined, and a three-level hierarchical classification of diseases with tau pathology has been suggested (Shi et al. 2021). Based on the knowledge of aetiology, tau-only pathology versus co-existence with a parenchymal amyloid made of another protein and the role of assembled tau in disease pathogenesis six groups of tau-related conditions have been proposed (Kovacs et al. 2022). Importantly, the terms tau immunoreactivity, tau pathology and tauopathy have been defined. Accordingly, the term tauopathy has been proposed to be used only if the following criteria are met: (1) Abundant filamentous tau inclusions made of either 3R, 4R or 3R+4R tau and (2) consistent and typical patterns of cellular tau pathologies in multiple cases that correlate with clinical signs and neurodegeneration (Kovacs et al. 2022). Main tauopathies include *MAPT*-tauopathies, PSP, CBD, GGT, AGD, PART and Pick's disease; a few examples of 'other' tauopathies include chronic traumatic encephalopathy, IgLON5-antibody-related tauopathy or Western Pacific amyotrophic lateral sclerosis parkinsonism dementia complex (Kovacs et al. 2022).

21.1.4 Immunomorphology of Pathological Tau Deposition in 'Main' and 'Extracellular Filamentous Deposit-RELATED TAUOPAThies'

Hyperphosphorylated tau is the major constituent of neuronal and glial inclusions. Ultrastructurally these are composed of filaments, which may vary in structure, such as paired helical filaments, straight filaments or twisted ribbons. According to the cellular distribution there is a spectrum from neuron-predominant through mixed neuronal and glial to glia-predominant forms of tauopathies (Fig. 21.1a–h)

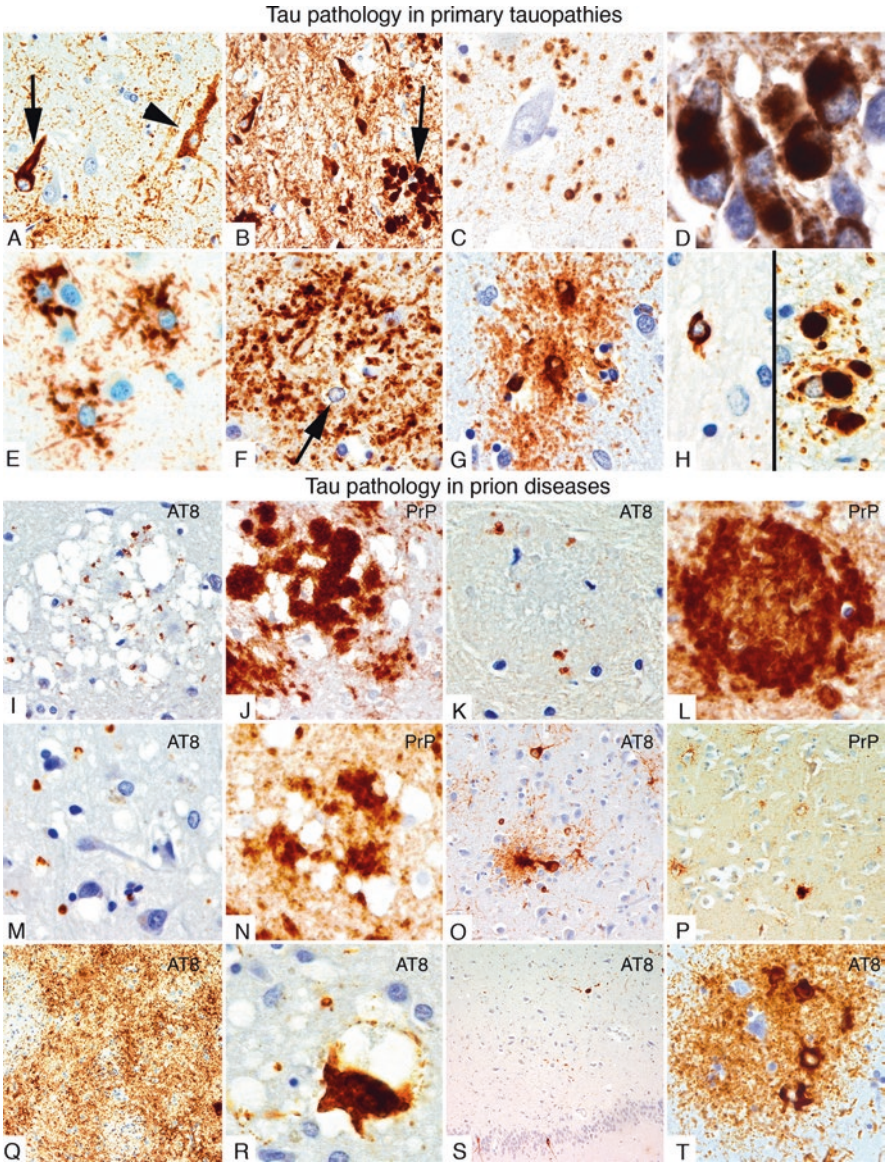

Fig. 21.1 Overview of tau pathology in primary tauopathies and prion diseases. (**a**) Neurofibrillary tangle (indicated by an arrow) and diffuse cytoplasmic neuronal immunoreactivity (indicated by an arrowhead) in Alzheimer's disease hippocampus sample. (**b**) Dystrophic neurites (indicated by and arrow) and neuropil threads in Alzheimer's disease hippocampus sample. (**c**) Grains in the hippocampus in argyrophilic grain disease. (**d**) Pick bodies in the granular layer of the dentate gyrus in Pick's disease. (**e**) Tufted astrocytes in the caudate nucleus in progressive supranuclear palsy. (**f**) Astrocytic plaque in the caudate nucleus in corticobasal degeneration. (**g**) Fine granular/fuzzy tau immunoreactivity in astrocytic processes in complex tauopathy of the elderly. (**h**) Oligodendroglial coiled body (left side of image) and globular glial inclusions (right side of image) in progressive

(Kovacs et al. 2010; Kovacs and Budka 2009b). Neuronal tau pathology predominates in AD and in Pick's disease. These comprise neurofibrillary tangles (NFTs) that are immunoreactive for both 4R and 3R tau isoform-,specific antibodies (e.g. in AD) and spherical inclusions called Pick bodies that are purely 3R isoform immunoreactive (e.g. in Pick's disease). In PSP and CBD, a mixture of neuronal and glial deposition of tau is observed, whereas the anatomical distribution and morphology of cellular inclusions distinguish the disorders. In addition to oligodendroglial coiled bodies seen in both, astrocytic plaques (tau accumulation in the distal segment of astrocytic processes) are features of CBD, and tufted astrocytes (tau deposition in the proximal segment of astrocytic processes) characterize PSP. AGD is a tauopathy where the tau immunoreactive argyrophilic grains and diffuse cytoplasmic granular tau immunoreactivity are neuron-related, but oligodendroglial coiled bodies are also important features, however, restricted to limbic areas. There are further tauopathies where glial tau, in particular in the white matter, is a major feature; these are mainly 4R predominant tauopathies (Bigio et al. 2001; Kovacs et al. 2008b; Powers et al. 2003). Recently, further complex tauopathies associated with dementia in the elderly have been described and expand the spectrum of tauopathies (Kovacs et al. 2011a).

One important feature of some neuronal and astrocytic tau pathologies is the maturation of inclusions. For example, diffuse neuronal cytoplasmic granular tau immunoreactivity cannot be detected using anti-ubiquitin immunohistochemistry; these lesions are not visible either using silver stainings (i.e. Gallyas or Bielschowsky), hence the name 'pretangle'. These are detected using antibodies against the 4R isoform of the tau protein. This morphology is followed by the typical NFT, which is argyrophilic (i.e. detected by silver stains) and ubiquitin immunoreactive (Bancher et al. 1989a; Bancher et al. 1989b). Furthermore, it shows both 3R and 4R tau isoform immunopositivity. A similar process was described also for astroglial tau pathology (Botez et al. 1999; Kovacs et al. 2011a).

To understand the complexity of tauopathies and to interpret tau pathologies, one must be familiar with the fact that some lesions show stages, which means that

Fig. 21.1 (continued) supranuclear palsy and white matter tauopathy with globular glial inclusions, respectively. (**i**) Tau immunoreactive neuritic profiles in the cerebral cortex in variant Creutzfeldt–Jakob disease (CJD). (**j**) PrP immunoreactivity in the corresponding area for image (**i**) (samples of variant CJD were kindly provided by Professor James Ironside, CJD Surveillance Unit, Edinburgh, UK). (**k**) Tau immunoreactive neuritic profiles in the cerebellum in Gerstmann–Sträussler–Scheinker disease (P102L mutation). (**l**) PrP immunoreactivity in the corresponding area for image (**k**). (**m**) Tau immunoreactive neuritic profiles in the cerebral cortex of sporadic CJD. (**n**) Patchy/perivacuolar PrP immunoreactivity in the corresponding area for image (**m**). (**o**) Tau immunoreactive neurons in genetic CJD (E200K mutation). (**p**) Perineuronal and synaptic PrP immunoreactivity in the corresponding area for image (**o**). (**q**) Abundant phospho-tau (AT8) immunoreactive threads in the caudate nucleus in genetic CJD (E200K mutation). (**r**) Globose neurofibrillary tangle with vacuolation in the nucleus accumbens in genetic CJD (E200K mutation). (**s**) Neuronal tau immunopositivity in the granular layer of the dentate gyrus (lower part of image) and the CA4 subregion of the hippocampus (upper part of image) in in genetic CJD (E200K mutation). (**t**) Tau immunopositive astrogliopathy in the amygdala in genetic CJD (V203I mutation)

certain anatomical pathways of the appearance of tau immunoreactive lesions can be recognized. This was originally described for the NFTs seen in AD and has become known as Braak and Braak stages: from the entorhinal cortex and hippocampus and subsequently the temporal cortex, it reaches subcortical structures and association cortices in six stages (Braak and Braak 1991). A similar progressive anatomical involvement has been proposed for PSP (Williams et al. 2007) or AGD (Saito et al. 2004) as well.

21.1.5 Spectrum of Tau Pathology in Other Conditions

Pathological tau may be present in normal aging or non-neurodegenerative disorders (summarized in Refs. (Goedert et al. 2006; Kovacs et al. 2010, 2022; Kovacs and Budka 2009b). In some cases, tau immunoreactivity using various antibodies has been described; however, the presence of filamentous tau inclusions has not been demonstrated (Kovacs et al. 2022). In several conditions, age-associated NFTs are observed. A recently described tau pathology is ageing-related tau astrogliopathy (ARTAG) that includes thorny astrocytes in subpial, subependymal, perivascular, and white matter locations, and granular fuzzy astrocytes in the gray matter (Kovacs et al. 2016).

21.1.6 How Is Tau Pathology in Prion Diseases to Be Characterized?

This requires an analysis of the following aspects:

- Is it within the frame of age-associated neurofibrillary degeneration?
- Is it compatible with a well-established main tauopathy as concomitant pathology, or does it represent a novel phenotype?
- What are the hallmark tau immunomorphologies; in particular, is it neuron or glial predominant, and what is the shape of the inclusions?
- What is the biochemical signature of insoluble tau and what is the ultrastructural feature of filaments?

21.2 Tau Pathology in Human Prion Diseases

Human prion diseases may be classified according to the etiology as idiopathic (sporadic) such as Creutzfeldt–Jakob disease (sCJD), acquired (iatrogenic-iCJD; variant CJD-vCJD), or genetic (familial, hereditary) CJD (gCJD), fatal familial insomnia (FFI), or PrP cerebral amyloidoses such as

Gerstmann–Sträussler–Scheinker disease (GSS) and PrP cerebral amyloid angiopathy (PrP-CAA). These disorders differ in brain pathology: spongiform encephalopathy in CJD, thalamic degeneration in FFI, and brain amyloidosis in the majority of GSS (Kovacs and Budka 2009a). This suggests that additional tau pathology may be influenced by several factors in prion diseases. For long, tau immunohistochemistry was not routinely performed during the neuropathological evaluation of prion diseases; thus, many novel aspects have been described only recently.

21.2.1 Tau Pathology in Sporadic CJD

According to the literature and our experience, a concomitant tau pathology or tauopathy in sCJD may be classified as follows:

1. *Neuritic tau pathology associated with deposition of disease-associated PrP*

This is the most frequent type of tau immunoreactivity. Its presence was underestimated for long, but a study (Reiniger et al. 2011) as well as our experience indicate strong correlation with the density of PrP immunodeposition but not duration of illness. It was proposed that the PrP load is the major triggering factor for tau phosphorylation (Reiniger et al. 2011). The presence of these neuritic profiles was reported to be not related to amyloid-β (Aβ, the protein component of plaques in AD), and the morphological appearance (granular or tiny rod-shaped) was also distinct (Reiniger et al. 2011). Further comprehensive biochemical characterization of tau pathology has not been reported for sCJD. Tau immunoreactivity was described also surrounding kuru-type plaques in a rare sCJD subtype (Sikorska et al. 2009). A recent report suggests that sCJD VV2 and MV2K subtypes show higher levels of p-tau in the cerebrospinal fluid when compared with other sCJD types, and this correlates positively with the amount of tiny neuritic tau pathology (Lattanzio et al. 2017).

2. *Co-existence of AD- and PART-related pathology and CJD*

This is observed in all larger CJD series, as both conditions preferentially occur in the elderly; however, tau pathology and other mixed pathologies (Kovacs et al. 2008a) are thought to be not consistent features of sporadic CJD. A comprehensive study indicated that, according to CERAD (Consortium to establish Registry for AD) criteria (Mirra et al. 1991), definite and probable AD constituted 10.9% of sCJD cases, somewhat lower as in the control group (19%) (Hainfellner et al. 1998). It was concluded that AD-type pathology in CJD is most likely age-related. Two forms of coexistence of CJD and AD in the same patient have been suggested (Tsuchiya et al. 2004): the first when AD patients develop CJD in the late stage of disease, and the second form when sCJD brains show AD pathological features without any clinical features typical of AD. It must be noted that the CERAD approach focuses on the density of neuritic plaques that consist of tau-immunoreactive dystrophic neurites; however, in these studies, other types of tau pathologies were not systematically evaluated using phospho-dependent tau antibodies. In variably

protease-sensitive prionopathy (Gambetti et al. 2008; Zou et al. 2010), NFTs were also reported corresponding to stage II according to Braak and Braak in a 76-year-old patient (Head et al. 2010). A recent study found that approximately 80% of sCJD cases show additional tau pathology in the medial temporal lobe compatible with PART, but in 40% of these, the tau immunoreactivity load was significantly different from the typical distribution of the Braak staging (Kovacs et al. 2017). Complementary to these observations, another study reported a lack of correlation between variables affecting CJD and those defining the AD/PART spectrum and suggested that, except for a tendency to increase the frequency of cognitive symptoms, AD/PART co-pathology did not significantly affect the clinical presentation of typical CJD (Rossi et al. 2019).

3. *Other tau pathologies in sCJD*

These include the rare presence of PSP or CBD-type pathologies and also the presence of AGD and widespread gray matter ARTAG, altogether seen in approximately 14% of sCJD cases (Kovacs et al. 2017).

21.2.2 Tau Pathology in Acquired CJD

Acquired forms comprise prion diseases with suspected or proven exposure to external prions. This includes kuru, related to historical ritualistic cannibalism in Papua-New-Guinea; iatrogenic CJD (iCJD), related to medical intervention (e.g. neurosurgery, deep electrodes, hypophyseal hormones, dura mater transplants); and variant CJD (vCJD), which represents dietary exposure to bovine spongiform encephalopathy (BSE) (Kovacs and Budka 2009a). Although tau-immunoreactivity around plaques has been described in a kuru brain (Sikorska et al. 2009), and Alzheimer-type senile plaques without NFTs have been reported in a single 28-year-old patient with iCJD (Preusser et al. 2006), comprehensive observations on tau pathology have been described only for vCJD: phospho-tau-immunoreactive neuritic profiles clustered around PrP amyloid deposits in vCJD patients in the absence of Aβ, not only in the cerebral cortex but also in the cerebellum (Giaccone et al. 2008). This was localized to perikarya and dendrites less constantly. The biochemical counterpart was the presence of phospho-tau in the detergent-insoluble fraction of cerebral cortex. A further study showed significant tau-immunopositive dystrophic neurites around the PrP-immunoreactive amyloid plaques together with some phospho-tau immunoreactive structures dispersed in the cerebral and, to a lesser degree, the cerebellar cortex (Sikorska et al. 2009). This was considered reminiscent of AD plaques but, in contrast to AD, no paired helical filaments were observed within dystrophic neurites in vCJD on electron microscopy (Sikorska et al. 2009). However, tau pathology seems to be a regular component of the neuropathology of vCJD.

21.2.3 Tau Pathology in Genetic CJD and FFI

Mutations in the *PRNP* associated with spongiform encephalopathy are termed genetic CJD (gCJD). There a tau pathology profile similar to sCJD may be expected and was indeed reported in some mutations (Reiniger et al. 2011). However, a more complex pathogenetic scenario has been suggested in a recent comprehensive evaluation of protein deposition in *E200K gCJD* cases, one of the most frequent *PRNP* mutations worldwide (Kovacs et al. 2011b). Accumulation of phospho-tau, alpha-synuclein, and Aβ was frequent, while TDP-43 immunoreactivity was not present. Moreover, Aβ plaques have been reported in E200K gCJD (Ghoshal et al. 2009). Our previous study on *E200K gCJD* provided the first evidence for a complex interrelation of neurodegeneration-related proteins triggered by a single *PRNP* mutation. Approximately 90% of cases exhibited *neuritic profiles*, mainly in areas with more prominent tissue pathology, PrP deposition, neuronal loss and spongiform change. This finding is consistent with the findings of another study on sCJD and few gCJD cases (Reiniger et al. 2011). Double immunolabeling studies suggested that most of the tau pathology is neuronal in origin (Kovacs et al. 2011b). Immunoblotting revealed bands characteristic of 3R tau. Roughly one-third of the patients showed *NFTs following Braak and Braak stages*. Usually, these were in a more developed stage than what would accord with the age of the patients. Immunoblotting revealed patterns similar to AD in the hippocampus sample, while 3R and fragments of tau were detected in several other regions where only neuritic tau immunopositivity was detected in tissue sections (Kovacs et al. 2011b). A further type of tau pathology, again in about one-third of the patients, comprised a peculiar constellation of tau pathologies that did not fulfill criteria of established sporadic tauopathy entities (Kovacs et al. 2011b). This could be further subdivided into two major types: (A) Cases with NFTs, diffuse cytoplasmic tau immunoreactivity (pretangle-like), and threads in the basal ganglia, brainstem (substantia nigra, dorsal raphe nucleus, and locus coeruelus) and less in the thalamus, including one with prominent involvement of neocortical regions. Globose tangles in subcortical areas were prominently 4R immunoreactive, while in neocortical areas and hippocampus both 3R and 4R immunopositivities were noted in NFTs. Abundant thread-like structures that were associated with neurofilaments, but not astrocytic processes, were mainly 4R immunopositive. There was lack of astrocytic plaques or tufted astrocytes, although some dot-like immunostaining of astrocytic processes was noted. Oligodendroglial coiled bodies were only occasionally seen. (B) Further cases exhibited an unusual distribution of neuronal and glial tau deposition in the hippocampus, which included NFTs and prominent diffuse neuronal granular cytoplasmic immunoreactivity in CA4, CA3 and CA2 subregions and dentate gyrus, but also in the CA1 subregion and subiculum, without or with scant NFTs in the entorhinal cortex. Argyrophilic grains were not seen, but some oligodendroglial tau immunopositivity and dot-like immunolabeling of astrocytic processes were observed. In addition, all of these cases showed NFTs in the noradrenergic locus coeruleus. In these cases, however, further biochemical evaluation of tau protein was not available.

An unusual pattern of tau pathologies was described in the *R208H gCJD* reminiscent of the type B pattern described above in E200K gCJD: few NFTs and neurones with stained cytoplasm (pretangles) in the CA1 region, and a small number of AT8-positive inclusions in oligodendrocytes and astrocytes (Roeber et al. 2005). In addition, tiny granules in the CA1 region and entorhinal cortex were also noted. Since immunoblotting revealed an additional 17-kDa PrP fragment, absent in two other cases with the same R208H mutation but without tau pathology, the possibility that the additional PrP band is related to tau protein pathology was raised (Roeber et al. 2005). Although a similar band was described in *V203I gCJD* recently, findings on tau immunohistochemistry were not reported (Jeong et al. 2010). Interestingly, a single V203I gCJD case in our collection (Höftberger et al. 2011) and a further case from France (Kovacs et al. 2017) exhibited features of an unusual pattern with a peculiar tau-astrogliopathy, originally described in non-prion diseased elderly demented patients (Kovacs et al. 2011a).

NFTs were also reported in *V180I gCJD*. This gene alteration may be present in elderly patients with spongiform encephalopathy; however, NFTs are not consistently reported. In an elderly patient, stage IV of NFTs according to Braak and Braak was noted; however, it was interpreted as similar to sCJD cases having AD pathological features without any clinical features typical of AD (Yoshida et al. 2010).

In *fatal familial insomnia* (FFI), there is also a paucity of systematic studies on tau pathology. However, a recent case report demonstrated neuropil threads and small neuronal inclusions in the anterior ventral and dorsomedial nuclei of the thalamus, the pulvinar, inferior olivary nuclei and striatum together with neuropil threads seen adjacent to the pigmented neurons of the substantia nigra (Jansen et al. 2011a). Distribution of the tau pathology did not follow Braak and Braak staging (Jansen et al. 2011a). This finding is particularly interesting since here PrP deposition is only mild as compared to other prion diseases.

21.2.4 Tau Pathology in Dominantly Inherited PrP Cerebral Amyloidoses

Brain PrP amyloidosis is characterised by the appearance of parenchymal (multicentric) amyloid plaques (GSS) in the brain or in the vessel walls (PrP cerebral amyloid angiopathy, CAA) (Ghetti et al. 1995, 2018). The biochemical hallmark of PrP cerebral amyloidosis is thought to be the presence of N- and C-terminal truncated proteinase K (PK) resistant PrP degradation products that range from approximately 7 to 15 kDa and a low molecular weight band in Western blot (WB) (Ghetti et al. 2003; Piccardo et al. 1998). Hallmark studies from Bernardino Ghetti and coworkers have outlined the complexity of tau pathology (Ghetti et al. 1989, 1995, 1996a, b; Giaccone et al. 1990) that is a very important component of the neuropathology of many cases with cerebral PrP amyloidosis. It is characterized by tau-immunoreactive dystrophic neurites surrounding PrP amyloid plaques and NFTs. However, not all related mutations associate consistently with NFTs.

The following mutations inconsistently show NFTs or other types of tau pathology; such cases tend to have a longer clinical duration:

- P102L-129M: Variably present in hippocampus and cerebral cortex together with neuropil threads, in some cases in correlation with the burden of PrP deposition (Ishizawa et al. 2002).
- P105L-129V: NFTs are present mainly in the cerebral cortex but may appear in the brainstem as well (Yamada et al. 1999; Yamazaki et al. 1999).
- A117V-129V: Described in the cerebral cortex and subcortical nuclei, including amygdala and thalamus with immunobiochemical profile similar to AD (Mohr et al. 1999).
- 168 base pair insertion with 129V: diffuse punctuate phospho-tau staining with sparse neuropil threads in cerebral cortex and also striatum and molecular layer of the cerebellum, but only a few NFTs in the hippocampus, frontal cortex and temporal cortex (Jansen et al. 2011b).

NFTs have been reported also in the following mutations with GSS phenotype: G131V-129M, S132I-129M, H187R-129V, D202N-129V, E211D-129V, Q212P-129M, Y218N-129V, Q227X-129V. NFTs as integral part of the clinicopathological phenotype has been reported in F198S-129V and Q217R-129V mutations with GSS or Y145X-129M, Q160X0129M, or Y163X-129M with PrP-CAA. Neuritic tau-positive dots have been described in Y226X-129V mutation associated with PrP-CAA. Further studies have indicated that the tau immunoreactivity profile and ultrastructure was very similar if not identical to AD (Ghetti et al. 1989, 1996b; Giaccone et al. 1990). The correlation of PrP deposition and tau pathology is reminiscent to that seen in other amyloidoses (Holton et al. 2001) and supports the idea that abnormal tau phosphorylation may accompany cerebral amyloid deposition regardless of the chemical composition of the amyloid. However, this is not always seen in subcortical regions in GSS.

Importantly, tau folds co-existing with various cerebral parenchymal amyloidosis such as PrP amyloid, Abri and ADan amyloid are identical to those of AD (Hallinan et al. 2021; Shi et al. 2021). It has been proposed that the Tauopathy seen in certain *PRNP* mutations associated with PrP amyloidosis should be included in the group of 'Tauopathy, obligatory association with extracellular filamentous deposits caused by genetically determined other proteinopathy' (Kovacs et al. 2022).

21.3 Concluding Remarks

21.3.1 Pathogenesis of Tau Deposition in Human Prion Diseases

The interaction of tau protein and PrP still needs more experimental data. There are a few investigations that provide a pathogenetic link between these two proteins, such as that using PrP 106–126 peptides that induced glycogen synthase kinase 3β

(GSK3β) mediated tau phosphorylation (Perez et al. 2003). A study in scrapie-infected hamsters showed that changes of profiles of phospho-tau correlate with illness (Wang et al. 2010), while gene knockout of tau did not contribute to the pathogenesis of prion disease in mice (Lawson et al. 2011). Since not all mutations with PrP amyloid associate with tau pathology, it might be theoretically possible that binding activities of a PrP–tau complex differ between mutations, as suggested by in vitro observations (Wang et al. 2008). Although there are several components of the tau–PP relation in tissue in parallel with observations in other amyloidoses (Holton et al. 2001), there are many exceptions to the rule. This may suggest differences in neuronal processing or genetic/epigenetic influences. A recent study found no evidence for an association between *MAPT* gene variations and sCJD, and only some weak evidence for an association with vCJD (Sanchez-Juan et al. 2007). Altogether these studies indicate a complex interaction of tau and PrP.

21.3.2 Relevance of Tau Protein as Biomarker in Human Prion Diseases

Examination of total tau and phospho-tau protein levels in the cerebrospinal fluid is an established method used in practice mainly for AD diagnostics. In sCJD, although protein 14-3-3 has traditionally been a most useful surrogate laboratory marker, total tau protein presents comparable levels of sensitivity and specificity (reviewed in Ref. (Quadrio et al. 2011)). Measurement of total tau in the CSF performs best in terms of both specificity and sensitivity for all sCJD types; furthermore, sCJD VV2 and MV2K types demonstrated higher CSF levels of p-tau when compared with other sCJD types (Lattanzio et al. 2017). Interestingly, a high rate of total tau levels was found in gCJD, while in GSS, only 40% of cases had tau levels above the cut-off level, and only a single FFI patient (from 14 investigated) had abnormal tau levels (Ladogana et al. 2009).

21.3.3 Summary: Classification of Tau Pathology in Human Prion Diseases

Tau pathology appears in practically all forms of human prion disease and is mainly neuron-related, while glial tau pathology is unusual. In addition to the rare co-occurrence of main tauopathy with CJD, tau pathology presents in the following patterns (summarized in Figs. 21.1i–t and 21.2):

1. *Small neuritic profiles* correlating with the density of PrP deposition and tissue lesioning. This type can be observed in all prion diseases with spongiform encephalopathy (sCJD and gCJD), but is rare in FFI.

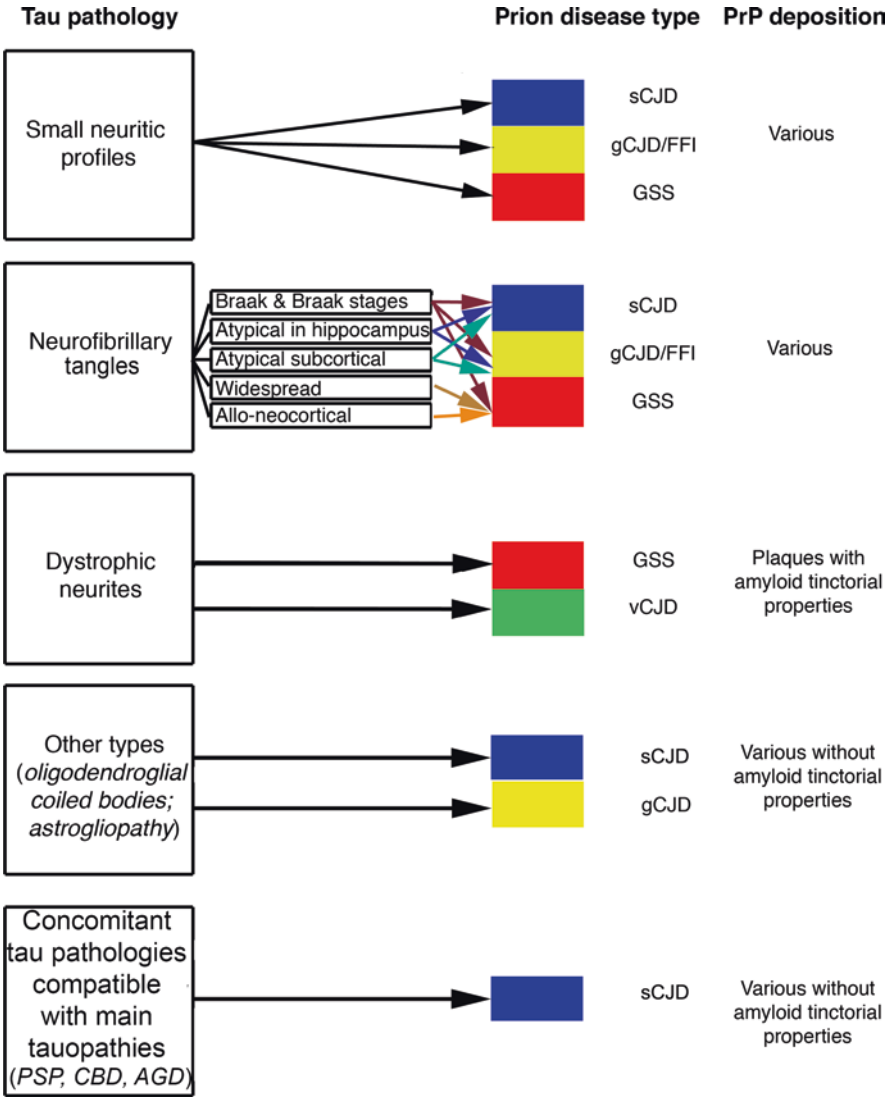

Fig. 21.2 Stratification of tau pathology according to morphology, prion disease type, and PrP immunoreactivity (see text for details)

2. *Larger dystrophic neurites and neuritic profiles* may be observed around multicentric PrP amyloid plaques as a feature of GSS, reminiscent of other brain amyloidoses including AD. Furthermore, it is prominent in the amyloid-plaque predominant vCJD.
3. *NFTs*, which can be further grouped as follows:

   (a) NFTs following the distribution described by Braak and Braak: this might be age-associated but may also appear in more advanced stage in younger patients in gCJD.

(b) NFTs restricted to the medial temporal lobe following or deviating from the Braak and Braak stages, i.e. sparing of the entorhinal cortex with more prominent NFT pathology and diffuse cytoplasmic neuronal immunoreactivity ('pretangles') in the CA4 subregion of the hippocampus or dentate gyrus (i.e. in gCJD).
(c) NFTs and diffuse cytoplasmic neuronal-tau immunoreactivity, together with variably prominent neuropil threads in subcortical regions (basal ganglia and brainstem), associated with PrP deposits lacking amyloid tinctorial properties in gCJD cases (i.e. E200K gCJD or FFI).
(d) Widespread NFTs in several subcortical, allo- and neocortical anatomical regions without predominance in the hippocampus. This is consistently associated with certain *PRNP* mutations associated with brain PrP deposits showing amyloid tinctorial properties (Ghetti et al. 2003); GSS or PrP-CAA phenotype.
(e) NFTs in allocortical and neocortical anatomical regions inconsistently present in certain *PRNP* mutations associated with GSS.

4. *Other types of tau pathologies* include the rare presence of glial tau immunoreactivity either in the form of oligodendroglial coiled bodies (usually restricted to the hippocampus) or tau astrogliopathy.
5. *Concomitant tau pathologies compatible with main tauopathies such as PSP, CBD or AGD.*

21.3.4 Perspectives

Recent widespread application of phospho-tau immunostaining has revealed a previously underrecognized spectrum of tau pathologies in human prion diseases. There are still several issues that merit further studies and clarification:

1. What is the relation between tau pathology and PrP deposition? Is there any evidence of cross-seeding between these two pathogenic proteins? Although small neuritic profiles correlate with the PrP load, the relation of further morphologies with PrP requires more studies (in particular in gCJD).
2. Why is the pattern of hippocampal tau pathology often deviating from the stages of NFTs described by Braak and Braak (1991)?
3. What further factors influence the appearance of tau pathology? In particular (i) why do GSS cases with various mutations, all by definition with prominent amyloidosis, considerably differ with regard to NFTs?; (ii) why does gCJD with the same single mutation (i.e. E200K) associate with a clearly distinct spectrum of tau pathologies, including subcortical and hippocampus predominant forms, while other cases show only small neuritic profiles?; (iii) why do a few cases with sCJD or gCJD show prominent astrocytic tau pathology in the gray matter without other features of main tauopathies?
4. How can the application of biomarkers for tau help to understand better the clinical relevance of concomitant tau pathologies?

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Chapter 22
Prion Protein Complex with mGluR5 Mediates Amyloid-ß Synaptic Loss in Alzheimer's Disease

Graham P. Roseman, Li Fu, and Stephen M. Strittmatter

Abstract Alzheimer's disease (AD) is the most common form of dementia affecting millions worldwide. The primary histopathological features of AD are amyloid-beta (Aβ) plaques and neurofibrillary tangles. Aβ oligomers (Aβo) are believed to be essential triggers of the cascade leading to the synaptotoxicity and cell death characteristic of the illness. Therefore, the mechanism of Aβo synaptotoxicity is central to AD pathophysiology. It is clear that the cellular prion protein (PrP^C) can act as a high-affinity binding partner for Aβo leading to memory and cognitive dysfunction, synaptic density impairment, long-term potentiation impairment, and neuronal dysfunction in AD transgenic mouse models. Moreover, mGluR5 physically associates with and mediates toxic signaling triggered by PrP^C and Aβo. Antagonizing PrP^C or mGluR5 with antibodies or antagonists reverses the memory deficits and restores synapse density in AD mouse models, indicating that targeting PrP^C or mGluR5 is a potential therapeutic target for AD.

Keywords Alzheimer · Amyloid β-peptide · Oligomer · Neurodegeneration · Signal transduction · Transgenic · Spatial memory · Long-term potentiation · Synaptic plasticity · Structural epitopes

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22.1 Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease and is responsible for 50–70% of all cases of dementia (Tiwari et al. 2019). An estimated 6.2 million Americans age 65 years and older are currently living with AD. However, this number could grow to an estimated 13.8 million by 2060 (Monica Moore et al. 2022). Seventy-two percent of those living with AD are over the age of 75 (Rajan et al. 2021). After onset of AD dementia, patients typically live between 3 and 8 years (Helzner et al. 2008). Currently, most of the available treatment options for AD are limited to partial efficacy and symptomatic control (Long and Holtzman 2019).

AD typically presents with amnestic memory loss, deteriorating language ability, and visual deficits (Cummings 2004). These symptoms are coupled with deposition of amyloid-β (Aβ) plaques composed of Aβ-peptide (Glenner and Wong 1984; Jack Jr et al. 2018) and tangles of the hyperphosphorylated microtubule-associated protein, tau (Kosik et al. 1986). The accumulation of these misfolded proteins is accompanied by synapse loss, innate immune activation, neurodegeneration, progressive cognitive decline, and eventually death.

The National Institute of Aging (NIA) and the Alzheimer's Association (AA) have created the Aβ, tau, neurodegeneration (ATN) research framework for the definition and diagnosis of Alzheimer's disease (Dubois et al. 2021; McKhann et al. 2011). It is believed that extracellular Aβ-oligomers (Aβo) initiate the early toxic signaling that results in the hyperphosphorylation of tau intracellularly (Bloom 2014). Thus, understanding how Aβo initiates neuronal damage is crucial for understanding early AD pathogenesis. This chapter focuses on the action of Aβo at the synaptic surface. Specifically, we review evidence that Aβo binds the cellular Prion Protein (PrP^C) to drive an interaction with the metabotropic glutamate receptor 5 (mGluR5) to initiate a signaling complex leading to synaptic dysfunction and synaptic loss.

22.2 Relevant Aβ Species in AD

Aβ-peptides are derived from the single-pass transmembrane protein amyloid precursor protein (APP). During AD pathology, APP undergoes sequential proteolysis by β-secretase and γ-secretase, generating Aβ-peptides primarily with a length of 40 (Aβ40) and 42 amino acids (Aβ42) (Goldgaber et al. 1987; Mills and Reiner 1999). Aβ40 and Aβ42 are present in healthy individuals, but the total peptide amounts accumulate to high levels in Alzheimer's-affected brain tissue. Moreover, there is an increase in the Aβ42 to Aβ40 ratio in the brain of those afflicted with AD (Hardy and Selkoe 2002).

The first 20–30 amino acids of Aβ-peptides are hydrophilic, whereas the more C-terminal amino acids are hydrophobic. These hydrophobic amino acids are

crucial to the aggregation-prone nature of these peptides, leading them to misfold into a variety of different amyloid species rich in β-sheet secondary structure. The formation of Aβo starts from alterations in the conformation of monomeric Aβ (molecular weight ~4 kDa). This leads to a variety of species from low-molecular-weight dimers (Garzon-Rodriguez et al. 1997; Hilbich et al. 1992; Roher et al. 1996; Walsh et al. 2000), trimers (Chen and Glabe 2006; Townsend et al. 2006; Walsh et al. 2000), tetramers (Chen and Glabe 2006; Walsh et al. 2000), pentamers and decamers (Ahmed et al. 2010), dodecamers, Aβ*56 (Lesne et al. 2006), globulomers (Barghorn et al. 2005), Aβ-derived diffusible ligands (ADDLs) (Chromy et al. 2003; Hepler et al. 2006; Klein et al. 2004; Lambert et al. 1998), to larger species including amylospheroids, annular protofibrils, prefibrillar aggregates or protofibrils. Extended insoluble fibrils aggregate and deposit in plaques. Among all these Aβ assemblies, smaller oligomers are the most damaging species in AD (Benilova et al. 2012; Lee et al. 2019b). There is a substantial correlation between the soluble Aβo level and the severity of synaptic loss and cognitive decline in AD (Caughey and Lansbury 2003; Chiti and Dobson 2006; Ferreira et al. 2007; Haass and Selkoe 2007; Klein et al. 2001; Kostylev et al. 2018; LaFerla et al. 2007).

The synaptic dysfunction and memory impairment observed in 3XTg-AD transgenic mice are associated with elevated soluble Aβo species (Haass and Selkoe 2007; McGowan et al. 2006; Yamin 2009). Additionally, memory dysfunction (Figueiredo et al. 2013; Reed et al. 2011; Walsh et al. 2005) and memory retention impairment (Lesne et al. 2006) measured by the Morris water maze (MWM) is observed after small quantities of Aβo is injected into the intracerebral ventricle of wild-type animals. Furthermore, long-term potentiation (LTP) is suppressed, and long-term depression is enhanced by either synthetic or brain-derived Aβo both in slice preparations and animal models (Klyubin et al. 2008; Lambert et al. 1998; Shankar et al. 2008; Townsend et al. 2006; Viola and Klein 2015; Walsh et al. 2005). These actions can impair memory acutely and are followed by synaptic loss more chronically.

22.3 Aβo Bind to PrP^C

To better understand the early mechanisms of AD pathogenesis, it is important to determine the cell surface receptors that bind selectively to Aβo over monomers or fibrils. An unbiased screen of over 220,000 clones expressed in COS-7 cells, PrP^C was the only isolated high-affinity binding site for Aβo (Lauren et al. 2009). PrP^C is unique in its high selectivity for the oligomeric state, as opposed to monomer or fibrillar Aß peptide (Chen et al. 2010; Fluharty et al. 2013; Haas et al. 2014; Kostylev et al. 2015; Lauren et al. 2009; Smith et al. 2018; Um et al. 2012). PrP^C is a glycosylphosphatidylinositol-anchored protein ubiquitously expressed throughout the nervous system and is best known for its role in transmissible spongiform encephalopathies. It was later demonstrated using primary neurons that Aβo cell surface binding is reduced upon genetic depletion of PrP^C (Lauren et al. 2009).

Using mutagenesis and antibody competition assays in COS-7 cells, Aβo were found to bind to the polybasic N-terminus (aa 23–27) (Chen et al. 2010) as well as around the central charge cluster (aa 95–111) of PrP^C (Lauren et al. 2009). Additionally, brain slices from PrP^C knockout mice do not exhibit Aβo-induced inhibition of LTP (Lauren et al. 2009).

Recent research has demonstrated in vitro binding of recombinantly expressed PrP^C to globulomeric Aβo drives the formation of a hydrogel phase that is separable from the supernatant by natural settling or accelerated by centrifugation (Kostylev et al. 2018). The Aβ-globulomers are known to consist of 12 Aβ-monomers per oligomer (Barghorn et al. 2005). The hydrogel is produced stoichiometrically with two PrP^C molecules and one Aβ-globulomer (Kostylev et al. 2018). Hydrogel formation is reversible with the addition of more Aβo. The hydrogel formed is dependent on Aβo binding to PrP^C lysine residues at both the extreme N-terminus and the central charge cluster. Fluorescence recovery after photobleaching (FRAP) measurements of COS7 cells transfected with SNAP-PrP^C demonstrate a reduced PrP^C lateral movement upon Aβo binding. Solid-state nuclear magnetic resonance (NMR) measurements of in vitro prepared PrP^C–Aβo hydrogel find that resonances of PrP^C's glycine and alanine amino acids, most of which are found in PrP^C's unstructured N-terminus, shift from random coil to α-helical secondary structure. Thus, upon hydrogel formation, the N-terminal domain of PrP^C is restructured.

Despite the plethora of literature emphasizing selective binding of Aβo to PrP^C and its role in AD (Purro et al. 2018), there is also some evidence for the binding of Aβ-fibrils and monomers to PrP^C. One study showed that recombinant PrP^C can bind to Aβ-fibrils, leading to inhibition of fibril elongation rather than the initial nucleation or secondary nucleation steps in the formation of Aβ-fibrils (Bove-Fenderson et al. 2017). Using kinetic models of fibril formation, the authors implied that PrP^C binds to the ends of the growing fibrils. Interestingly, this study also found that the binding event was dependent on the presence of both the N- and C-terminal domains of PrP^C. A follow-up study used super-resolution fluorescent microscopy to show that PrP^C selectively binds to the rapidly growing end of Aβ-fibrils (Amin and Harris 2021). PrP^C binds to these fibrils in a similar fashion to Aβo, suggesting a similar structure of the ends of fibrils to Aβo. In a different study looking at PrP^C-dependent Aβ endocytosis using a soluble, and presumably monomeric, Aβ-peptide consisting of amino acids 1–30 [Aβ(1–30)], was able to demonstrate stereoselective endocytosis (Foley et al. 2020). Additionally, solution NMR showed broadening of amino acids of PrP^C near the central charge cluster, demonstrating that this peptide can bind to PrP^C and that this potentially contains the binding site for PrP^C on Aβ-peptide.

In AD transgenic mice expressing APPswe and PSen1ΔE9 (APP/PS1), Aβ plaques accumulate, and the mice have an impaired hippocampal-dependent spatial learning and memory as measured by the MWM (Radde et al. 2006; Serneels et al. 2009). These mice also have early synaptic dysfunction (Dickey et al. 2003; Heiss et al. 2017). It was found that transgenic APP/PS1 animals devoid of PrP^C performed better in the MWM, showed an increase in synaptic markers, and had a

longer survival time as compared to APP/PS1 mice with PrP^C (Gimbel et al. 2010). Additional work revealed that synaptic responsiveness in hippocampal slices from young adult PrP^C null mice is normal, but the Aβo-induced inhibition of LTP is absent (Lauren et al. 2009). Moreover, PrP^C deletion blocked Aβo-driven synaptic loss in 12-month-old APP/PS1 transgenic mice indicating the critical role of PrP^C in mediating learning and memory deficits in this AD mouse model (Gimbel et al. 2010). These AD mice show degeneration of serotonergic axons, which is prevented by PrP^C deletion. Moreover, conditional deletion of PrP^C rescued APP/PS1 memory and synaptic deficits after deficits were established (Salazar et al. 2017). Overall, this demonstrates that PrP^C expression is at least partially required for the memory deficits and synaptic changes seen in these Aβ positive AD model mice (Corbett et al. 2020; Gimbel et al. 2010; Haas et al. 2016; Heiss et al. 2017; Lauren et al. 2009; Purro et al. 2018; Salazar et al. 2017).

22.4 Therapeutic Strategies Blocking the Aβo-PrP^C Interaction

Therapeutic targeting of the PrP^C–Aβo complex for use as potential AD treatments has been studied extensively (Purro et al. 2018). Aβo-induced inhibition of LTP was blocked by the PrP^C antibody 6D11, targeting the central charge cluster (Lauren et al. 2009), and ICSM-18, which targets alpha helix one of PrP^C (Freir et al. 2011). Early experiments using intraperitoneal injection of the PrP^C antibody 6D11 targeting– showed a rescue of behavioral deficits found in APP/PS1 mice but did not show a reduction in Aβ-plaque burden (Chung et al. 2010). An additional study demonstrated that intravascular injection of the fully humanized ICSM-18 antibody, PRN100, into rats was able to block brain injected soluble AD brain extract induced inhibition of LTP (Klyubin et al. 2014). More recently, the use of the PrP^C antibody AZ59, which targets the Aßo binding site in the N-terminus, was also able to rescue behavior deficits, memory function, and hippocampal synaptic density in a preclinical mouse model of AD (Cox et al. 2019). Importantly for clinical significance, intraperitoneal dosages of AZ59 reached brain concentrations well above its affinity for PrP^C, demonstrating the efficacy of potential antibody treatments.

Using a small molecule library screen of about 10,000 compounds aimed at blocking Aβo from binding to PrP^C, a degradation product of the antibiotic cefixime, named compound Z, was found to strongly inhibit the binding (Gunther et al. 2019). Although the exact chemical structure of compound Z was not elucidated, it behaved as a poly-anion. From structure–activity relationships, it was determined that compounds with poly-anionic functional groups interspersed with hydrophobic moieties was able to block Aβo binding to PrP^C. The optimized 17 kDa polymer poly (4-styrenesulfonic acid-co-maleic acid) (PSCMA) blocked Aβo from binding to PrP^C transfected COS7 cells or primary neurons with an IC_{50} of 3.4 nM and 32 nM, respectively. FRAP measurements demonstrated that unlike Aβo, PSCMA

does not lead to a reduced lateral diffusion of cell surface PrP^C. Furthermore, oral gavage treatment of PSCMA in APP/PS1 mice demonstrated that PSCMA can pass through the blood–brain barrier and block Aβo binding to PrP^C. Notably, PSCMA treatment led to rescue of memory deficits and synapse density of APP/PS1 mice in the MWM and synaptopathology.

22.5 mGluR5 Facilitates Aβo-PrP^C-Induced Toxicity

PrP^C is anchored to the outer leaflet of neurons, implying that there must be a transducer of the toxic signal across the neuronal cell membrane leading to AD-related molecular markers such as the activation of Fyn kinase. A screen of most transmembrane proteins enriched in the postsynaptic density revealed that only mGluR5 allowed for an Aβo-PrP^C-dependent activation of Fyn-kinase in HEK293T cells co-transfected to express PrP^C and Fyn-kinase (Um et al. 2013) (Fig. 22.1). mGluR5 is a Class C G-protein coupled receptor with a very large extracellular domain that upon glutamate binding causes large conformational changes leading to changes in the transmembrane domain and subsequent activation of intracellular signaling. In transfected cells, mGluR5 and PrP^C co-immunoprecipitate together. Furthermore, the addition of Aβo to cells co-expressing mGluR5 and PrP^C led to an increase in the amount of mGluR5 co-immunoprecipitated with PrP^C. Chimeric constructs of mGluR5 and mGluR8, which do not bind to PrP^C, show a reduction in PrP^C

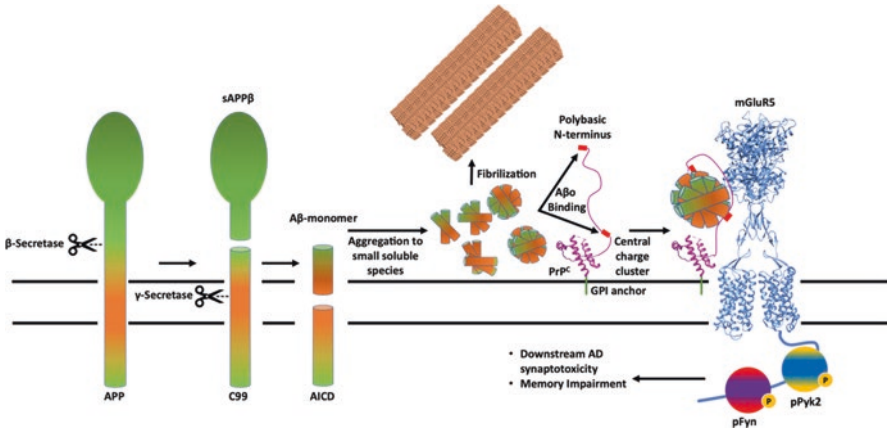

Fig. 22.1 Model for Aβo Interaction with PrP^C and mGluR5 in Alzheimer's. Aβ-peptide is first generated by sequential proteolysis of APP by β-secretase and γ-secretase. Aβ-peptides aggregate together to form various soluble oligomers, eventually leading to insoluble fibrils. However, the oligomers interact with PrP^C on the synaptic membrane to drive an interaction with mGluR5 leading to Fyn and Pyk2 phosphorylation and ultimately downstream AD synaptotoxicity and memory impairment. The PrP^C and mGluR5 ribbon structures are from PDB 6DU9 and PDB 6 N51, respectively

co-immunoprecipitation (Haas et al. 2014), suggesting the PrP^C–mGluR5 interaction surface spans multiple mGluR5 domains. Furthermore, antibody competition binding and deletion studies (Haas et al. 2014) have demonstrated that PrP^C binds to mGluR5 via amino residues 91–153 of PrP^C.

When detergent-solubilized mGluR5 is included in the PrP^C-Aβo hydrogel formation reaction, mGluR5 is also driven into the hydrogel phase with PrP^C and Aβo (Kostylev et al. 2018). Additional FRAP measurements of CLIP-mGLuR5 transfected COS-7 cells showed that Aβo treatment induced a reduction in lateral movement of mGluR5, which is dependent on co-expression of PrP^C. Treatment of cells co-expressing PrP^C and mGluR5 with the PrP^C antibody AZ59 caused a reduction in the Aβo-induced association of PrP^C and mGluR5 (Cox et al. 2019). Overall, this suggests that Aβo toxicity at the synapse occurs via elevating the levels of PrP^C–mGluR5 interaction complex.

Not only are mGluR5 and PrP^C enriched in the postsynaptic density, the two proteins physically associate in the brain (Um et al. 2013). On the other hand, Aβo binding to the cell surface is exclusively PrP^C dependent and is unaltered upon mGluR5 co-expression. Thus, there is a pair-wise physical interaction network of Aβo–PrP^C, PrP^C–mGluR5, and mGluR5–Fyn (Um et al. 2013).

Downstream signaling initiated by the complex of Aβo–PrP^C–mGluR5 involves Fyn kinase, protein tyrosine kinase 2 (Pyk2) kinase, Homer, eukaryotic elongation factor 2 (eEF2), Ca2 +/calmodulin-dependent protein kinase II (CamKII), phospholipase C, inositol trisphosphate, and intracellular calcium. Each of these may contribute to the deleterious effects on synaptic transmission and neuronal maintenance during AD progression. mGluR5 is crucial for linking Aßo–PrP^C to Fyn and Pyk2 kinases (Beraldo et al. 2016; Bhakar et al. 2012; Haas et al. 2014, 2016, 2017; Hamilton et al. 2014, 2016; Heidinger et al. 2002; Hu et al. 2014; Kaufman et al. 2015; Lee et al. 2019b; Lesne et al. 2006; Li et al. 2009; Luscher and Huber 2010; Nicodemo et al. 2010; Salazar et al. 2019; Um et al. 2012, 2013; Zhang et al. 2017). Synaptic plasticity is regulated by the phosphorylation of NR2A and NR2B by Fyn (Grant et al. 1992; Nakazawa et al. 2001; Suzuki et al. 1995). Aβo induces phosphorylation of NR2B in a manner dependent on PrP^C and Fyn expression (Um et al. 2012).

A key mediator of Aβo signaling through PrP^C and mGluR5 is activation of the tyrosine protein kinase Pyk2, encoded by *PTK2B*. Genetic variation of *PTK2B* is linked to late-onset AD risk (Beecham et al. 2014; Jansen et al. 2019; Kamboh et al. 2012; Kunkle et al. 2019; Lambert et al. 2013; Li et al. 2016), providing human genetic validation to this pathway. The physical association of Pyk2 with mGluR5 is decreased by Aβo-induced PrP^C signaling and allows for Pyk2 activation (Haas et al. 2016). Pyk2 is known to regulate synaptic plasticity, to interact directly with Fyn, and to be fully activated after phosphorylation by Fyn (Collins et al. 2010a, b; Park et al. 2004). Moreover, Pyk2 is required for memory deficits and synaptic loss in AD transgenic mice (Salazar et al. 2019). At least one mechanism for Pyk2 function in AD-related dendritic spine loss is through the adaptor Graf1 and RhoA GTPase activation (Lee et al. 2019a).

The phosphorylation of the protein translation regulator, eEF2, by Aβo in cortical neurons is also dependent on Fyn signaling. Moreover, the Fyn inhibitor saracatinib fully blocks Aβo-induced eEF2 phosphorylation and dendritic spine loss (Um et al. 2013) as well as memory impairment in APP/PS1 mice (Smith et al. 2018). Furthermore, eEF2 signaling occurs downstream of Fyn signaling and depends on the expression of PrP^C and mGluR5 (Um et al. 2013). Future studies are needed to assess the relative roles and interplays among Fyn, eEF2, Pyk2, CamKII, and intracellular calcium as mediators of the pathophysiology of the Aβo–PrP^C–mGluR5 complex.

22.6 Targeting mGluR5 with Allosteric Modulators as Novel Therapy for AD

Early studies focused on treating Aβo-PrP^C dependent AD neuropathogenesis by targeting PrP^C with antibodies. With mGluR5 acting as a physical transducer of Aβo-PrP^C-driven neurotoxicity, use of allosteric modulators of mGluR5 provides an additional avenue for AD treatment amenable to small molecule targeting. The negative allosteric modulator (NAM) of mGluR5, MTEP, led to a reduction in Aβo-induced synaptotoxicity in primary neurons as well as rescue of learning deficits in APP/PS1 mice (Haas et al. 2014; Um et al. 2013). Another study showed that chronic, but not acute, treatment with the negative allosteric modulator CTEP, reversed memory deficits and AD pathology in two different AD mouse models (Hamilton et al. 2016). However, MTEP and CTEP both inhibit normal glutamate signaling through mGluR5, leading to abnormal synaptic physiology and memory impairments in wild-type mice (Haas et al. 2017). Thus, dosing with NAMs is problematic because slightly higher doses impair glutamate function and impair learning and memory in healthy brains.

The discovery of mGluR5 silent allosteric modulators (SAM) that do not show any antagonist or agonistic effects with regards to basal or glutamate signaling provides the means to avoid NAM related toxicity and greatly broaden the therapeutic window for mGluR5 targeting in AD. Several mGluR5 SAMs potently inhibit AD signaling. Initially, the mGluR5 SAM 3,3′-Dichlorobenzaldazine was shown to reduce the Aβo-induced PrP^C–mGluR5 interaction (Haas et al. 2014). However, it has low potency for mGluR5. The SAM, BMS-984923, has sub-nanomolar mGluR5 affinity and also reduces Aβo-induced interaction of PrP^C and mGluR5 (Haas et al. 2017; Huang et al. 2016). BMS-984923 rescued Aβo-induced inhibition of LTP in wild-type hippocampal slices. Additionally, BMS-984923 rescues synaptic density loss and memory deficits in APP/PS1 transgenic mice. Similar to previous results using PrP^C-directed antibodies, BMS-984923 does not affect plaque burden or gliosis in APP/PS1 mice. All of the above indicates the potential for the use of mGluR5 SAM drugs at blocking the PrP^C–mGluR5-dependent toxicity seen in AD.

22.7 Future Outlook

Widely used and approved AD therapies treat the symptoms but do not modify disease course. At this point, there is an abundance of literature implicating the Aβo–PrP^C–mGluR5 complex as a key mediator initiating downstream synaptotoxicity observed in AD. Pharmacological use of antibodies, polymers, and small molecules aimed at disrupting this PrP–mGluR5 complex reverses AD-related memory deficits and synaptopathologies despite the persistence of Aβ plaque and brain inflammation. The BMS-984923 is now in clinical trials for AD (ClinicalTrials.gov Identifier: NCT04805983). The atomic structure of mGluR5 has been solved by cryo-electron microscopy (Koehl et al. 2019; Nasrallah et al. 2021). Coupled with structures of Aβ and PrP^C, rational drug design for AD may be optimized. Future work on the downstream signaling cascade driven by the Aβo–PrP^C–mGluR5 complex may discover additional therapeutic avenues. Lastly, characterization of the different Aβo species able to bind to PrP^C and transmit a toxic signal through mGluR5 may further aid the design of effective interventions.

Competing Interests S.M.S. is founder, equity holder and paid consultant of Allyx Therapeutics, which seeks to develop mGluR5 compounds for Alzheimer's disease and holds a license for BMS-984923.

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Chapter 23
Prion and Cancers

Wei Xin

Abstract Prion protein (PrP) is a glycosylphosphatidylinositol (GPI)-anchored, highly conserved, and ubiquitously expressed glycoprotein. In human, the PrP is first synthesized as a pre-pro-PrP and then processed as pro-PrP with both ends removed. Finally, the mature GPI-anchored PrP will be formed with the addition of a GPI anchor and two N-linked glycans. PrPs are up-regulated in many cancer types. In breast, stomach, and colorectal cancers, PrPs exert effects on drug resistance, invasiveness, and protect the tumor cells by regulating the apoptosis pathway. In pancreas cancer and melanoma, the main form of PrP is pro-PrP not normal mature PrP. As pro-PrP and PrP have different biological functions, Pro-PrP binds to Filamin A (FLNA) and interacts with Notch1, forming a PrP/FLNA/Notch1 complex. The inhibition of PrP decreases Notch1 expression and Notch1 signaling, which exhibited decreased proliferation, xenograft growth, and tumor invasion but showed increased tumor apoptosis. Targeting PrP combined with anti-Notch would have a synergic effect on inhibiting PDACs. Furthermore, since high levels of soluble PrP are detected in the culture supernatants of the PDAC cell lines, the detection of pro-PrP from fecal materials or pancreatic ductal fluids may provide an early and noninvasive method for detecting PDAC.

Keywords Prion · PrP · Notch · Pancreatic cancer · Melanoma

23.1 Introduction of Prion Protein

Transmissible spongiform encephalopathies (TSE) or prion diseases are a group of fatal neurodegenerative disorders that affect both humans and animals. In humans, TSE include Creutzfeldt–Jakob disease, fatal insomnia, and Gerstmann–Sträussler–Scheinker disease. In animals, TSE include scrapie in sheep and goat, bovine spongiform encephalopathy (known as Mad Cow Disease), chronic wasting disease in

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elk and deer, transmissible mink encephalopathy and transmissible spongiform encephalopathy of domestic and captive zoo animals (Bolton et al. 1982; Diener et al. 1982; Prusiner 1982, 1991).

Griffith was the first to propose that the pathogen for TSE is a protein (Griffith 1967). Griffith proposed three mechanisms by which this might happen: a protein that turns on its own transcription; an altered form of a protein that catalyzes the conversion of the normal form into the same altered form through formation of an oligomer-like crystal seed; and an antibody that stimulates its own production. However, it was Prusiner and his colleagues who made the fundamental discovery that led to the current understanding of TSE. Prusiner and colleagues identified and sequenced the pathogen, which was subsequently found to be an aberrant form of a highly conserved normal protein in mammals. They named this agent as a proteinaceous infectious particle, prion. Since then prion diseases have been used synonymously with TSE. All three forms of prion diseases, the infectious, the inherited, and the sporadic forms, are believed to share the same pathogenic mechanism that is based on the conversion of the normal PrP into the pathogenic, scrapie PrP (PrP^{Sc}) (Prusiner 1996).

The human prion gene, *PRNP,* is located on chromosome 20, at 20p13, with a three-exon structure. The third exon contains the entire open reading frame of the protein, which encodes prion protein (PrP). PrP is a glycosylphosphatidylinositol (GPI)-anchored, highly conserved, and ubiquitously expressed glycoprotein (Kretzschmar et al. 1986; Harris 1999).

In human, the PrP is first synthesized as a pre-pro-PrP of 253 amino acids in the cytosol (Fig. 23.1). The first 22 amino acids at the N-terminus contain the leader peptide sequence, while the last 22 amino acids at the C-terminus encompass the GPI anchor peptide signal sequence (GPI-PSS). Both these sequences are removed in the endoplasmic reticulum and thus are not present in the mature GPI-anchored PrP. Addition of a GPI anchor and two N-linked glycans co-translationally completes the synthesis of a mature GPI-anchored and glycosylated PrP.

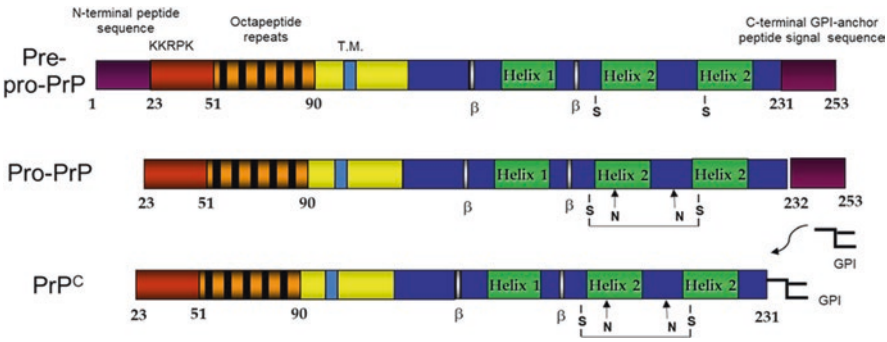

Fig. 23.1 The post-translational modification of the normal cellular PrP protein. Starting from Pre-pro-PrP, then Pro-PrP to the final mature product of PrP

The mature product of PrP contains 209 amino acids from residue 23 to 231 and divided into three major domains based on the structural motifs. The N-terminal domain includes the first 90 amino acids and is thought to be unstructured. This region also has a highly conserved motif of five repeating octapeptides. The central domain is located between amino acids 110 and 130. The C-terminal region contains a well-defined, globular domain that has two potential N-linked glycosylation sites and a disulfide bridge (Donne et al. 1997; Prusiner et al. 1998; Safar and Prusiner 1998; Williamson et al. 1998). The protein backbone of the PrP has a molecular weight of approximately 23 kDa. However, with the post-translational modification, the addition of two N-linked glycans, and a GPI anchor, the final completed PrP will have an approximate molecular weight of 34–39 kDa. Despite the fact that PrP is a relatively small protein, the synthesis, processing, and transit of PrP are complex, cell-context dependent, and not completely understood (Brown et al. 1997; Hope 1999; Hunter 1999; Kretzschmar 1999; Pergami et al. 1999).

In addition to cells in the CNS, PrP is expressed in many cell types (Pergami et al. 1999). The octapeptide repeats at the N-terminus of PrP contain four binding sites for divalent cations, such as Cu^{2+} and Zn^{2+}. Evidence suggests that PrP functions as a metal transporter (Viles et al. 1999; Wadsworth et al. 1999; Whittal et al. 2000). Some reports found that when PrP binds copper it has anti-oxidant activity. The N-terminus of all mammalian PrP also contains a glycosaminoglycan (GAG)-binding motif. Binding of GAG has been speculated to be important in prion disease pathogenesis (Brown et al. 1997; Aguzzi 2000; Aguzzi et al. 2000; Aronoff-Spencer et al. 2000; Bonomo et al. 2000).

Like many other GPI-anchored proteins, PrP is present in a micro-domain on the cell surface commonly referred to as lipid rafts. Lipid rafts are special domains on the cell surface where signaling protein complexes are organized. PrP is detected in caveolae in association with caveollin-1. However, both neurons and lymphocytes express PrP, but these cells lack caveolae (Harmey et al. 1995; Vey et al. 1996; Massimino et al. 2002; Prado et al. 2004). Therefore, the arrangement of PrP on the cell surface is likely to be cell-type dependent.

In addition to binding metals and GAG, PrP also interacts with laminin receptors, N-CAM, lipids, heat shock proteins, nucleic acids, chaperon protein, stress-inducible protein and transcription factors (Martins and Brentani 2002; Martins et al. 2002). PrPs have a putative nuclear localization signal and thus can function as a nuclear transport protein (Jaegly et al. 1998; Gu et al. 2003). PrP is expressed on bone marrow progenitor cells. $PrP^{-/-}$ stem cells are less efficient in engrafting irradiated host, suggesting that PrP is critical during hematopoietic development (Dodelet and Cashman 1998).

PrP has been reported to possess pro-apoptotic activity in primary murine neurons and in human HEK293 cells. PrP controls the functions of p53 at transcriptional and translational levels (Paitel et al. 2002; Kim et al. 2004a, b). On the other hand, other laboratories have reported that PrP has anti-apoptotic activities in breast cancer cell lines (Roucou et al. 2005; Bounhar et al. 2006) and neuroblastoma cells (Zafar et al. 2017) by anti-staurosporine-induced apoptosis. PrP-expressing neuronal cell lines are more resistant to apoptosis than PrP-negative cell lines (Kuwahara et al. 1999). PrP

transduces neuroprotective signals (Brown et al. 2002). PrP inhibits the functions of Bax and thus protects human neurons against Bax-mediated apoptosis in breast cancer cell lines (Bounhar et al. 2001, 2006). These differences may reflect the use of neurons from different species or the natures of the cell types studied.

23.2 PrP and Cancers

23.2.1 Breast Cancer

Since the discovery of PrP, most of the PrP studies have been focused on the role it plays in neurodegenerative disease. With the finding of PrP regulating apoptosis, more and more studies have been shifted on the possible role of this protein involving in cell survival and proliferation.

With the development of modern technology, such as cDNA microarray technique, one group found that PrP was up-regulated at both transcriptional and translational levels in TNF (tissue necrotizing factor)-resistant breast cancer cell lines compared to that of TNF-sensitive breast cancer MCF7 cell lines (Diarra-Mehrpour et al. 2004). They identified many genes in PI3K/Akt pathways involving the TNF resistance. Also interestingly, they showed that over-expression of PrP at both transcriptional (mRNA, 17-fold) and translational levels (protein, 10-fold) in TNF-resistant cell lines compared to those of TNF-sensitive cell lines. By using recombinant adenoviruses, they could convert TNF-sensitive cells into TNF-resistant cells. The study suggested that PrP might induce cancer cell's resistance to TNF by involving the PI3K/Akt pathway. They also found that PrP might regulate P53 expression and suggested that the resistant process might be related to the apoptotic cascades involving P53. Thus, it appears that the over-expression of PrP could protect breast cancer cells from TNF-mediated injury.

Another study suggests that suppression of PrP expression may facilitate the activation of proapoptotic Bax by down-regulation of Bcl-2 expression and thus reduces the resistance to TRAIL-induced apoptosis in breast cancer cells. These investigators studied the relationship between the resistance to the pro-apoptotic action of TNF-related apoptosis inducing ligand (TRAIL) and PrP function. They compared a TRAIL-sensitive MCF-7 human breast adenocarcinoma cell line with two TRAIL-resistant sublines: 2101 and MCF-7/ADR to Adriamycin, an apoptosis-inducing agent. It was found that the down-regulation of PrP by small interfering RNA increased the sensitivity of Adriamycin- and TRAIL-resistant cells to TRAIL but not to Epirubicin/Adriamycin. They also found that Bcl-2 expression was substantially decreased after PrP inhibition but the levels of Bcl-X(L) and Mcl-1 were not affected and the down-regulation of Bcl-2 expression was accompanied with Bax relocalization. Based on these findings, these investigators concluded that the inhibition of PrP expression promotes the activation of proapoptotic Bax by down-regulation of Bcl-2 expression, thereby abolishing the resistance of breast cancer cells to TRAIL-induced apoptosis (Meslin et al. 2007b).

Expression of PrP was also associated with the resistance to adjuvant chemotherapy in patients with estrogen receptor (ER)-negative breast cancer. In this study, the investigators found that by immunohistochemical staining PrP was mainly expressed by myoepithelial cells in normal breast tissue. The tissue microarray analysis from 756 breast tumors showed that PrP was associated with ER-negative breast cancer subsets ($P < 0.001$). The administration of the anthracycline-based adjuvant chemotherapy was not associated with a significant risk reduction for death in patients with ER-negative/PrP-positive disease, but it decreased the risk for death in patients with ER-negative/PrP-negative tumors. They concluded that the ER-negative/PrP-negative phenotype is associated with an enhanced sensitivity to adjuvant chemotherapy (Meslin et al. 2007a; Mehrpour and Codogno 2010). Another study also revealed that silencing PrP in breast cancer cells could affect chemotherapy susceptibility by interfering P53 pathway (Yu and Jiang 2012).

Over-expression of PrP also showed the ability to increase the ability of invasion and migration of breast cancer cell line. The inhibition of PrP expression by siRNA could inhibit breast cancer cell migration and invasion in vitro through the regulation of ERK pathway and MMP-9 involvement (Gil et al. 2016). Another study also showed that anthracyclines could be sequestered by secreted PrP and blocking their cytotoxic activity and led to chemotherapy resistance. The inhibition of PrP expression restored the cytotoxic activity (Wiegmans et al. 2019).

23.2.2 Gastric Cancer

Fan and his colleagues were the first group to report that PrP was over-expressed in some gastric carcinoma cell lines. Subsequently, this group of investigators reported that over-expression of PrP in gastric cancer cell lines was associated with the resistance to both P-glycoprotein (P-gp)-related and P-gp-nonrelated drugs. Inhibition of the PrP expression by antisense or RNAi partially reversed the multidrug resistance. PrP also suppresses adriamycin-induced apoptosis by altering the expression of Bcl-2 and Bax (Du et al. 2005). The inhibition of the PrP expression by RNAi in the gastric cancer cell line could suppress ROS and slow down apoptosis in these cells. They proposed the mechanism by which PrP modulates the apoptotic pathway, functioning as an anti-apoptotic protein through Bcl-2-dependent pathways (Liang et al. 2006).

By immunohistochemical staining, gastric adenocarcinoma with increased PrP expression also correlated with the clinical staging. PrP was over-expressed in metastatic gastric cancers compared to non-metastatic cancer. Expression of PrP promotes the adhesion, invasion, and in vivo metastasis of gastric cancer cell lines SGC7901 and MKN45 in xenograft models. Mechanistically, PrP appears to increase the promoter activity and the expression of MMP1. It was suggested that the N-terminal region of PrP might promote the invasion and metastatic ability of the tumor cells partially through activation of MEK/ERK pathway and consequently by transactivation of MMP11. They also reported that over-expression of PrP might

promote the tumorigenesis and proliferation of gastric cancer cells partially through the activation of PI3K/Akt pathway, activation of CyclinD1 to regulate the G1/S phase transition. It was reported that the octapeptide repeat region might play an role in promoting the proliferation of gastric cancer cells, as cancer cells proliferation with more octapeptide repeats have a more rapid proliferation rate (Pan et al. 2006; Liang et al. 2009). One study also showed that the co-expressions of PrP and MGr1-Ag/37LRP, a protein interacting with PrP, indicate the poor prognosis in gastric cancer and poor response to therapy (Zhou et al. 2014).

23.2.3 Colorectal Cancers

By using expression microarray, a study showed that PrP was over-expressed in colorectal cancer. Along with other proteins, PrP had a significant difference in the expression levels between the right colonic and the rectal cancers. PrP expression constituted an independent prognostic factor of the 3-year survival in multivariate analysis (Antonacopoulou et al. 2008).

The above studies all showed that PrP involved in breast and gastric cancers through anti-apoptosis. Another group utilized different antibodies against different PrP regions to investigate whether these antibodies could induce apoptosis and be utilized in the treatment of these cancers. They found that different antibodies against PrP had varying degrees of anti-proliferative activity, and some antibodies were particularly potent and afforded >40% reduction in proliferation. In combination therapy experiments, antibodies to PrP could induce apoptosis and variably enhanced the anti-tumoral effect of irinotecan, 5-FU, cisplatin and doxorubicin. In different colon cancer cell lines, antibody effectiveness correlated with tumor aggressiveness. The administration of PrP antibody in vivo nude mouse could inhibit human HCT 116 xenografts (McEwan et al. 2009). A proteomics study on surface proteins of CRC cell lines revealed GLUT1 and PrP along with some other proteins could be used as biomarkers for adenoma-to-carcinoma progression (Wit et al. 2012). The role of PrP in colon carcinogenesis is similar to that of in breast cancer by inhibiting apoptosis. The overexpression of PrP was shown to increase cell proliferation, increase cell motility and invasiveness in vitro (Chieng and Say 2015). PrP also has the target for the therapeutic purpose and some in vitro study showed that the inhibition of PrP expression could enhance the chemotherapy effect (Yun et al. 2016).

23.2.4 Pancreatic Ductal Carcinoma

Pancreatic ductal carcinoma (PDAC) is one of the most deadly solid cancer with a 5-year survival rate of about 6%. In a microarray study, expression of *PRNP* was up-regulated in 5 out of 5 pancreatic cancer cell lines. At the protein level, our group

found that all human pancreatic ductal adenocarcinoma (PDAC) cell lines ($n = 7$) in our study expressed PrP. On the other hand, in normal pancreas, only islet cells have detectable PrP; neither acinar cells nor ductal cells, which are thought to be the precursors of PDAC, have detectable PrP (Li et al. 2009, 2010; Sy et al. 2010). However, the PrP in pancreatic cancer cells is different from the normal forms, and the PrP was neither glycosylated nor GPI-anchored; it existed as pro-PrP retaining its GPI-PSS. Unexpectedly, we also found that the PrP GPI-PSS has a filamin A-binding (FLNa-binding) motif and interacted with FLNa. FLNa is an actin-binding protein that integrates cell mechanics and signaling. Binding of pro-PrP to FLNa disrupted cytoskeletal organization. Inhibition of PrP expression by shRNA in the PDAC cell lines altered the cytoskeleton and expression of multiple signaling proteins; it also reduced cellular proliferation and invasiveness in vitro as well as tumor growth as xenografts in vivo.

A subgroup of human patients with pancreatic cancer was found to have tumors that expressed pro-PrP. Most importantly, PrP expression in tumors correlated with a marked decrease in patient survival. Therefore, the binding of pro-PrP to FLNa perturbs FLNa function, thus contributing to the aggressiveness of PDAC. Prevention of this interaction could provide an attractive target for therapeutic intervention in human PDAC.

23.2.4.1 GPI-PSS Has a Specific Biological Function

The finding that in PDAC PrP exists is Pro-PrP is fascinating. Due to efficient processing and competent quality control system in normal cells, pro-PrP is undetectable in normal cells. What is the significance of the accumulation of pro-PrP in PDACs? The GPI modification pathway is complex and not completely understood. In human, there are approximately 0.5–1% of the genome that encodes for GPI-anchored proteins (Ikezawa 2002; Maeda et al. 2006; Orlean and Menon 2007; Wiedman et al. 2007). More than 24 genes are involved in this process, and the biosynthesis of the GPI anchors and their attachment to proteins are complex, protein specific and depend on the cell context. The common core structure of the GPI anchor is synthesized in the endoplasmic reticulum in a stepwise mechanism. First catalyzed by a a1–6 GlcNAc transferase complex, which is composed of seven gene products: PIG-A, PIG-C, PIG-H, GPI-1, PIG-Y, PIG-P and DPM2; it transfers *N*-acetyl-glucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) to form GlcNAc-PI. Second, the compound is de-*N*-acetylated by PIG-L to generate GlcN-PI. Then, three mannose residues are sequentially added. The last step is helped by transamidase, the formed GPI complex will be attached to the pro-protein, with the simultaneously cleavage of the C-terminal GPI-PSS, at a site known as the w site, which is confined to amino acids glycine, serine, cysteine, alanine, aspartic acid and asparagine. There is no other obvious motif in the GPI-PSS that signals the transamidase reaction.

The specific function of GPI-PSS has been previously studied on human carcinoembryonic antigen (CEA). One study showed that ectopic expression of various

members of the family of intercellular adhesion molecules in murine myoblasts either blocks (CEA, CEACAM6) or allows (CEACAM1) myogenic differentiation. CEA and CEACAM6 are GPI anchored, whereas CEACAM1 is transmembrane anchored. Over-expression of GPI-linked neural cell adhesion molecule (NCAM) accelerated the myogenic differentiation. After forming chimeric protein by exchanging C-terminal hydrophobic domains of CEA, CEACAM1, and NCAM, it was reported that the presence of the GPI-PSS from CEA in the chimeras was sufficient to convert both CEACAM1 and NCAM into differentiation-blocking proteins. Conversely, CEA could be converted into a neutral protein by exchanging its GPI anchor for the TM anchor of CEACAM1. These results suggest that biologically significant functional information resides in the processed extreme C-terminus of CEA and in the GPI anchor that it determines (Screaton et al. 2000).

Another study also showed that exchanging the GPI-PSS of NCAM for the GPI-PSS of CEA generates a mature protein that has a NCAM external domain, but CEA-like tumorigenic activity. Based on these findings, it is postulated that the GPI-PSS posses a functional biological information that specifies the addition of a particular GPI anchor that, ultimately, determines the final function of the mature protein (Nicholson and Stanners 2007).

CDC91L1 is the gene encoding CDC91L1 [also called phosphatidylinositol glycan class U (PIG-U)], a transamidase complex unit in the GPI anchoring pathway. The germline mutation of translocation 20q11 in bladder cancer causes the CDC91L1 protein to over-express, which could malignantly transform NIH3T3 cells in vitro and in vivo. Over-expression of CDC91L1 also resulted in upregulation of the urokinase receptor (uPAR), a GPI-anchored protein, and in turn increased STAT-3 phosphorylation in bladder cancer cells. CDC91L1 could function as an oncogene in bladder cancers and implicate the GPI anchoring system as a potential oncogenic pathway (Guo et al. 2004).

Evidence also showed that two other GPI transamidase complexes were involved in human breast cancer, PIG class T (PIG-T) and GPI anchor attachment 1 (GPAA1). The over-expression of PIG-T and GPAA1 transformed NIH3T3 cells in vitro and increased tumorigenicity and invasion of these cells in vivo (Reference). Suppression of PIG-T expression in breast cancer cell lines led to inhibition of anchorage-independent growth. In addition, PIG-T and GPAA1 expression levels could positively correlate with paxillin phosphorylation in invasive breast cancer cell lines. Furthermore, suppression of PIG-T and GPAA1 expression led to a decrease in paxillin phosphorylation with a concomitant decrease in invasion ability. These data suggest that the GPI transamidase complex has functions of oncogenes (Wu et al. 2006).

In head squamous cell carcinomas, in addition to PIG-U, other proteins in the same family such as GAA1, PIG-T, were also found to be significantly up-regulated at transcriptional and translational levels, which further suggests the GPI anchor process involved in tumorigenesis (Jiang et al. 2007).

PrP in pancreatic cancer cell lines is neither glycosylated nor GPI anchored; it exists as pro-PrP retaining its GPI-PSS (Fig. 23.1). This deficiency is not caused by a general defect of the GPI anchor process in the PDAC cell lines, as the two control

GPI-anchored proteins, CD55 and flotillin 1, remain GPI anchored in the PDAC cell lines. Despite lacking a GPI anchor, the pro-PrP is present on the PDAC cell surface, using the GPI-PSS as a transmembrane domain, as the model proposed before (Waneck et al. 1988). Our immunoblotting results with multiple anti-PrP monoclonal antibodies (mAbs) suggest that pro-PrP is the only detectable PrP in the PDAC cell lines.

The underlying reason that the GPI-PSS of PrP is not cleaved in the PDAC cell lines has not been elucidated so far. On the genetic level, we did not find any mutation in the coding region of the PRNP after sequencing all six PDAC cell lines. It is interesting to note that the GPI-PSS of PrP is intrinsically inefficient compared with other GPI-anchored proteins (Chen et al. 2001). Thus, a slight defect in the GPI anchor assembly machinery in PDAC may have a more dramatic effect on PrP than other GPI-anchored proteins with a more efficient GPI-PSS, such as CD55, which is GPI-anchored in the PDAC cell lines. A defect in lipid metabolism, which limits the availability of the GPI anchor precursor, can also impact the modification of PrP. In addition to defects in GPI anchor modification and lipid metabolism, defects in the quality-control system in the endoplasmic reticulum or in the removal of the unprocessed pro-PrP, presumably by the proteasomal degradation machinery, may also contribute to the accumulation of pro-PrP.

In the PDAC cell lines, PrP is also not glycosylated. Although the presence of the N-linked glycans on PrP is not required for GPI anchor modification (Cancellotti et al. 2005; Wiseman et al. 2005), the presence of a GPI anchor has been reported to influence the glycosylation of Thy-1, a GPI-anchored protein (Devasahayam et al. 1999). Thus, failure to remove the GPI-PSS may modulate PrP glycosylation. The lack of N-linked glycans may then alter the metabolism or transit of pro-PrP, contributing to its accumulation in the PDAC cell lines.

23.2.4.2 Filamin A and PrP Binding

Filamin A (FLNa) gene is located on chromosome Xq28, and encodes a protein connecting cell surface molecules to the actin filaments of the cytoskeleton and, thus, integrates signaling events with cellular mechanical forces (Stossel et al. 2001). FLNA has a molecular mass of 280 kDa. After binding actin filaments, FLNa promotes high-angle branching of actin filaments to maintain a cytoskeletal network responsible for cell-shape maintenance and migration. In males, FLNa deficiency caused by a null mutation is embryonic lethal. In females, depending on the nature and local of the mutation, it causes several developmental syndromes involving neuronal, skeletal and connective tissues (References).

Native FLNa is a homodimer and each subunit contains an N-terminal ABD followed by the 24 long rod-like β-sheet, interrupted by two roughly 30-amino-acid, flexible loops that are proposed to form hinge structures (Fig. 23.2). The C-terminal last domain 24 is responsible for the dimerization forming a V-shaped flexible structure that is essential for function (Feng and Walsh 2004).

FLNa interacts with numerous proteins, including proteins involving in signal-transducing pathway, adhesions, and growth factor receptors. Most of the proteins bind to domain 10 to domain 24 at C-terminal Ig-like domains of FLNa.

By co-immunoprecipatation filamin A (FLNa) co-purified with PrP and vice versa in pancreatic cell lines. Further in vitro studies shows that FLNa only binds pro-PrP but not mature PrP, which lacks the GPI-PSS. In the PDAC cell lines, the binding of Pro-PrP and FLNa is stable, as PrP and FLNa colocalize in the cancer cells by immunofluorescent staining and observed in a confocal microscope (Li et al. 2009).

The presence of an FLNa-binding motif in the GPI-PSS appears to be specific for PrP. We examined 14 GPI-PSS from other normally GPI-anchored proteins, and we found that only the GPI-PSS of PrP has the FLNa-binding motif. Therefore, even if some other normally GPI-anchored proteins also exist as pro-proteins, retaining their GPI-PSS, they are not expected to bind FLNa.

More recent studies using recombinant proteins in vitro reveal that pro-PrP has multiple binding sites at the C-terminal Ig-like domains of FLNa, including domains 10, 16, 17, 18, 20, 21 and 23. This finding is not unexpected because the Ig-like domains are highly conserved. However, whether all these binding sites are available for pro-PrP binding in native, dimeric, FLNa is not known. On the other hand, we found that the last five amino acids at the C-terminal end of the PrP GPI-PSS are critical for FLNa binding. Removal of these five amino acids completely eliminates its FLNa-binding capacity. The data suggest that the GPI-PSS of PrP is able to transverse the membrane bilayer and binds FLNa.

Inhibition of PrP expression by PrP-specific shRNA in the PDAC cell lines did not affect the expression level of FLNa; however, it did interfere with the spatial distribution of FLNa (Li et al. 2009, 2010). Compared to control cells, in PrP down-regulated cells, FLNa is more concentrated in the cytosol, away from the inner-membrane leaflet in the leading edges. Therefore, it appears that pro-PrP by binding to FLNa is able to concentrate FLNa closer to the inner membrane leaflet. As expected, down-regulation of PrP also alters the organization of the actin filaments (Li et al. 2010). These morphological changes have significant behavior alterations, as PrP down-regulated cell lines proliferate more slowly and are less invasive than control cells. Most importantly, the growth of the PrP down-regulated tumor cells in nude mice was significantly diminished. Thus, the binding of pro-PrP to FLNa enables the PDAC cell lines to proliferate faster and more invasive. The binding of pro-PrP may physically remove FLNa from its normal environment and prevent its

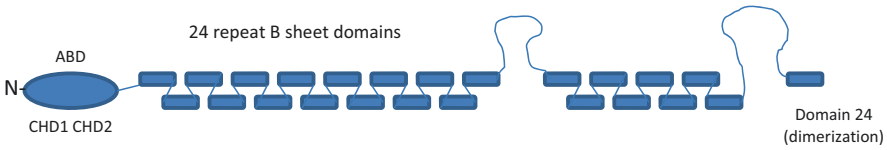

Fig. 23.2 The structure of Filamin A. CHD1 and CHD2 are two calponin homology domains. Actin-binding domain (ABD) is actin binding domain

normal physiological function. Alternatively, the binding of pro-PrP may compete for binding sites on FLNa that are normally occupied by its interaction partners.

At the molecular level, PrP down-regulated cell lines have reduced levels of p-cofilin-1, a critical regulator of the actin filament polymerization. On the other hand, the levels of p-Rac1, a Rho-GTPase; p-ERK-1/2 and p-MEK-1, two serine/threonine kinases in the MAPK pathway; and p-Fyn, a *Src* family tyrosine kinase, are markedly increased in the PrP down-regulated cells. Therefore, reducing the expression of PrP in the PDAC cell lines appears to have effects on multiple signal transduction pathways. As more than 40 proteins bind to FLNa, the aberrant binding of pro-PrP to FLNa will have rippling effects on the binding of FLNa to some of its binding partners, such as integrins, which are known to play critical roles in cellular adhesion, invasion and migration (Li et al. 2010).

The formation of pro-PrP is simply due to single gene mutation and attributed to multiple gene irregularity (Yang et al. 2016).

23.2.4.3 Expression of pro-PrP Is a Marker of Poorer Prognosis in Pancreatic Cancer

Pancreatic cancer is the fourth leading cancer death in the USA and responsible for more than 30,000 deaths a year in this country. Nearly 90% of pancreatic cancers are pancreatic ductal adenocarcinoma (PDAC). PDAC is still a lethal disease with a dismal overall median survival of 6 months and less than 10% of the 5-year survival rate. Progression of human PDAC correlates with a series of histological changes from a flat, normal columnar epithelium to a flat/papillary mucinous epithelium, with increasing complexity of cellular architecture and cytological atypia (Warshaw and Fernandez-del Castillo 1992; Hruban et al. 2001a, b). These precursor lesions are defined as pancreatic intraepithelial neoplasia (PanIN), which includes: PanIN-1, −2 and − 3, based on the cytological atypia and complex architecture, as well as accompanied with the increasing numbers of corresponding genetic mutations.

The molecular pancreatic carcinogenetic pathways are complex and not fully clarified; many genetic mutations been identified. The most common genetic lesions found in human PDAC are mutations in *KRAS*, *TP53*, *DPC4* and *CDNK2A* (Hruban et al. 2001a, b). It is now generally accepted that the *KRAS* mutation is one of the earliest, and most important genetic lesion in the development of PDAC; the majority of PDAC cases have a mutation in codon 12 of *KRAS*, substituting a glycine with aspartate, valine or arginine. However, many benign pancreatic lesions also have increased K-ras mutations.

In normal human pancreas, only islet cells demonstrated PrP immunoreactivity; neither acinar nor ductal epithelial cells stained for PrP. PrP was also undetectable in the duct cells in chronic pancreatitis, and PanIN-1 and -2. Approximately 13% PanIN-3 specimens showed weak PrP staining. However, among the 83 PDAC cases, 34 (41%) showed strong PrP staining. All PDAC tumor cells reacted strongly with the anti-GPI-PSS antiserum. Thus, as in the PDAC cell lines, PrP exists as pro-PrP in human PDAC lesions (Li et al. 2009).

Most importantly, the over-expression of pro-PrP is present only in a subset of pancreatic cancers associated with poorer clinical prognosis. PDAC patients with over-expression of PrP had a median survival time of 360 days, while those without PrP expressions had a median survival time of over 1000 days. Furthermore, this association is independent of other clinical parameters, such as age, gender, size or histological differentiation of the tumor. The PDAC tumors with PrP may have a growth advantage as in cell culture and, thus, are more aggressive.

Although there was a study that reported that PrP was up-regulated in BxPC 3, Capan 1 and five other PDAC cell lines (Han et al. 2002), the result could not be validated by other groups. We could not detect the genetic alteration of Prnp in cell lines and in human tissues. The study was performed by cDNA microarray, and the data were not validated. In contrast, other gene profiling studies have not identified PrP as a contributing factor in human PDAC (Aguirre et al. 2004; Holzmann et al. 2004; Bashyam et al. 2005). Other genetic mutations, especially DPC4 and TP53 interacting with PrP, have not been fully studied. As we know already, PrP could regulate TP53 in other cancer cells, and there is also a potential TP53 binding site in the promoter region of *PRNP*As (Guillot-Sestier et al. 2009). It is logical to study the mutual interaction of P53 and PrP in pancreatic cancer. Our unpublished data already showed that PrP and P53 have a synergic effect on PDAC progression, and the co-expression would deliver a much worse prognosis compared to that of either PrP or P53 alone.

23.2.4.4 Interacting with Nocth1 Signal Transduction Pathway

Recent study also indicated that PrP involving PDAC carcinogenesis thru interaction with Notch1 signal transduction pathway. Filamin A (FLNA), PrP interacts with Notch1, forming a PrP/FLNA/Notch1 complex. The inhibition of PrP decreases Notch1 expression and Notch1 signaling, which exhibited decreased proliferation, xenograft growth and tumor invasion but showed increased tumor apoptosis. The overexpression of PrP in PDAC with low PrP expression increased Notch1 expression and signaling, enhanced proliferation, and increased tumor invasion and xenograft growth that could be blocked by a Notch inhibitor. Targeting PrP combined with anti-Notch would have a synergic effect on inhibiting PDACs (Wang et al. 2016).

23.2.5 PrP and Melanoma

Filamin A is an integrator of cell signaling and mechanics and, thus, participates in many biological responses. However, despite its importance in cellular responses, FLNa is dispensable for cell-autonomous survival. Some human melanoma cell lines, such as M2 and − 3 do not express FLNa (Byers et al. 1991). Since FLNa is important in actin organization, cells lacking FLNa are devoid of actin fiber bundles

and, thus, are impaired in their cellular migration in vitro. This deficiency is rescued A7 cells by the transfection of a plasmid encoding FLNa into M2 cells (Cunningham et al. 1992).

More recently, we found that both M2 and A7 cells express pro-PrP.

Inhibition of PrP expression in A7 cells alters the spatial distribution of FLNA as in pancreatic cancer, and reduces the cell spreading and migration. In A7 cells, FLNA, PrP, and integrin beta1 exist as two independent, yet functionally linked, complexes; they are FLNA with PrP or FLNA with integrin beta1. Reducing PrP expression in A7 cells decreases the amount of integrin beta1 bound to FLNA. A PrP GPI-PSS synthetic peptide that crosses the cell membrane inhibits A7 cell spreading and migration. Thus, in A7 cells FLNA does not act alone; the binding of pro-PrP enhances the association between FLNA and integrin beta1, which then promotes cell spreading and migration.

Human in situ melanoma cells growing along the dermal-epidermal junctions, as single cells were largely FLNA negative, whereas tumor cells in nests and dermal components showed strong FLNA staining (Bouffard et al. 1994). It was postulated that FLNA might promote melanoma cell motility during tissue invasion from the epidermis to the dermis. With regard to integrin expression, it was reported that in situ melanoma stained either uniformly positive or uniformly negative for a2b1; the expression of this protein correlated with the later stages of melanoma progression (Duncan et al. 1996). On the other hand, in normal human skin, only epithelial cells and sporadic mononuclear cells within the dermis demonstrated weak PrP immunoreactivity (Pammer et al. 1998). We found that Pro-PrP is undetectable in normal melanocytes but is detected in melanoma in situ, and with the significant upregulation of pro-PrP in invasive melanoma. The binding of pro-PrP to FLNA, therefore, also contributes to melanomagenesis. Nevertheless, PrP also can promote melanoma migration through an FLNa independent pathway (Ke et al. 2019).

Immunostaining for pro-PrP, integrin and FLNa in melanoma biopsies may provide new insights into the role these molecules play in human melanoma tumorigenesis.

23.3 Conclusion and Future Perspective

Multiple studies have shown that PrPs are up-regulated in many cancer types including breast, gastric, colorectal and pancreatic cancers as well as melanoma. In breast, stomach and colorectal cancers, the data suggest that PrPs exert effects on drug resistance, invasiveness and protect the tumor cells by regulating the apoptosis pathway. However, it should be noted that it is not clear whether in these tumor cell lines the PrP exists as a normal GPI-anchored PrP or pro-PrP as we have demonstrated in the PDAC cell lines as well as in melanoma cell lines.

In pancreas cancer and melanoma, the main form of PrP is pro-PrP not normal mature PrP (Fig. 23.3). The pro-PrP is present on the cell surface as well as in the cytosol. As pro-PrP and PrP have different biological functions, identifying the

forms of PrP in these cancers will provide insights into the mechanisms PrP modulates tumor cell biology. In pro-PrP form, identifying the underlying mechanisms that cause the retention of the GPI-PSS on PrP in cancer cell lines will help us understand the cell biology of the GPI-anchor modification pathway and the roles it plays in tumor biology. Furthermore, since high levels of soluble PrP are detected in the culture supernatants of the PDAC cell lines, therefore, soluble pro-PrP may be present in the circulation or body fluid of patients with PDAC. Detection of pro-PrP from fecal material or pancreatic ductal fluids may provide an early and noninvasive method for detecting PDAC. In addition, prevention of the interaction between pro-PrP and FLNa could provide a novel target for therapeutic intervention in PDAC.

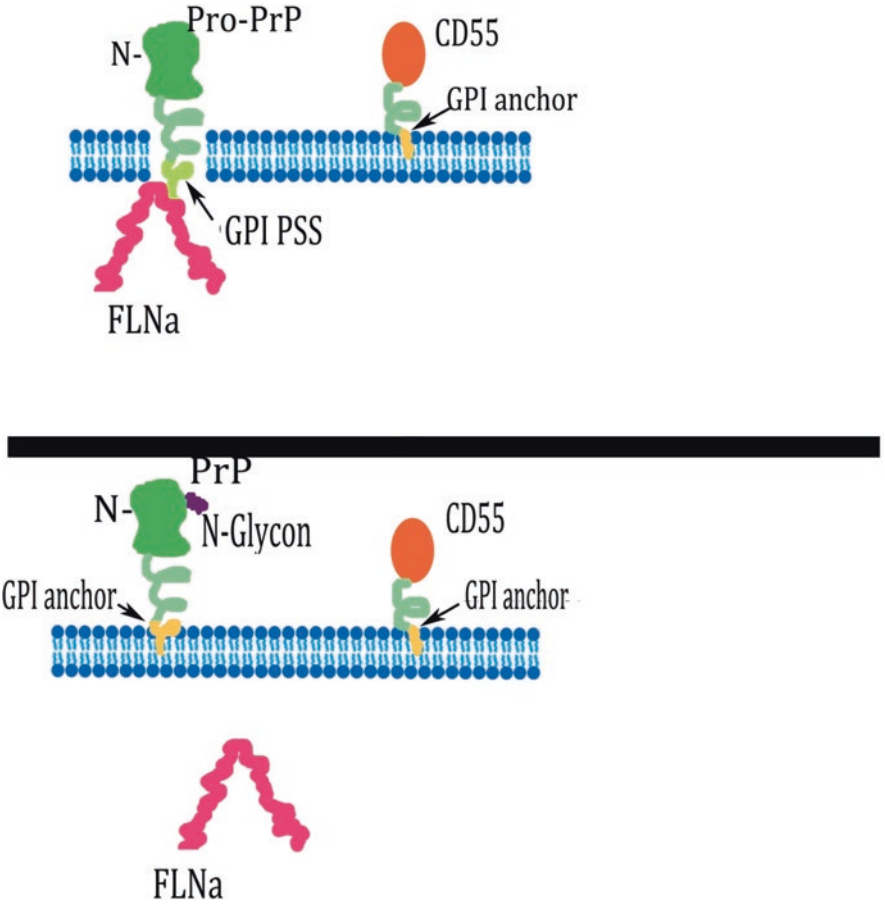

Fig. 23.3 Pro-PrP and PrP with FLNa. The top panel shows Pro-PrP in PDAC binds to FLNa. The GPI-PSS functions as the transmembrane domain. Other GPI anchor protein CD55 has the GPI anchor. The bottom panel shows normal PrP with GPI anchor and has no reaction with FLNa. Normal CD55 with GPI anchor

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Chapter 24
Protective Role of Cellular Prion Protein in Tissues Ischemic/Reperfusion Injury

Zerui Wang and Wen-Quan Zou

Abstract Normal cellular prion protein (PrP^C) is well documented to be the precursor of the infectious pathogenic prion protein that plays a critical role in the pathogenesis of prion diseases, a group of fatal transmissible spongiform encephalopathies or neurodegenerative diseases. However, the physiological functions of PrP^C remain poorly understood. Recent studies have revealed that PrP^C plays a protective role in tissue ischemia/reperfusion injury (IRI), an event caused by a reduced blood supply followed subsequently by restored blood back to infarct ischemic tissue. The brain, heart, and kidneys are the most common targeted organs prone to IRI. This chapter reviews the PrP^C-related protective role in ischemia/reperfusion of patients, cell and animal models, as well as discusses the possible pathways involved in these events.

Keywords Cellular prion protein (PrP^C) · Ischemia/reperfusion injury (IRI) · Brain · Heart · Kidneys · Hypoxia · Reactive oxygen species · knockout (KO)

24.1 Introduction

Cellular prion protein (PrP^C) is a glycoprotein attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor and expressed mainly in the brain and slightly in other peripheral organs (Kretzschmar et al. 1986a). The pathologically misfolded infectious prion protein (PrP^{Sc}) derived from PrP^C via a conformational

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transition can cause prion diseases, a group of fatal transmissible spongiform encephalopathies or neurodegenerative diseases in animals and humans (Bolton et al. 1982; Prusiner 1998). However, the physiological functions of PrP^C remain to be determined. Recent studies have shown that PrP^C plays a variety of physiological roles in neurons and non-neuronal cells, including copper ion uptake (Perera and Hooper 2001), cell signal transduction and neuron survival (Martins et al. 1997; Bounhar et al. 2001), cell transportation (Lee et al. 2001; Negro et al. 2001), as well as cell adhesion and differentiation (Graner et al. 2000). It has also been observed that PrP^C can protect nerve tissue from oxidative stress (Brown et al. 1999, 2002). Ischemia/reperfusion injury (IRI) is a serious condition that undergoes a decrease in blood supply-induced cellular dysfunction and death and is followed by restoration of blood flow to the ischemic tissues, which further damages the tissue involved. Recent studies have shown that PrP^C may play a protective role in the IRI process, providing a potential new target for effective therapeutic intervention. On the one hand, PrP^C expression has been observed to be increased in neurons of ischemic human and animal brains, and the size and damage of the infarct area are significantly larger and more severe in PrP^C-knockout (KO) mice than in wild-type (WT) mice (McLennan et al. 2004). On the other hand, overexpression of the PrP^C gene is also able to alleviate cerebral ischemic injury in rats and improve their neurological dysfunction (Shyu et al. 2005). A recent study indicated that PrP^C could also reduce cardiac oxidative stress and cell death caused by reperfusion after ischemia, while the downregulation of PrP^C can increase oxidative injury during ischemic preconditioning and hydrogen peroxide perfusion (Zanetti et al. 2014). In the kidney IRI model, PrP-KO mice showed more severe renal insufficiency and structural damage than WT mice (Zhang et al. 2015). Moreover, significant differences in renal dysfunction, histological changes, oxidative stress, and mitochondrial complex between WT and KO mice were observed (Spudich et al. 2005). These findings suggest that PrP^C could play a protective role in ischemia/reperfusion injury.

24.2 The Physiological Function of PrP^C

PrP^C exists in different topological forms including the major form that is attached to the cell surface through the GPI anchor (Stahl et al. 1987; Lehmann and Harris 1995) and the less common transmembrane forms that span the various cell membranes (Hegde et al. 1998; Hay et al. 1987; De Fea et al. 1994; Stewart et al. 2001). In addition to being mainly expressed in the central nervous system, PrP^C is also expressed in various peripheral tissues throughout the body, including the heart, muscle, lymphoid tissue, kidney, gastrointestinal tract, skin, and endothelial tissues (Bendheim et al. 1992; Aguzzi and Heikenwalder 2006; Petit et al. 2013). Among the cell types of the central nervous system, PrP^C has been identified in neurons, extraneural cells, and glial cells (Kretzschmar et al. 1986b; Moser et al. 1995; Ford et al. 2002). Within the brain, high expression of PrP^C is found in specific brain areas, including the olfactory bulb, striatum, hippocampus, and prefrontal cortex (Fournier 2001; Salès et al. 1998). Moreover, PrP^C in mouse CA1 and hippocampal dentate gyrus is predominately

located in the secretory pathway, endosomal compartments, and plasma membranes as observed by the quantitative ultrastructural studies by electron microscopy (Mironov Jr et al. 2003; Lainé et al. 2001). The protein is also found in the cytoplasm of the neocortex, hippocampus, and thalamus neurons but not in the cytoplasm of cerebellar neurons (Aguzzi and Heikenwalder 2006).

The physiological functions of PrP^C have not been fully elucidated. The current understanding of its functions is derived from research work with cell-free and cell models, animal models, and epidemiological studies. Previous studies have observed no significant and consistent physiological defects in PrP^C-KO mice. However, some studies reported that PrP^C-KO mice may have neurological abnormalities such as circadian rhythm disorders, cognitive and olfactory deficits, as well as immunological changes (Aguzzi and Heikenwalder 2006). Several lines of studies have demonstrated that PrP^C binds to Cu(II) with high affinity, indicating that it could be a copper transporter (Brown et al. 1997; Viles et al. 1999; Evans et al. 2016; Nguyen et al. 2019). Due to its ability to interact with Cu(II), PrP^C is considered an antioxidant that can potentially reduce reactive oxygen species in cells (Brown et al. 2002; Vassallo and Herms 2003). Primary cultured neurons can be protected against Bax-mediated cell death by overexpressing PrP^C (Bounhar et al. 2001), the indication of an anti-apoptotic activity. The anti-apoptotic activity of PrP^C is believed to be associated with the caspase-dependent apoptotic pathways in mitochondria since the apoptosis of *PRNP*$^{0/0}$ cells can be prevented by ectopic expression of PrP^C and Bcl-2 (Brown and Besinger 1998; Kim et al. 2004). PrP^C may participate in regulating intracellular Ca^{2+} homeostasis through the Scr-like tyrosine kinase Fyn and inositol triphosphate (IP3) pathways. It is also linked to the functional regulation of ionotropic glutamate receptors (Gavín et al. 2020). PrP-KO mice exhibited an increase in neuronal susceptibility to glutamate agonists and AMPA/KA receptor dysfunction, which is responsible for excitotoxicity (Rangel et al. 2007; Carulla et al. 2015). Moreover, since the protein can bind a variety of other molecules associated with different physiological roles including cell survival and neurite outgrowth (Graner et al. 2000; Chen et al. 2003), the protein is proposed to be a cell surface scaffold protein (Linden 2017). The interaction of PrP^C with other molecules is also implicated in its role in different signaling pathways.

24.3 Protective Effect of PrP^C on Cerebral Ischemia/Reperfusion Injury

Despite high expression of PrP^C in the brain, as mentioned above, knockout of PrP^C in animals may exhibit only subtle phenotypic abnormalities under physiological conditions. However, subsequent studies revealed that under stress conditions that increase cellular energy requirements, the presence of PrP^C turns out to be crucial (McLennan et al. 2004; Steele et al. 2007). The anti-hypoxic role of PrP^C has been consistently observed in the brain of ischemia-induced hypoxia. PrP^C accumulation was observed in the penumbra of ischemic brains in both human adults and experimental rodents, and the infarct size was significantly greater in PrP^C-KO than in the

wild-type mice (McLennan et al. 2004). The extent of PrP^C upregulation was dependent on the severity of ischemia (Weise et al. 2004). Moreover, both cell and animal models revealed that the N-terminal octarepeat region plays a lead role in PrP^C neuroprotection against oxidative stress involving the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (Vassallo et al. 2005; Weise et al. 2006; Mitteregger et al. 2007). In addition, a study exposing wild-type PrP^C, PrP^C-KO and PrP^C-overexpressing mice to focal cerebral ischemia followed by 28 days of reperfusion suggested that PrP^C induces post-ischemic long-term neuroprotection, neurogenesis, and angiogenesis by inhibiting proteasome activity (Doeppner et al. 2015). As a result, the neuroprotective effect against hypoxic injury is one of the PrP^C functions that is believed to be the best-supported (Ramljak et al. 2016). The transcription factor hypoxia-inducible factor 1α (HIF-1α) could transactivate the hypoxia response elements (HREs) in the promoter region of LDH-A in hypoxia (Semenza et al. 1996). The stabilization of HIF-1α enables adaptive responses to hypoxia and other stress conditions (Semenza 2000; Dery et al. 2005). Thus, the stabilization of HIF-1α could protect astrocytes from glutamate-induced damage during severe hypoxia (Badawi et al. 2012). In addition, the HIF-1α expression could be significantly reduced in $PRNP^{-/-}$ mice while increased in $PRNP^{+/+}$ mice after 24-h-stroke (Doeppner et al. 2015), suggesting that PrP^C may play a neuroprotective role by directly regulating hypoxic injury in vivo or indirectly modulating HIF-1α, thereby regulating LDH-A/lactate levels.

For the lactate levels regulation, it has been proved that PrP^C has been involved with Na^+/K^+ ATPase in regulating lactic acid transport in astrocytes through MCT1 (Kleene et al. 2007). Moreover, astrocytes can express lactate-released MCT1 and MCT4 types, while neurons mainly express MCT2 type, which is conducive to lactate uptake (Dimmer et al. 2000; Pellerin et al. 2005; Rosafio and Pellerin 2014). It was also found in cell models that overexpression of PrP enhanced MCT1 expression under normoxic conditions (Ramljak et al. 2015). In the PrP-KO animal model, the $PRNP^{-/-}$ mice showed a 100% increase in lactic acid content in the hippocampus and cerebellum (Cudalbu et al. 2015), indicating an impairment in the regulation of lactic acid without PrP.

It has also been reported that the early growth reactive-1 (EGR-1) is a rapidly induced transcription factor initiated by hypoxia that could deactivate the HIF-1α promoter region (Sperandio et al. 2009) and acts independently of HIF-1α (Yan et al. 1999). The binding of EGR-1 to conserved intron sequences and regulation of *PRNP* gene expression has been found in mouse neurons (Keilani et al. 2012). Studies in mouse brains have shown that prion disease dysregulated several microRNAs (miRNAs), and EGR-1 is one of the gene promoters homologous to these miRNAs (Shapshak 2013). In addition, the neurotoxic PrP peptide 106–126 could induce the synthesis of EGR-1 in primary cortical neurons (Gavín et al. 2005). Since hypoxic conditions could protect neuroblastoma cells from the PrP peptide neurotoxicity by activating the Akt signaling pathway (Xu et al. 2010), it suggests that hypoxic conditions are involved in pathogenetic prion-induced neuronal damage. It is also reported that the PrP peptide 106–126 could promote endogenous PrP^C aggregation into the form of amyloid protein similar to the PrP^{Sc} isoform

(Singh et al. 2002). Furthermore, it has been proved that the ubiquitin–proteasome system is central to the pathogenesis of neurodegenerative diseases (Takalo et al. 2013). These current findings suggest that hypoxia could be one of the survival processs and prevent the further formation of protein aggregates.

Vassallo et al. have proved that PrP^C could activate the anti-apoptotic PI3K/Akt pathway (Vassallo et al. 2005), while PrP^C deficiency inhibits the PI3K/Akt pathway by reducing phosphorylated-Akt expression (Weise et al. 2006). The PI3K/Akt pathway is required by HIF-1α stabilization in the early stage of hypoxia (Pellerin et al. 2005). In addition, it is known that phosphorylated Akt could inhibit glycogen synthase kinase 3β (GSK-3β) activity, leading to HIF-1α stabilization and increased HIF-1 transcriptional activity (Mottet et al. 2003). GSK-3β, known as a component of the polyprotein-destroying complex, is part of the Wnt/β-catenin signaling pathway (Rosafio and Pellerin 2014) and involved in the crosstalk between the two pathways. The phosphorylation of Akt suppresses GSK-3β activity and stabilizes β-catenin, promoting transcription of Wnt target genes, such as pyruvate dehydrogenase kinase 1 (PDK-1), LDH, along with TCF/LEF transcription factors (MacDonald et al. 2009; Pate et al. 2014). In addition, HIF-1 directly targeting PDK-1 inhibits mitochondrial function by restricting pyruvate entry into the TCA cycle (Kim et al. 2006). This kinase phosphorylates and reduces the assembly of the mitochondrial pyruvate dehydrogenase (PDH) complex (Papandreou et al. 2006), which could further inhibit the conversion to acetyl-CoA and promote the conversion to lactic acid. Additionally, Wnt can also enhance LDH activity, which further promotes glycolysis (Roche et al. 2001). PrP^C could interact with β-catenin and upregulate the transcriptional activity of the β-catenin/TCF complex (Chafey et al. 2009). It has been shown that Wnt/β-catenin signaling was impaired in mice infected with itching bacteria, and phosphorylated GSK-3β levels were significantly reduced, leading to enhanced activity (Besnier et al. 2015) and degradation of β-catenin. In addition, dysfunction of the PI3K-Akt-GSK-3 pathway is widely involved in models of prion disease (Sun et al. 2015). Thus, the role of PrP^C in the hypothetical synergistic network between EGR-1- PrP^C-HIF-1α-LDH under hypoxic tension is crucial for further investigation (Fig. 24.1).

Since PrP^C could be associated with neuronal stress responses (Riek et al. 1996), compared to the normal expression level of PrP^C, the PrP^C knockout neurons exhibit increased susceptibility to oxidative stress when exposed to ischemic injury. In order to elucidate the protective role of PrP^C in the injured ischemic brain (McLennan et al. 2004), Spudich et al. compared PrP KO mice, wild-type controls, and PrP^C transgenic mice to see how the expression level of PrP^C impacts brain injury level (Zanetti et al. 2014). They indicated that PrP^C knockout could lead to an increase in ischemic damage in the brain, while the transgenic PrP^C could restore viability after stroke compared to the wild-type PrP^C mice. They also found out that during 72 h of observation, the worsened brain infarct of knockout mice is associated with the increased activities of ERK-1/-2, STAT-1, JNK-1/-2 and caspase-3, suggesting that these signaling factors might be involved in neuronal death induced by the PrP-KO condition.

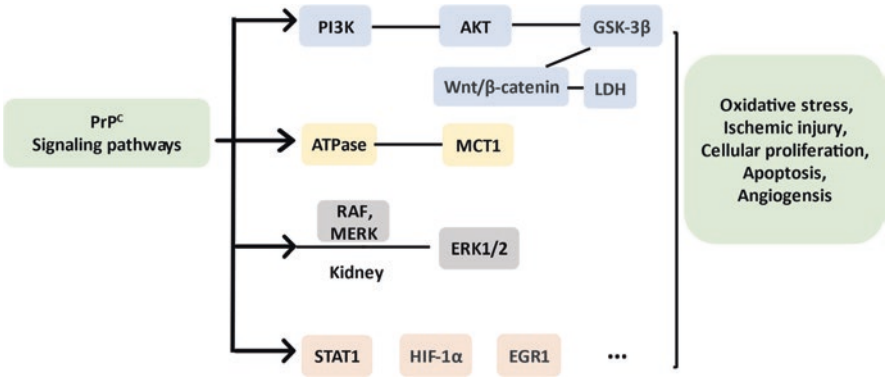

Fig. 24.1 Diagram of the PrPC signaling pathways and binding partners. Summary of the downstream signaling pathways mediated by PrP^C and interactions with binding partners. These pathways are mainly characterized in neuronal cells leading toward oxidative stress, ischemic injury, cellular proliferation, apoptosis, and angiogenesis in targeted cells/tissues

While the above studies mainly focused on the acute injury process, Doeppner et al. (Doeppner et al. 2015) then investigated the role of PrP^C in the post-ischemic brain. They used wild-type PrP^C, PrP^C KO, and PrP overexpressing mice to evaluate the effect of PrP^C on neurological recovery, ischemic injury, neurogenesis, and angiogenesis. They observed that PrP^C induced long-term neuroprotection in the post-acute stroke phase and promoted neurological recovery. The proteasome activity, which was found to augment ischemic brain injury via oxidative stress and HIF-1α degradation (Doeppner et al. 2012), was reduced in PrP overexpressing mice in ischemic brains while increased in ischemic PrP KO mice, suggesting that the restorative effects of PrP^C are mediated by proteasomal deactivation. They also found that neurogenesis and angiogenesis that are closely linked in the ischemic brain were both increased in ischemic PrP^C overexpress and knockout mice, suggesting that PrP^C can promote neural progenitor cell migration and trafficking. Therefore, the PrP^C–proteasome–HIF-1α axis might represent a promising target for restorative stroke therapies.

Recently, the protective role of PrP^C in peripheral immune responses after cerebral IRI was investigated (Zhang et al. 2021). They showed that PrP^C expression was altered in CD4+ T cells after the cerebral IRI, and different levels of PrP^C expression regulate the proportion of splenic Th1/2/17 phenotypes and the secretion of relevant pro- and anti-inflammatory cytokines. PrP^C knockout could induce an elevation in the Th1/17 cells and pro-inflammatory cytokines, which deteriorated I/R injury. In comparison, PrP^C overexpression induced an increase in Th2 cells and anti-inflammatory cytokine expression, which alleviated the illness. PrP^C deficiency also aggravates the apoptosis of OGD/R HT-22 neurons, while overexpression protects the neurons. Hence, PrP^C may serve as a neuron protector in the CNS when IRI occurs by peripheral immune responses and defend against stroke-induced apoptosis (Fig. 24.2).

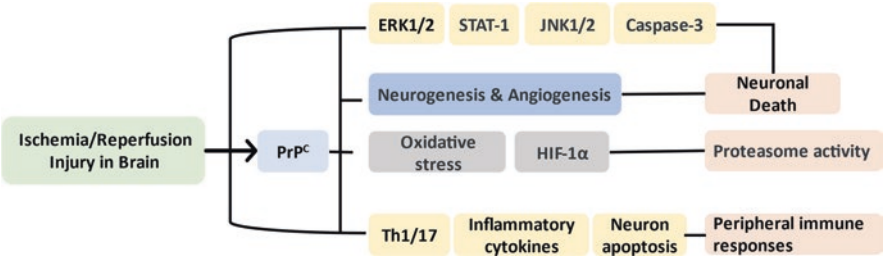

Fig. 24.2 Diagram of PrPC in cerebral ischemia. Schematic for the effect of PrP^C on the pathophysiology of brain ischemia in cell signaling processes. PrP^C is associated with several protective functions against ischemic injury through affecting the ERK1/2 transduction pathway, reducing oxidative stress, increasing neurogenesis and angiogenesis, and modulating T cells through peripheral immune responses

24.4 The Protective Effect of Normal Prion Protein on Cardiac Oxidative Insults

Higher levels of oxidized lipids and proteins and reduced antioxidant activity were observed in cardiac muscles from PrP knockout (KO) mice than in the wild-type (WT) (Klamt et al. 2001). Zanetti et al. further determined whether PrP^C could protect against oxidative stress in the heart (Zanetti et al. 2014). They compared hearts isolated from mice expressing different amounts of PrP^C-WT and PrP-KO mice and mice with a threefold expression of PrP^C (PrP-OE). The heart under different PrP expression conditions were examined for the accumulation of reactive oxygen species (ROS), oxidation of myofibrillar proteins, and cell death. To test the molecular basis of PrP^C antioxidant action, they also analyzed proteins involved in heart antioxidant responses. They observed that PrP^C is involved in the cell mechanisms protecting cardiomyocytes from oxidative injury. A close insight into PrP^C antioxidant features was accomplished by comparing the response of hearts isolated from mice with different PrP genotypes (WT, OE, and KO). These models highlighted the physiological significance of PrP^C antioxidant properties under stress conditions. While ischemia (40 min) followed by reperfusion (15 min) failed to underscore differences in cell death between WT and PrP-KO hearts, PrP-OE hearts showed a significantly reduced cell damage and lower amounts of ROS and oxidized myofibrillar proteins (Zanetti et al. 2014).

Further insight into the protective property of PrP^C came from the perfusion with H_2O_2, an oxidative challenge devoid of ischemic injury (Zanetti et al. 2014). It was found that the PrP-KO phenotype was disclosed by 14–15 min H_2O_2-based perfusion, which is similar to the response in WT. In contrast, PrP-OE hearts exhibited a lower extent of cell death and oxidized proteins. The above findings (Zanetti et al. 2014; Klamt et al. 2001) suggest that physiological PrP^C is essential to protect the heart from oxidative injury (Fig. 24.3).

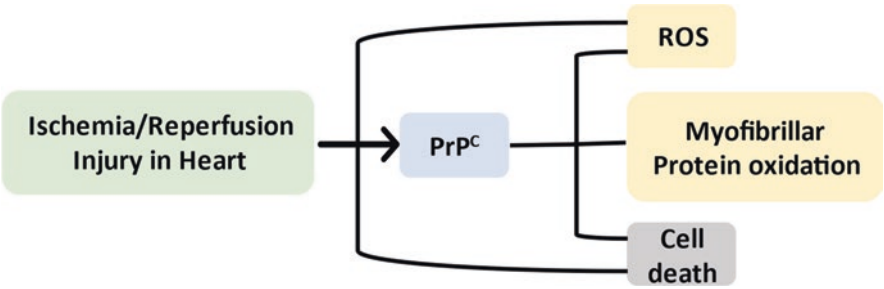

Fig. 24.3 Diagram of PrPC in cardinal ischemic. The function of PrP^C in cardinal ischemic model. Low expression level of PrP^C could induce aggravating ROS, myofibrillar protein oxidation and cardinal cell death

24.5 The Protective Effect of Normal Prion Protein on Renal Ischemia/Reperfusion

The main characteristic of renal IR injury is acute tubular damage characterized by brush margin loss, tubular dilatation/cavitation, apoptosis, and necrosis (Brezis and Rosen 1995; Chiao et al. 1997). Several theories have been carried out about the mechanism of renal IR injury. In addition to reduced glomerular filtration and white blood cell accumulation (Chiao et al. 1997), oxidative stress is thought to play an important role in IR-induced kidney injury (Heemann et al. 2000; Aragno et al. 2003). Specifically, both the production of reactive oxygen/nitrogen species and the loss of antioxidant defense mechanisms are associated with the pathogenesis of IR-induced tissue injury. Given that the underlying mechanisms of IR-associated AKI are not fully understood, elucidation of other factors involved in regulating oxidant/antioxidant balance in animals and humans may lead to further improving our understanding of the pathogenesis of AKI.

To determine whether PrP^C also protects the kidney that is also vulnerable to ischemic injury, as in the brain and heart, the effect of PrP^C on wild-type and PrP^C-KO mouse models subjected to renal IR injury was examined (Zhang et al. 2015). The renal function and histological changes induced by reperfusion after 30 min of ischemia for one, two, or three days were monitored and compared between the two types of animals. They observed that more severe renal damage occurred in KO mice than in wild-type mice, and these changes were associated with oxidative stress, mitochondrial respiratory chain complex, and IR-related signaling pathways.

Zhang et al. found that the levels of serum creatinine, which were used to monitor renal dysfunction during and after renal IR/I, were much higher in PrP-KO than in WT mice (Zhang et al. 2015). More severe renal pathological changes were found in the KO than in wild-type mice, and PrP levels in the IR-injured kidney of wild-type mice were increased compared to that of the normal wild-type mice. The levels of nitrotyrosine and CML, which are oxidative stress markers, were higher in KO

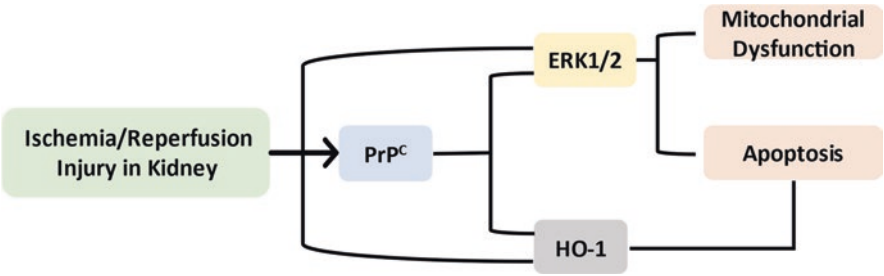

Fig. 24.4 Diagram of PrPC in renal IRI. PrP^C plays a protective role in renal ischemia/reperfusion injury through affecting the ERK1/2 transduction pathway and heme oxygenase-1 (HO-1) and further induce mitochondrial dysfunction and apoptosis

than in WT kidneys, indicating that deletion of PrP^C results in a profound loss of anti-oxidative stress capability of the KO kidney. Additionally, the mitochondrial respiratory chain complexes I and III were significantly lower in KO than in WT, suggesting that increased oxidative stress found in the KO kidney compared to WT may be associated with the decrease in CI and CIII. Moreover, the immunostaining of phosphorylated ERK (pERK) was restricted within renal tubular cells of KO mice, while it was much higher in WT, suggesting that the ERK pathway is actively involved in renal tubular damage of KO mice. These findings implied that PrP^C and its signaling pathways are involved in the event of renal IR injury. This study revealed that a more severe degree of acute renal failure was observed in KO mice than wild-type mice treated with ischemia/reperfusion injury (Fig. 24.4).

24.6 Conclusion

The general functions of PrP^C in the IRI model have been investigated via *PRNP* overexpress and knockout gene experiments. Since the downregulation of PrP^C occurs before and during the IRI incubation period, the loss of PrP^C function may underlie the pathogenesis of the injured area, suggesting that PrP^C could be considered a potential therapeutic target for IRI. Although it is still unclear whether PrP^C regulates the pathways in IRI directly, many signaling events have been reported to be inhibited or activated via PrP^C. To investigate how PrP^C affects its downstream processes involved in IRI pathways, further studies are warranted to elucidate the crosstalk between different pathways and the expression of PrP^C and its binding partners. Mapping the entire regulatory network for physiological PrP^C could help identify more accurate cellular pathways associated with PrP^C during IRI. Thus, it would be essential to clarify the role of PrP^C and its associated pathways to discover therapeutic targets for IRI.

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Conflict of Interest The authors declare no conflict of interest.

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Part VII
Animal Prion Diseases

Chapter 25
Bovine Spongiform Encephalopathy

Gianluigi Zanusso and Salvatore Monaco

Abstract Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease of cattle caused by foodborne exposure to prions. First described in 1986, this novel disorder was clinically characterized by altered behavior, sensory changes, and locomotor signs. For almost two decades, BSE, now named classical BSE (C-type BSE), has been regarded as the only and exclusive prion disorder of cattle. The introduction of an active surveillance system for BSE in 2001 allowed the identification of two additional atypical forms of BSE, named H-type and L-type BSEs, because of distinct conformations of the pathological prion protein, or PrP^{Sc}, with higher (H-type) or lower (L-type) electrophoretic mobility of the unglycosylated protease-resistant PrP^{Sc} fragment. Up to 2019, a total of 69 L-type BSE and 62 H-type BSEs have been detected in European Union (EU) reporting countries, in addition to 2 H-type BSE in Norway and Switzerland, as opposed to 190,469 C-BSE cases. The clinical phenotypes of atypical BSE forms are only partially known in field animals, although indirect information has been obtained from intraspecies transmission studies. Transmission studies to mice show that C-type, H-type, and L-type BSE forms display distinct molecular properties, consistent with the occurrence of three different prion strains. Intriguingly, upon serial passages, H-type and L-type BSEs may acquire C-type properties, hence suggesting their possible role in the origin of BSE epidemics, and, in addition, are transmissible to mammalians, including non-human primates, issues that raise public health concerns.

Keywords Amyloid · Atypical BSE · Bovine amyloidotic spongiform encephalopathy · Bovine spongiform encephalopathy · Creutzfeldt–Jakob disease (CJD) · H-type BSE · L-type BSE · Prion strains

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In 2021, the OIE published a map with countries with a negligible BSE risk status with a controlled BSE risk, limited to Chinese Taipei, Ecuador, France, Greece, and the UK, and others with a negligible BSE risk status. This new scenario mirrors the efficacy of the adopted measures of BSE spreading based on prevention as well as on active surveillance.

25.1 Introduction

The story of bovine spongiform encephalopathy (BSE) began on December 1984, when a UK farmer called a veterinary surgeon to look at "a cow that was behaving unusually". Seven weeks later the cow died. Early in 1985, more cows from the same herd developed similar clinical signs (O'Brien 2000). Afterward, the BSE outbreak started.

25.2 BSE Epidemics in the UK

BSE epidemics originated from the exposure of cattle to a dietary protein-rich supplement, meat and bone meal (MBM), prepared from rendered carcasses of livestock. This intensive practice of nutrition was introduced in 1940s to increase protein content in animal diets, particularly in dairy herds. The preparation of protein-rich supplements followed a rendering process, whereby the slaughterhouse refuse (offal) was separated into tallow and a defatted mixture of concentrated proteins, following sequential boiling, milling, and fat extraction with hydrocarbon organic compounds. Changes in the rendering process, in particular the omission of the use of organic hot solvent extraction and solvent recovery steps, resulted in an increase of the fat content in MBM, and inefficient inactivation of the infectious agent.

Between November 1986 and November 2000, confirmed cases of BSE in the UK were more than 180,000, but if included the asymptomatic cattle over 30 months (OTM), preemptively slaughtered and destroyed, the number of animals was nearly four and a half million (Brown et al. 2001).

To forefront the outbreak, the UK government issued a series of preventive measures. In July 1988, the prohibition to use and/or supply ruminant-derived proteins in ruminant feed was started, in addition to compulsory slaughtering and destruction of animals suspected of having BSE. In November 1989, specified bovine offal (SBO), the most infective parts, including the brain, spinal cord, tonsil, thymus, spleen, and intestines, were excluded from the animal and human food chains. The aim of this relevant public health measure was also focused to manage the risk of exposure to potentially infected tissues from clinically healthy animals, given the evidence that 1 g of BSE-infected brain material was an effective pathogenic oral dose (Wells et al. 1998). Moreover, BSE was successfully transmitted by parenteral

route to pigs challenged with brain material from a clinically affected cow (Dawson et al. 1990), although subsequent experiments showed that pigs are not susceptible to BSE following high doses of BSE by oral exposure (Wells et al. 2003). The positive effects of these measures of prevention were observed in 1993, when the BSE curve of epidemics downturned.

In April 1996, concurrently with the first report of variant Creutzfeldt–Jakob disease (vCJD) in ten young adults (Will et al. 1996), mammalian MBM preparations were definitely banned from feeding all farm animal species, horses, and fish (Collee and Bradley 1997). In addition, to reduce the risk of human exposure to the BSE agent, the UK government decided that no British cattle over 30 months (OTM) should be consumed, and from 1996 to 2000, 4.5 million cattle were incinerated. In 2005, the OTM rule was replaced by a mandatory BSE screening test for OTM cattle slaughtered for human consumption.

25.3 BSE in Europe

BSE spread to the Continent through the exportation of BSE-affected livestock and of contaminated foodstuff. In the critical period after 1985, more than 50,000 pure bred breeding cattle, as well as large quantities of contaminated MBM, were exported worldwide. In European countries, a total of 34 BSE cases were ascertained in the UK imported cattle, while cases of BSE in native-born cattle, assumedly exposed to MBM meal of UK origin, were first reported in 1989 in Ireland, and thereafter in Switzerland, Portugal, France, Belgium, Luxemburg, Netherlands, Lichtenstein, Denmark, Germany, and Spain (Cachin et al. 1991; Coles 1991; Smith and Bradley 2003). By the beginning of 2000, only 9 European countries reported new BSE cases in the native cattle population (Ducrot et al. 2008); however, 16 additional countries reported BSE cases during the following years, after the introduction of an active surveillance system.

After the earliest reports of BSE outside the UK, only in 1990, the European Commission stopped the importation of live cattle and MBM/SBO preparations for ruminant feeding, thus allowing for almost 2-year importation of MBM from the UK (Butler 1996). This is in contrast with the French ban prohibiting UK meals for ruminant feed in August 1989. Notwithstanding, UK exports continued to grow through increased sales of MBM to communities outside the EU. In 1991, Israel imported 10,000 tons and Thailand 62,000 tons of UK feed (Butler 1996).

Since July 1994, the EU prohibited the use of proteins derived from mammalians in ruminant feed in the whole community, although some member states had implemented such a ban before that date. However, the persistence of BSE cases in native-born animals suggested large cross-contamination of ruminant feed, still authorized in other species such as pigs or poultry. Therefore, in January 2001, mandatory measures were implemented by prohibiting processed animal proteins to all farmed animals, birds, and fishes.

25.4 The Impact of the BSE Surveillance System and the Emergence of Atypical BSE Forms

The identification of BSE-affected cattle by a passive surveillance system was one of the first measures set up in the UK and in European countries. The real effectiveness of this measure, based on the mandatory reporting of clinically suspected BSE cases by veterinarians, was questionable, since it depended on the appropriateness of the case definition, the variability of clinical signs, the disease awareness of the veterinarian or the cattle owner and the quality of ante mortem slaughter inspection; this, in addition to the paved loss of the entire herd as a consequence of BSE reporting, the inadequate compensation and the stigmatization of the cattle owner (Ducrot et al. 2008; Doherr et al. 2001).

The true efficacy of mandatory reporting of clinical BSE suspects was unknown until diagnostic confirmatory tests were available. The BSE test was a reliable control measure for estimating the number of BSE positive cases among clinically affected cattle or cattle subpopulations with a higher BSE incidence, as well as asymptomatic animals. Finally, the analysis of the active surveillance results showed that BSE positive cases were eight times higher in the at-risk cattle population (downer cattle and at emergency slaughter) than at routine slaughter, indicating that if correctly pursued passive surveillance would be a safe measure of prevention.

25.5 The Active Surveillance

In 1999, Switzerland was the first country to introduce the measure of an active surveillance system for the ascertainment of BSE in adult cattle. While maintaining a passive surveillance system, the entire population of cattle over 24 months "at risk," including dead on farm animals, euthanized cases, emergency slaughter, or downer cattle were tested (Doherr et al. 2001). Moreover, 3% of adult cattle sent to routine slaughter were randomly sampled and tested.

In 2000, also France initiated BSE active surveillance of at-risk stocks in its three most affected regions, including Basse-Normandie, Bretagne, and Pays de Loire (Morignat et al. 2002).

In January 2001, the European Union implemented BSE surveillance by statutory active surveillance program based on systematic testing of all slaughtered bovines over 24 months of age in France, Germany, and Italy, and over 30 months in other countries; Austria, Finland, and Sweden randomly tested 10,000 cattle per year, since they were classified by the "Office International des Epizooties" at level II risk, that is, "unlikely, but not excluded" (Bird 2003). Portugal, Greece, and Belgium had the lowest rate of surveillance on routinely slaughtered bovines. Non-EU countries, including Canada and the United States, maintained passive surveillance. In Japan, active surveillance began in April 2001 on all clinical BSE suspects and fallen stock (Yamanouchi and Yoshikawa 2007).

After the establishment of active surveillance, several countries, including Italy, that did not report BSE cases in native-born cattle before 2001, found BSE cases. Accordingly, Italy reported 48 cases, whereas Spain reported an increase of 41 times, Belgium 20, France 17, Germany 18, The Netherlands 10, and Switzerland 12 times.

In 2001, the active surveillance in the Europe system snapshots the real occurrence of BSE, which was the likely consequence of BSE contamination during the period 1995–1996, in accordance with the estimated incubation period of 5 years for BSE. The reduction of BSE cases in several European countries during the following years, suggests that the 2001–2002 period corresponds to the peak of BSE epidemics in Europe.

Thereafter, the number of BSE cases progressively declined, and in 2011 only 15 cases were reported. Based on these results, in 2009 EU member states increased the age limit for testing from 30 months to 48 months for healthy slaughtered cattle, and from 24 months to 48 months for at-risk bovines. Since July 2011, the active surveillance system has been restricted to healthy slaughtered animals over 72 months, and to at-risk cattle over 48 months. Additional relaxation measures have been prospected for 2013, including the testing of at-risk cattle population and randomly healthy slaughtered cattle. In particular, the European Food Safety Authority (EFSA), given the progressive decrease of BSE cases, recommended to carry out testing only in subpopulations of at-risk cattle (dead animals, emergency slaughter, and suspected clinical cases) and not in slaughtered healthy animals (https://www.oie.int/en/disease/bovine-spongiform-encephalopathy/).

25.6 The Detection of Atypical BSE Forms by Routine Testing

Since 1999, the EU validated three BSE screening tests, which were based on the detection of protease-resistant PrP^{Sc} by ELISA (Platelia® and Enfer test®) or by Western blot analysis (Prionics-Check®), thus allowing a rapid and large-scale analysis of BSE cases (Schaller et al. 1999). Testing was carried out on brainstem samples obtained at the slaughtered house, and all brain samples testing positive were further investigated by additional confirmatory tests, namely Western blot and immunohistochemistry.

While the aforementioned validated tests shed light on the dimension of underreported BSE cases by assessing the presence of PrP^{Sc}, the use of the confirmatory Western blot provided a qualitative analysis of PrP^{Sc} conformation, hence allowing the detection of variant PrP^{Sc} conformers. In 2003 two novel forms of BSE were found in France and Italy, which were characterized by a pathological prion protein differing in gel mobility and glycotype from C-type BSE. The three French cattle showed a PrP^{Sc} migrating "higher" as compared to C-BSE PrP^{Sc} (H-type BSE), whereas the two Italian cattle had a PrP^{Sc} migrating "lower" than C-BSE (L-type

BSE) (Biacabe et al. 2004; Casalone et al. 2004) (Fig. 25.1a). L-type BSE was

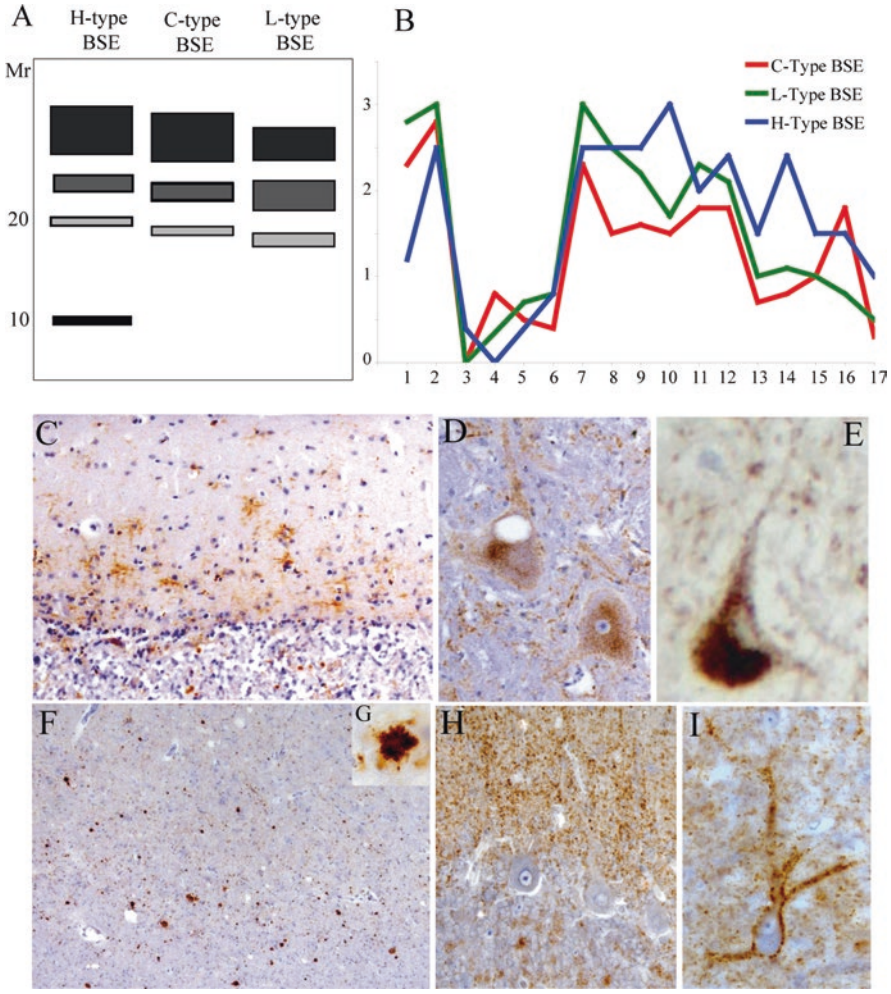

Fig. 25.1 Biochemical features, lesion profile, and pathological phenotypes of classical and atypical BSE forms. (**a**). Electrophoretic patterns of protease-resistant PrP in C-type and atypical BSE forms (H-type and L-type); (**b**). Histograms of the lesion load in H-type, C-type, and L-type BSEs; numbers in abscissa denote brain areas (1, nucleus of the solitary tract; 2, nucleus of the spinal tract of the trigeminal nerve; 3, hypoglossal nucleus; 4, vestibular nuclear complex; 5, cochlear nucleus; 6, cerebellar vermis; 7, central gray matter; 8, rostral colliculus; 9, medial geniculate nucleus; 10, hypothalamus; 11, nucleus dorsomedialis thalami; 12, nucleus ventralis lateralis thalami; 13, frontal cortex; 14, septal nuclei; 15, caudate; 16, putamen; 17, claustrum); numbers in ordinate denote vacuolation score (1, mild; 2 moderate; 3, severe); (**c**–**i**). Patterns of PrP deposition in different BSE forms. Typical stellate PrP pattern in the molecular layer of the cerebellum (**c**), and intraneuronal PrP staining in C-type BSE (**d**); intraneuronal PrP staining in H-type BSE (**e**); immunohistochemistry in L-type BSE showing PrP-amyloid plaques in the frontal cortex (**f** and **g**), granular and axonal PrP deposition in the cerebellum (**h**), and perineuronal PrP deposition (**i**)

originally named "bovine amyloidotic spongiform encephalopathy" (BASE) to highlight the unprecedented neuropathological phenotype, characterized by the abundance of amyloid-PrP plaques in brain tissues.

During the following years, atypical BSE cases were found in almost all European countries, in the United States, in Canada, and in Japan (Table 25.1). Eight years after their identification, a common phenotypic characteristic of atypical BSE forms is the relatively old age of affected cattle, as compared to cattle with classical BSE, and the apparent absence of clinical signs, with a few exceptions (Brown et al. 2006; Jacobs et al. 2007; Dudas et al. 2010).

In 2019, seven BSE cases were reported in the EU, all atypical BSE belonging to the subpopulation at risk. Six H-type (two by Spain and four by France) and one L-type by Poland. Outside the EU a single H-type case was reported in Brazil in a 17-year-old beef cow found dead during the ante mortem inspection at the abattoir. These atypical BSE cases were detected in cattle older than 11 years, in line with the average age at detection of 11.85 years (range: 5.5–18.5 years). However, one cow with H-type BSE was unusually young, that is, 5.5-year-old, and as such the

Table 25.1 Typical and atypical BSE cases detected worldwide from 1989 to 2019

	C-type			
Country	1989–2000	2001–2019	L-type	H-type
Austria	–	5	2	1
Belgium	19	114	–	–
Canada	–	19	1	1
Czech Republic	–	29	–	1
Denmark	1	15	1	–
Finland	–	1	–	–
France	95	873	20	24
Germany	7	409	3	2
Ireland	507	1057	–	1
Israel	–	1	–	–
Italy	–	142	5	–
Japan	–	36	2	–
Luxembourg	1	2	–	–
Netherlands	8	84	3	1
Poland	–	60	13	2
Portugal	522	556	–	7
Slovakia	–	27	–	–
Slovenia	–	9	–	–
Spain	2	797	10	11
Sweden	–	1	–	1
Switzerland	366	98	–	1
UK (GB)	179,087	2568	9	7
United States	–	2	–	2
Total	*180,615*	*6905*	*69*	*62*

youngest atypical BSE case ever reported. Although the number of BSE cases shows a significant decreasing trend in the occurrence of C-BSE no significant trend for the two atypical BSE forms is found (www.efsa.europa.eu/efsajournal18 *EFSA Journal* 2020;18(11):6303). Taken together, these findings further support the assumption that atypical cases of BSE represent cases of naturally occurring BSE in cattle. Thus, the observation of unexpected young cases might be not an exception but part of disease occurrence in the sporadic prion disease cases.

25.7 Disease Phenotypes of Classical BSE and Atypical Forms of BSE

After its original description, the clinical phenotype of classical BSE has been largely reported, being characterized by an insidious onset of altered behavior, with nervousness or apprehension, followed by sensory changes, including over-reactivity to external stimuli, spontaneous or evoked startle responses, hypersensitivity to external stimuli, and by locomotor signs such as tremor, hypermetria, ataxia, and recumbency (Wells et al. 1987). The neuropathological profile of BSE was clearly defined in over 600 cases at the beginning of the epidemic in the UK. The distribution and the score of vacuolar changes in different brain areas were examined by Scott and coworkers, who showed the highest lesion load in the medulla, midbrain, and thalamus, whereas cerebellum, hippocampus, cerebral cortex, and basal ganglia were relatively less involved (Scott et al. 1990). Spongiform degeneration was invariably observed in two medulla oblongata nuclei, that is, the solitary tract nucleus and the spinal tract nucleus of the trigeminal nerve, allowing a 100% diagnostic specificity (Wells et al. 1989), in addition to the central gray matter of the midbrain. Spongiform changes were located in the neuropil, albeit intracellular vacuoles, either in neuronal perikarya or in their axonal extensions, were observed in addition to astrocytic proliferation. Exclusive intraneuronal vacuolation, but not neuropil spongiosis, was considered not diagnostic.

In contrast to BSE, the clinical phenotype of atypical BSE forms is not clearly defined in field cases, albeit H-type and L-type BSE cases have been reported among fallen stock, and these animals might have displayed unreported clinical abnormalities; an exception is the Japanese L-type case, which exhibited dystasia at the abattoir (Dudas et al. 2010; Masujin et al. 2008).

Available data on the clinical features of atypical BSEs have been obtained by experimental transmission studies. We firstly reported that the clinical phenotype in BASE-affected cattle was characterized by dullness, hypersensitivity to facial stimuli, and weight loss, followed by fasciculations and amyotrophy, in the absence of cerebellar signs (Lombardi et al. 2008); conversely, H-type BSE was characterized by loss of weight, deteriorating body condition, low head carriage, high sensitivity to acoustic and visual stimuli, and slight hind limb ataxia (Balkema-Buschmann et al. 2011a; Okada et al. 2011). Hence, the prevalence of behavioral changes and

constitutional signs in atypical BSEs may in part explain the lack of recognition of these forms at slaughter inspection.

The neuropathological lesion profile of atypical BSE forms differed from C-BSE (Fig. 25.1b), and also immunohistochemical analysis showed patterns of PrP deposition, distinct from PrP deposits of granular type (in the neuronal cytoplasm or in gray matter neuropil), linear type (thick, thread- like profiles), and glial type, observed in C-BSE (Fig. 25.1c, d). In H-type BSE, PrP immunohistochemistry disclosed a prevailing intraneuronal and intraglial pattern of deposition (Fig. 25.1e), whereas in L-type BSE, or BASE, perineuronal synaptic staining, accompanied by abundant amyloid PrP deposition, was observed in deep gray nuclei and in the white matter (Fig. 25.1f–i) (Fukuda et al. 2009; Balkema-Buschmann et al. 2011a, b; Buschmann et al. 2006; Richt et al. 2007; Gavier-Widén et al. 2008). Interestingly, PrP^{Sc}-positive plaques, but not amyloid deposits, have been reported in H-type BSE (Okada et al. 2011).

25.8 Prion Strain Properties in Typical and Atypical BSEs

In addition to providing valuable information on the disease phenotype in its natural host, intraspecies transmission studies showed that atypical BSE forms displayed biological properties diverging from C-type BSE. Accordingly, cattle exposed to atypical BSEs, either H-type or L-type, had disease duration significantly shorter than C-type BSE, while the incubation period was longer (Lombardi et al. 2008; Balkema-Buschmann et al. 2011a).

Moreover, experimental studies in transgenic bovinized mice (Tgbov) challenged with H-type, L-type, and C-type BSEs showed an incubation period significantly shorter in animals inoculated with L-type BSE as compared to mice exposed to C-type BSE; conversely, TgBov mice inoculated with H-type BSE showed the longest incubation period, findings which favor the occurrence of different strains of the BSE agent (Buschmann et al. 2006).

Further, experimental transmission studies of atypical BSEs and C-type BSE to wild-type mice have provided intriguing results. At the first passage, the L-type isolate failed to transmit the disease to wild-type mice (C57Bl/6 or SJL), while H-type BSE transmitted to C57Bl/6, although with features differing from C-type BSE. Intriguingly, after serial passages in inbred mice or in a transgenic mouse model overexpressing ovine PrP (tg338), the L-type BSE strain acquired biological properties and phenotypic characteristics of the C-type BSE strain. Similar results were observed in C57Bl/6 mice serially challenged with H-type BSE, in which C-type BSE type properties were observed in some of the infected mice while others maintained the H-type BSE properties (Capobianco et al. 2007; Béringue et al. 2008; Baron et al. 2011).

25.9 On the Origin of BSE

The enigma of the BSE epidemic is still unsolved. Although it is clear that infected tissues had been included in MBM fed to cattle, several possibilities have been proposed as to the ancestral culprit of foodstuff contamination, including scrapie or genetic BSE. The hypothesis of an origin from scrapie is the more circumstanced. In the UK, sheep is the only recognized natural reservoir of the scrapie agent in the ovine population, with a prevalence of about 2 cases per 1000 (Morgan et al. 1990). Further, cattle have been shown to be susceptible to scrapie infection (Gibbs et al. 1990; Konold et al. 2006) and it might be reasonable to assume that the BSE epidemic started when the scrapie agent entered the food chain crossing the sheep-cow species barrier (Fig. 25.2a).

Another possibility remains the unapparent endemic presence of cattle BSE, or the occurrence of spontaneous cases of BSE in the cattle population (Kimberlin 1993; Brown 1998) (Fig. 25.2b). Several lines of evidence indicate that atypical BSE forms might be sporadic forms of BSE due to strict analogies with sporadic Creutzfeldt–Jakob disease (sCJD) in humans (Brown et al. 2006). These include the incidence of 1.9 cases per million of atypical BSEs in healthy slaughter cattle, the late age of disease onset, the occurrence of two distinct biochemical PrP^{Sc} types, and the presence of distinct patterns of PrP deposition (synaptic-type in H-type and amyloid-forming plaques in L-type) (Biacabe et al. 2008).

A study of oral inoculation of the L-BSE agent to calves demonstrated that the infectivity of the L-BSE agent was lower than that of the C-BSE agent. Calves were exposed orally to 1, 5, 10, or 50 g of brain homogenate of L-type BSE. A single animal exposed to 50 g showed clinical symptoms at 88 months while another was clinically healthy until 94 months post-exposure. In contrast, other animals exposed to a lower dose of inoculum did not develop clinical symptoms at around 90 months post-exposure. Although the molecular characteristics of proteinase K-resistant PrP^{Sc} in the brain, such as the molecular weight and the glycoform profile, were identical to the inoculum, the neuroanatomical distribution of PrP^{Sc} differed from that reported in natural cases of BSE L-type. In particular, PrP^{Sc} showed a higher distribution in the caudal medulla oblongata and the spinal cord but relatively lower in the cerebral cortices and the olfactory bulb (Okada et al. 2017). These findings indicate that although the biochemical profile of PrP^{Sc} remains identical, PrP^{Sc} distribution in orally exposed animals displays a modification of strain properties toward C-BSE. Likely, to demonstrate the origin of C-BSE from a "naturally occurring" strain, such as H-type in a cow exposed orally, more passages are needed to detect changes from the L-type BSE strain to the C-BSE strain (Okada et al. 2017).

Recently, a pathogenic E211K mutation has been reported in a cow with H-type BSE, but the biological relevance of this finding is still unclear (Fig. 25.2c) (Richt and Hall 2008). A subsequent study, in which H-type BSE associated with the E211K was inoculated intracerebrally into a calf of the same genotype, did not finally prove that E211K polymorphism was causative by itself, but suggests that

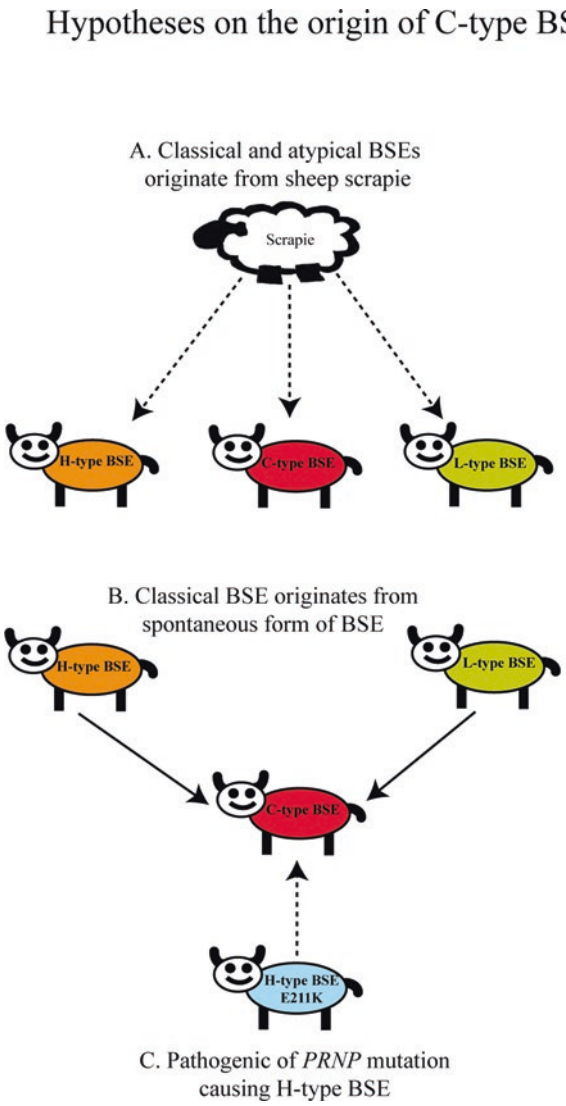

Fig. 25.2 Hypotheses on the origin of C-type BSE. (**a**) Classical and atypical BSEs originate from sheep scrapie. (**b**) Classical BSE originates from spontaneous form of BSE. (**c**) Pathogenic of *PRNP* mutation causing H-type BSE

cattle with the K211 PrP mutation are predisposed to a more rapid onset of BSE-H type (Greenlee et al. 2012).

25.10 Cattle BSE and Human Prion Diseases

In the original description of BASE (Casalone et al. 2004), we argued that BASE had molecular and pathological features similar to the MV2 molecular subtype of sCJD since both conditions shared the biochemical type of PrP^{Sc} and were characterized by PrP amyloid plaques in the nervous tissues.

The potential link between sCJD and BASE had been partially addressed in in vivo experimental models, by challenging transgenic humanized mice (TgHu) and non-human primates. Kong and collaborators showed that BASE was transmitted to TgHu mice overexpressing human PrP Met/Met at codon 129 (Tg40 mice), with an attack rate of 60% (Kong et al. 2008). The biochemical type of PrP^{Sc} observed in Tg40 mice was "monoglycosylated dominant," as observed in sCJD. In another study, TgHu mice (*tg650*) were intracerebrally inoculated with C-type, H-type, and L-type BSEs. In the first passage, all mice exposed to L-type BSE developed the disease, while mice inoculated with C-type BSE had an attack rate of 100% only in the second passage; in contrast, BSE H-type agent failed to transmit the disease (Béringue et al. 2008). The above studies indicate that the C-type BSE is less efficiently transmissible as compared to the L-type BSE. Therefore, a zoonotic risk is potentially higher for the BASE than for classical BSE and H-type BSE, as a likely effect of different species barrier properties (Béringue et al. 2008).

Furthermore, experimental infection of a single non-human primate with the L-type BSE isolate showed an incubation period shorter than that observed in animals exposed to C-type BSE; moreover, L-type and C-type infected animals displayed distinct disease phenotypes and PrP^{Sc} conformations (Comoy et al. 2008).

It is still not possible to assess whether the BASE strain is more pathogenic than C-type BSE for primates (including humans). Likewise, data are still too incomplete to prove a link between BASE and sporadic human CJD. However, results so far obtained justify some concerns about a potential human health hazard from atypical forms of BSE. In this context, it would be of help to monitor epidemiological data of sCJD as well as the occurrence of atypical sCJD phenotypes.

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Chapter 26
Classical and Atypical Scrapie in Sheep and Goats

Christine Fast and Martin H. Groschup

Abstract Scrapie is a naturally occurring transmissible spongiform encephalopathy (TSE) in sheep, goats and moufflons almost worldwide and is known for about 270 years. It is characterised by the accumulation of an abnormal isoform (PrP^{Sc}) of host-encoded prion protein (PrP^{C}) in the central nervous system which leads to progressive neurodegeneration and death. Scrapie represents the prototype of the so-called prion diseases. It is observed to date as two types, classical and atypical scrapie. The susceptibility to both types is modulated by polymorphisms of the prion protein gene. Whereas classical scrapie is clearly a naturally occurring contagious disease, atypical scrapie is most probably non-contagious and caused by an age-related spontaneous misfolding of the prion protein. This review gives an overview on the current knowledge of classical and atypical scrapie in sheep and goats with special emphasis on epidemiology, clinical and pathological signs, genetic susceptibilities, diagnosis and scrapie prion strains.

Keywords Atypical scrapie · Classical scrapie · Pathological prion protein · Prions · Scrapie · TSE

26.1 Overview

Scrapie is the most common name for transmissible spongiform encephalopathy (TSE), which affects sheep, goats and moufflons almost worldwide. Like all other prion diseases, scrapie is a neurodegenerative progressive and eventually fatal

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disease. Scrapie is associated with a number of clinical signs ranging from subtle behavioural abnormalities to more obvious neurological signs. The clinical diagnosis needs to be confirmed by the demonstration of pathognomonic spongiform lesions which are associated with an immunodetection of pathological prion protein (PrP^{Sc}) depositions in the central nervous system (CNS) primarily (OIE 2009). PrP^{Sc} depositions can be revealed by immunohistochemical and biochemical methods (see Chap. 33). To date, two distinct scrapie types are known: classical and atypical scrapie.

26.2 History

Scrapie is not only the prototype of TSEs but also the prion disease with the longest history of publication. The first authentic report on scrapie was written in Germany and dates back to the year 1750 (Leopoldt 1750). However, a later publication (Comber 1772) even mentions cases in England that occurred already in 1732. Several authors at later times even referred to much earlier time periods, spanning from Roman times up to the seventeenth century, but without giving corresponding references (for a detailed review, see Schneider et al. 2008). Moreover in former times, many sheep diseases were confused with scrapie. Other difficulties were the various names that were used to describe this disease throughout Europe: “Goggles”, “Ricketts”, “Rubbing Disease” and “Trotting Disease” in England, “Scratchie” and “Yeukie pine” in Scotland, “Basqvilla Disease” in Spain, “La maladie convulsive”, “La Tremblante” and “Prurigo lumbaire” in France, “Rida” in Iceland, “Gnave-og travesjuke” in Norway and “Gnubberkrankheit”, “Petermännchen”, “Traber” or “Reiberkrankheit” in Germany. Altogether, at least 42 different names were used in Europe and India (Schneider et al. 2008) for this disease in small ruminants.

The infectious nature of scrapie was already reckoned in the eighteenth century (Leopoldt 1750). In the following decades and centuries, different transmission routes were discussed in which sexual intercourse was the most suspected modus. However, among other causes like atmospheric disturbances, a few authors proposed a mere coexistence of infected and non-infected animals or a spontaneous origin of the diseases (Schneider et al. 2008). In addition, broad consent existed already in the nineteenth century concerning the role of hereditary factors for scrapie. Initially, a hereditary predisposition and the transmission by asymptomatic animals were assumed (Thaer 1821; von Richthofen 1821) and even the existence of hereditary and non-hereditary scrapie forms was postulated (von Richthofen 1826).

A number of experimental transmission studies were subsequently carried out in order to clarify the origin and transmission routes of scrapie. These experiments included contact studies with infected and non-infected sheep and subcutaneous and intravenous inoculation studies using different tissues and bodily fluids from infected animals. However, most of these studies were terminated prematurely and therefore failed due to the long incubation period of scrapie (for a detailed review, see Schneider et al. 2008). However, in 1936, the transmissibility of scrapie was first

time proven by experimental inoculation of healthy animals with the brain and spinal cord of diseased sheep. In this experiment, the inoculated animals were kept for longer periods of time, and sheep could develop scrapie after incubation periods of up to 2 years (Cuille and Chelle 1936, 1938a, b).

Since the 1930s, scrapie research was intensified when substantial financial losses to the sheep industry were caused by increasing numbers of cases. These losses prompted also studies on the true nature of the infectious agent. Besides parasites (M'Gowan 1914) and bacteria (Bastian 1979) as causative agents, virus infection was the most commonly proposed theory, already formulated in 1938 (Cuille and Chelle 1938a, b). In 1954, the term "slow virus infection" was first introduced (Sigurdsson 1954). However, already in 1966, an alternative to the virus origin was postulated as the causative agent, that is, polysaccharides (Alper et al. 1966, 1967; Field 1966) or lipids (Alper et al. 1978). In 1967, for the first time, a protein was assumed as infectious agent (Pattison and Jones 1967) and the first "protein-only-hypothesis" was enunciated (Griffith 1967) followed in the 1970s by the "virino" theory (Dickinson and Outram 1979). Finally, based on the resistance of the pathogen, in 1982, the term "proteinaceous infectious particle" (acronym: prion) was introduced (Prusiner 1982) and the conversion of a normal cellular protein (PrP^{c}) into a pathological isoform (PrP^{Sc}) as a key event of TSE pathogenesis was postulated shortly after (Oesch et al. 1985). PrP^{Sc} is currently considered to be the biochemical marker and the causative agent of TSEs. However, the prion theory is still debated since PrP^{Sc} is not always infectious and the phenomenon of strains is still an enigma (Lasmezas et al. 1997; Piccardo et al. 2007).

In 1998, the atypical form of scrapie, termed Nor98, was first time discovered in Norwegian sheep (Benestad et al. 2003). However, retrospective studies revealed atypical scrapie cases in the UK already in the late 1980s. Therefore, this disease is not considered as a new emerging form of TSE (Bruce et al. 2007). Atypical scrapie is distinguished from classical scrapie by the genotypes affected, the clinical and epidemiological characteristics as well as by the molecular pattern and the (neuro) anatomical spread of PrP^{Sc} (for review see Cassman and Greenlee 2019; EFSA 2021a). The disease can be found in sheep and goats and shows clear characteristics of a rare disease with a homogenous prevalence across countries, surveillance streams and year of examination (Fediaevsky et al. 2008). It is not rare compared to classical scrapie in most countries. Based on current knowledge a recent Scientific Opinion from EFSA (2021a) concluded that atypical scrapie is more likely a non-contagious rather than a contagious disease. Scrapie in goats was initially described after an experimental exposure in 1939 (Cuille and Chelle 1939) and the first natural case was reported a few years later (Chelle 1942). The first experimental challenge of goats with sheep scrapie showed 100% susceptibility suggesting that goats are highly susceptible (Pattison et al. 1959; Cuille and Chelle 1939). Like classical scrapie, atypical scrapie cases were reported also in goats (Fediaevsky et al. 2008, for a detailed review, see Vaccari et al. 2009) but showed a lower prevalence as compared to sheep (EFSA 2010).

In moufflons, only classical scrapie was reported at least in six natural cases so far (Wood et al. 1992a, b).

26.3 Geographical Distribution and Surveillance

At the turn of the millennium, classical scrapie cases were still widespread in small ruminants in Europe, and thousands of animals died because of the disease every year. As a result of surveillance, eradication and resistance breeding programmes, however, the number of cases has since fallen significantly. As a result, in 2020, classical scrapie cases were recorded only in Spain (273), Greece (203), Italy (115), Iceland (53), Romania (57), Bulgaria (13) and the United Kingdom (2). East Timor, Israel, Ivory Coast, Japan, Palestinian Autonomous Territories and the USA reported also scrapie cases (atypical and/or classical) in the last 5 years. Only individual atypical scrapie cases were documented on the Falkland Islands and New Zealand (World Animal Health Data Base (OIE-WAHIS)).

An introduction of classical scrapie via imported sheep from the UK was suspected in countries like Australia and New Zealand (1952–1954), South Africa (1964–1972), Colombia (1968–1971) and Kenya (1970). After thorough eradication by slaughtering the imported sheep and their flock mates, Australia and New Zealand remained free of classical scrapie to date (OIE-WAHIS).

However, the true scrapie status of many countries remains unknown because there is usually only an inadequate passive surveillance system in place to detect infected animals. It is nearly impossible to establish freedom from infection without establishing an active surveillance system, which includes the examination of fallen stock and emergency slaughter (Detwiler and Baylis 2003, OIE 2009). This is exemplified by the introduction of a harmonised active surveillance programme for scrapie in sheep and goats throughout the EU in 2003. In the context of this programme, defined numbers of sheep and goats over 18 months of age (fallen stock, emergency slaughter, as well as healthy slaughtered animals) were examined for TSE.

26.4 Prion Protein Gene and Susceptibility

It has been shown in several epidemiological studies that the successful transmission of classical scrapie requires genetically susceptible sheep. In the year 1968, the effect of a so-called *Sinc*-gene (scrapie incubation gene) on the length of the incubation period of experimentally infected mice and a synonymously so-called *Sip*-gene (scrapie incubation period gene) in sheep were proposed (Dickinson et al. 1968a, b). Eventually, different polymorphisms of the prion protein gene (*Prnp*) were matched in the 1980s and 1990s with the *Sip*-/*Sinc*-genes (Oesch et al. 1985; Westaway et al. 1987; Goldmann et al. 1991; Moore et al. 1998; Hunter et al. 1996).

The murine Prnp consists of two alleles, s7 and p7, which differ in their PrP amino acid sequence at codons 108 and 189 and are associated with short or prolonged incubation times after infection with particular (i.e., ME-7) experimental strains. However, infections with other strains (i.e., 22A) showed reversed results

(Dickinson et al. 1968a). Thus, the susceptibility and incubation period is determined at least by two factors: the genotype of the host and the agent strain. Similar results were obtained in sheep.

The ovine Prnp is located on chromosome 13 (Iannuzzi et al. 1998) and the functional length of the PrP gene is approximately 21 kb and is composed of three exons, from which exon III contains the complete uninterrupted open reading frame (ORF). The length of the unprocessed precursor protein is 256 amino acids. After post-translational modifications, about 210 amino acids remain in the mature protein (for a detailed review see Goldmann 2008).

Ovine PrP polymorphisms influence not only the susceptibility to the disease but also modulate the progression including the incubation period and clinical signs. The vast majority of polymorphisms are due to single nucleotide polymorphisms (SNP) in the DNA, which often cause single amino acid changes. Of particular interest are polymorphisms at codons 136, 154 and 171 within the ORF, which are clearly linked to scrapie susceptibility in sheep (Goldmann 2008). Standard abbreviations describe the alleles in reference to the three codons:

- A136V in which alanine (A) is associated with resistance and valine (V) is associated with susceptibility (Goldmann et al. 1991; Hunter et al. 1994).
- Q171R in which arginine (R) is associated with resistance and glutamine (Q) is associated with susceptibility (Westaway et al. 1994; Clouscard et al. 1995; O'Rourke et al. 1997).
- R154H in which histidine (H) is associated with resistance (Goldmann et al. 1991; Laplanche et al. 1993).

The polymorphisms mentioned above result in five different alleles (ARQ, VRQ, AHQ, ARR and ARH), leading to 15 different genotypes, which are the only alleles with significant distribution worldwide (Goldmann 2008). Some further genotypes, ARK and TRQ among others, are known (Gombojav et al. 2003; Guo et al. 2003; Billinis et al. 2004), but due to their low frequencies, they are not included in a TSE genotype classification system (Dawson et al. 1998). This five-group risk classification (Table 26.1) is the basis for breeding and scrapie eradication programmes applied in the EU. The highest risk to develop scrapie carry VRQ/VRQ animals, the highest genetic resistance is associated with ARR/ARR sheep (Belt et al. 1995; Hunter et al. 1996; Hunter 1997). However, this classification is subject to restrictions as, for example, at least two ARR/ARR sheep from different flocks in France and Germany have been shown to be subclinical carriers of classical scrapie (Groschup et al. 2007). Additionally, ARQ/ARQ animals, classified in R3, can be at the highest risk in flocks where the VRQ allele is absent for example due to breed (Goldmann 2008).

Furthermore, several polymorphisms are described at other positions, for example 25% of all ARQ alleles revealed additional polymorphisms (Goldmann 2008). However, it is unclear whether such polymorphisms have a profound effect on the disease. Some studies refer to resistance and/or prolonged incubation times in sheep carrying for example AC151RQ, AT137RQ or ARQK176 (Vaccari et al. 2009; Acin et al. 2004; Thorgeirsdottir et al. 1999).

Table 26.1 Ovine five-group risk classification system

Risk group	Genotype	Susceptibility
1	ARR/ARR	Highest genetic resistance
2	ARR/AHQ	Genetic resistance
	ARR/ARH	
	ARR/ARQ	
3	AHQ/AHQ	Low genetic resistance
	AHQ/ARH	
	AHQ/ARQ	
	ARH/ARH	
	ARH/ARQ	
	ARQ/ARQ	
4	ARR/VRQ	Genetic susceptibility
5	AHQ/VRQ	Highest genetic susceptibility
	ARH/VRQ	
	ARQ/VRQ	
	VRQ/VRQ	

The classification system described above and in Table 26.1 does not work for atypical scrapie. In contrast to classical scrapie in most of the atypical cases, animals of PrP genotype risk groups R1-3 (Benestad et al. 2008) are affected. Most frequently found in such cases are haplotypes such as AHQ/ARQ, AHQ/ARR and ARR/ARR, respectively (for review see EFSA 2021a). It has been shown that polymorphisms at codons 141 (L/F) and 154 (R/H) are linked to susceptibility. Genotype AF141RQ for example encoded for a higher susceptibility than the AL141RQ allele or even the AHQ genotype (Goldmann 2008).

Although the wild-type amino acid sequence of goat and sheep PrP are similar, the PrP genetics in goats is much more variable, yet without polymorphisms at codons 136 and 171 surprisingly. In goats, more than 70 other polymorphisms of the caprine Prnp, resulting in amino acid changes, have been found in different countries and breeds (Vaccari et al. 2009; Goldmann et al. 2011, Fast unpublished results). At least some of them seem to be associated with a certain degree of TSE resistance (for a detailed review see Vaccari et al. 2009 and EFSA 2017):

- I142M haplotypes show an incomplete resistance with a lengthened incubation period after experimental inoculation and an increased resistance to classical scrapie under natural conditions. However, some classical scrapie cases have been detected in such goats (Goldmann et al. 1996; Barillet et al. 2009; EFSA 2017).
- G145D was detected in resistant goats in an Italian study (Maestrale et al. 2015), combining experimental and epidemiological approaches. However, the examined animal group was small and no further data exist.
- R154H haplotypes are associated with some resistance to classical scrapie in different breeds and countries (Barillet et al. 2009; Billinis et al. 2002; Papasavva-Stylianou et al. 2007; Vaccari et al. 2006).

– N146S/D is associated with profound resistance to classical scrapie in field and experimental studies; however, intracerebral inoculation results in high attack rates but with a significant increase in the incubation period (EFSA 2017). Unfortunately, N146D is confined to goats in Cyprus (Papasavva-Stylianou et al. 2007).
– R211Q haplotypes have shown to convey an increased resistance to classical scrapie in French case–control studies (Barillet et al. 2009), but positive cases can be regularly found. There seems to be partial resistance and prolonged incubation periods, which might be strain dependent (EFSA 2017).
– Q222K haplotype is associated with protection against classical scrapie in several breeds and countries, demonstrated in epidemiological and experimental studies. However, protection against intracerebral challenge is incomplete, and few heterozygous animals are reported to be naturally infected (Acutis et al. 2006; Barillet et al. 2009; Vaccari et al. 2006; EFSA 2017).

In summary, in the Scientific Opinion published by EFSA (2017) a ranking of alleles was given, which is based on "weight of evidence" and "strength of resistance". This is, from high to weak scrapie resistance: K222 > D146 = S146 > Q21 1 = H154 = M142. As a consequence, both haplotypes 146S/D and 222 K can now be used for TSE resistance breeding and eradication programmes for goats. Most interestingly, Benestad et al. reported a single goat with a PrP-null allele in Norway (2012), which is resistant to scrapie (Salvesen et al. 2020).

Atypical scrapie in goats is most frequently associated with homozygous H154H and heterozygous R154H genotypes (Moum et al. 2005; Arsac et al. 2007; Seuberlich et al. 2007).

Taken together, the number of factors modulating the susceptibility to scrapie is high. The success of infection depends not only on the genotype of the host and the infectious agent strain but also on individual flocks, breeds and geographical location, not to forget the dose and route of inoculation effects.

26.5 Epidemiology of Scrapie

Summarising the prevalence of TSE infections in small ruminants worldwide is a difficult task in the face of the long incubation periods, the missing availability of a practical antemortem test (which prevents the detection of subclinical-infected animals), the variable clinical signs (which may result in unidentified animals), the potentially unknown host-encoded genetic components (which influence both the risk of infection and the incubation period) and the not yet fully understood routes of transmission (for detailed reviews concerning the epidemiology of classical and atypical scrapie see Hoinville 1996; Detwiler and Baylis 2003; Benestad et al. 2008; EFSA 2021a).

26.5.1 Prevalence in the EU

A comprehensive overview of the prevalence of classical and atypical scrapie in the European Union is given by Fediaevsky et al. as well as by EFSA (Fediaevsky et al. 2008, 2010; EFSA 2010, 2014, 2021a).

Following the introduction of active surveillance programmes for TSEs in sheep and goats in the EU in 2002, clearly defined epidemiological data were obtained for the first time. Most importantly, it has been shown that the prevalence of TSE in sheep/goats and the geographical distribution were much higher than originally assumed (EFSA 2014) and the number of cases in fallen stock was significantly higher as compared to healthy slaughter animals (Fediaevsky et al. 2008). Additionally, the prevalence of classical and atypical scrapie showed different patterns with more variation seen in classical scrapie (Fediaevsky et al. 2008). Moreover, the incidence rates of classical scrapie in geographical areas were non-uniform and clustered at the flock level (McIntyre et al. 2008). In some countries only a few, if any, cases were detected, whereas other EU member states experienced large epidemics (EFSA 2014). In 2002, a breeding and scrapie eradication programme was applied in the EU, and the results are heterogeneous (for a detailed review see EFSA 2014). From 2002 to 2012 only in six Member States a significantly decreasing trend was found, which was in all cases associated with an effective implementation of genetic and non-genetic measures for the control of the disease. Thus, a successful classical scrapie eradication policy cannot rely on post-mortem testing and depopulation programmes solely, but should also include breeding programmes for resistance to classical scrapie. However, due to the long incubation time and particular pathogenesis of classical scrapie, an underestimation of the real prevalence may apply and substantial numbers of undetected cases (up to 17%) were reported (Jeffrey et al. 2002; Ligios et al. 2006; Reckzeh et al. 2007; González et al. 2009).

The distribution of atypical scrapie cases is remarkably homogenous in space and time as compared to classical scrapie and no infection clusters were observed in positive flocks. In most cases, individual animals are affected instead (Fediaevsky et al. 2008, 2010). The animals (sheep as well as goats) are significantly older usually as compared to animals affected by classical scrapie. In eight EU countries between 2007 and 2009, the incidence of atypical scrapie in healthy slaughtered sheep was similar to or higher than the incidence of classical scrapie. These data suggest that atypical scrapie represents a significant proportion of TSE-infected small ruminants (EFSA 2010). However, relying on the prevalence in the EU and on scientific data it can be concluded that atypical scrapie is most likely a non-contagious disease or has very low transmissibility under natural conditions (Fediaevsky et al. 2010; EFSA 2021a). The prevalence seems to be stable within the EU, with >100 cases on average per year for sheep and 10 cases for goats (EFSA 2020). Atypical scrapie is most frequently detected by active surveillance in fallen stock animals. Due to the generally used passive surveillance approach, atypical scrapie may also be underreported. Other problems in estimating the exact

prevalence of atypical scrapie include the age-dependent variations and the inconsistent detection of atypical scrapie by using brainstem samples (Benestad et al. 2008), the low sensitivity of some rapid tests for atypical scrapie (EFSA 2005) and the absence of detectable pathological prion protein in the lymphoreticular tissues.

26.5.2 Transmission Routes in Scrapie

In the last centuries, a lengthy discussion about the mode of transmission of scrapie took place (Schneider et al. 2008), and even up to now the exact transmission routes are not resolved entirely. It is known that classical scrapie can transmit laterally between sheep under natural conditions. Such transmissions occur either via direct contact or through contamination of the environment. The oral route is most efficient (Jeffrey and Gonzalez 2007; van Keulen et al. 2008). Scrapie in goats is often found in mixed herds with sheep, but it has also been observed to spread from goat to goat (Wood et al. 1992b).

The main source of infection is the infectious placenta. Infectivity and PrP^{Sc} have been detected in the foetal parts of the placenta, depending on the genotype of the offspring (Pattison et al. 1972; Onodera et al. 1993; Race et al. 1998; Andreoletti et al. 2002; Alverson et al. 2006; Lacroux et al. 2007; O'Rourke et al. 2011). More recently, PrP^{Sc} and infectivity have also been detected in foetal tissue samples (Garza et al. 2011; Spiropoulos et al. 2014). The placenta and the amniotic fluid (Hoinville 1996) are shed into the environment during lambing and their ingestion by other sheep (and goats) is still assumed to be the most important infection mode within the flock (Pattison et al. 1972; Hoinville 1996). Moreover, it has been shown that scrapie agent remains infectious even after years in the environment (Brown and Gajdusek 1991; Seidel et al. 2007). Anecdotal data indicate even survival of infectivity for more than 16 years (Georgsson et al. 2006). Additional results indicate that released PrP^{Sc} may be sequestered near the soil surface and bound on soil minerals, which may then be ingested during grazing of farm animals (Johnson et al. 2006, for a detailed review, see Smith et al. 2011). Even more interestingly, amplifiable classical scrapie PrP^{Sc} has been detectable on surfaces of farm fomites and such fomites (i.e. water troughs) are sufficient to transmit the disease to naive sheep even after decontamination of barns, indicating that PrP^{Sc} in the dust was the source (Konold et al. 2015; Gough et al. 2015, 2018).

Besides the placenta, faeces (Terry et al. 2011) and milk (Konold et al. 2008; Lacroux et al. 2008; Maddison et al. 2009; Madsen-Bouterse et al. 2018) have been shown to contain PrP^{Sc} and/or infectivity. Recent results revealed PrP^{Sc} also in the oral cavity of scrapie-infected sheep (Maddison et al. 2010; Gough et al. 2011) and PrP^{Sc} and/or infectivity in urine was demonstrated in experimental scrapie models in hamsters and mice (Seeger et al. 2005; Gonzalez-Romero et al. 2008; Gregori et al. 2008). More artificial routes demonstrated in several experimental infections include transmissions via subcutaneous inoculation (Stamp et al. 1959; Kratzel et al. 2007), conjunctival exposure (Haralambiev et al. 1973), skin scarification

(Taylor et al. 1996) and blood transfusions (Houston et al. 2008). Some scrapie infections were consequences of iatrogenic transmissions due to contaminated vaccines (Gordon 1946; Caramelli et al. 2001).

In most flocks, only a single case of atypical scrapie is found. The transmission mode of atypical scrapie under natural conditions is not understood at all and it is even questioned whether this disease is contagious under all circumstances. The intracerebral route of infection has been clearly established in both rodent and susceptible (AHQ/AHQ) sheep models (Le Dur et al. 2005; Simmons et al. 2007, 2010), but silent wild-type carriers (ARQ/ARQ) are also known (Okada et al. 2016). Under experimental conditions, an oral challenge of newborn lambs within 24-h post-partum was successful in AHQ homozygous sheep (Simmons et al. 2011). Epidemiological data obtained by active surveillance programmes indicate that the capacity of atypical scrapie to transmit disease within the herd under field conditions is quite low and most probably non-existent (Fediaevsky et al. 2009, 2010). This could be due to the fact that affected sheep shed little or no PrP^{Sc} infectivity, as atypical scrapie does not spread or only very little in peripheral tissues compared to classical scrapie (EFSA 2021a). However, also cohort cases of atypical scrapie are reported in flocks and also coinfections with classical scrapie in some herds (Konold et al. 2007a, b; Onnasch et al. 2004; Orge et al. 2010). Taken together, these data support the theory of a spontaneous origin of the disease, which might be associated with a very low or absent natural transmissibility (Benestad et al. 2003; Moum et al. 2005; Hopp et al. 2006; Green et al. 2007). Retrospective studies indicate that large flock sizes (>1000 sheep), over-average animal exchanges within flocks and vitamin and mineral feed supplements may be risk factors for atypical scrapie (Hopp et al. 2006; Green et al. 2007).

26.5.3 Incubation Period

The incubation time of scrapie depends on the infection route and the animal's age at infection, its genotype, the involved agent strain and the infectious dose. Interestingly, certain strains proliferate easier in specific genotypes. Iatrogenic infections lead to slightly shorter incubation periods (Caramelli et al. 2001). In classical scrapie, sheep come down with clinical disease usually between 2 and 5 years of age, with an average age of 47.8 months in the time period 2002–2020 (EFSA 2020). Although both sexes appear to be equally affected, disease manifestations in rams occur often at a slightly younger age (Parry 1983; Wineland et al. 1998; Lühken et al. 2007; McIntyre et al. 2008). However, also shorter and longer incubation periods ranging between 1 and up to 11 years are reported (Parry 1983). Scrapie-diseased animals younger than 18 months are fairly rare (Dickinson and Stamp 1969). However, it is usually not possible to tell the time of infection in older scrapie-diseased sheep (Detwiler and Baylis 2003).

The frequency of atypical scrapie cases increases with the age of the animals, thus the average age of atypical scrapie cases in sheep and goats is significantly

higher than that of classical scrapie (EFSA 2021b). Atypical cases in sheep are on average 82 months of age (EFSA 2021b). In a German (Lühken et al. 2007) and in a larger pan-European (20 countries) study, almost 60% or 70% of the atypical scrapie cases were 5 years or older, respectively (Fediaevsky et al. 2008).

As for sheep, the incubation period of goats is influenced by the genotype (Goldmann 2008). Data concerning the age distribution of TSE-infected goats are rare but indicate similarities to the distribution in sheep scrapie. The mean age of goats affected by classical scrapie from 2002 to 2020 was 51.6 months (EFSA 2021b); however, cases up to 10 years of age were also reported (Brotherston et al. 1968; Hourrigan et al. 1969; Harcourt and Anderson 1974; Wood et al. 1992b; Capucchio et al. 1998; Konold et al. 2007b; Papasavva-Stylianou et al. 2010; Niedermeyer et al. 2016). Atypical scrapie cases in goats from 2002 to 2020 have a mean age of 84 months (EFSA 2021b).

26.5.4 Pathogenesis and Tissue Distribution of PrP^{Sc} and/or Infectivity

The pathogenesis of TSEs is discussed separately in Chap. 27. Nevertheless, the most important facts are summarised here and in Fig. 26.1.

After oral uptake, it still remains an enigma how the infectious agent overcomes the mucosal barrier of the gut (for a detailed review see Mabbott and MacPherson 2006). The first results indicate that the genotype does not affect this process (Jeffrey et al. 2006). M cells within the follicle-associated epithelium of the gut and specialised for the transport of macromolecules are important sites of PrP^{Sc} uptake (Donaldson et al. 2012). Transport across the villous enterocytes (Jeffrey et al. 2006; Akesson et al. 2011) and a direct uptake by processes of dendritic cells extending into the gut lumen (Rescigno et al. 2001) are further options. In this regard, there is experimental evidence that dendritic cells play a crucial role in the transport of PrP^{Sc} towards follicular dendritic cells (FDC) within Peyer's patches (PP) (Bradford et al. 2017). After crossing, the mucosal barrier PrP^{Sc} was found within 15 min after inoculation in the lacteals of the villi (Jeffrey et al. 2006; Akesson et al. 2011). The first accumulation of PrP^{Sc} was seen in the gut-associated lymphoid tissues (GALT) of the tonsil and PP in the intestines in lambs as early as 21-day post-partum (Andreoletti et al. 2000, 2002; van Keulen et al. 2002). Experimental infections indicate a rapid transport of inoculum into the GALT and corresponding lymph nodes, but replication and accumulation of de novo PrP^{Sc} were not seen before 1-month post-infection (Jeffrey et al. 2006). Experimental data demonstrate that FDC is the target cell for prion replication in lymphoid tissues (McCulloch et al. 2011). As shown in naturally infected lambs, the accumulation of PrP^{Sc} is restricted to the GALT and mesenteric lymph nodes for the first 2 months of age (Andreoletti et al. 2000, 2002; van Keulen et al. 2002). Subsequently in lambs older than 2 months, a spread to all lymph nodes of the lymphoreticular system (LRS) takes place and the amount of PrP^{Sc} in the LRS increases with age up to a

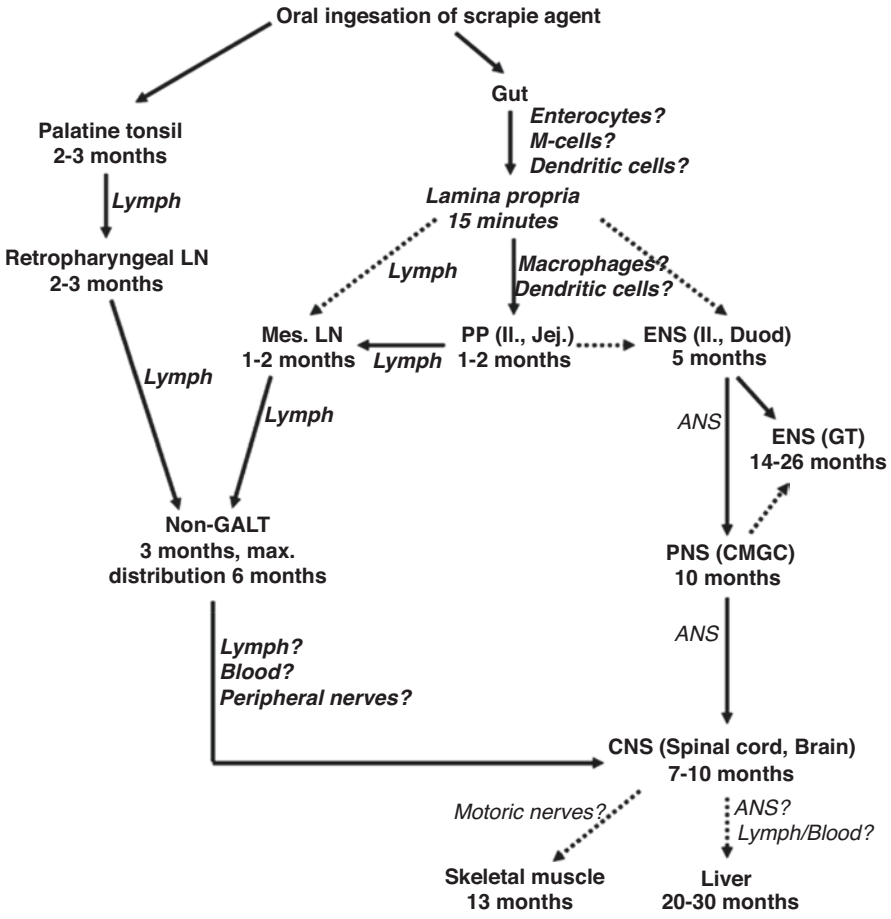

Fig. 26.1 Schematic illustration of the most common theories concerning the pathogenesis of classical scrapie (Modified from van Keulen et al. 2002; Sisó et al. 2010). The time periods stated are from different studies showing PrP^{Sc} accumulation by immunohistochemistry (Andreoletti et al. 2000, 2002, 2004; van Keulen et al. 2000, 2002; Jeffrey et al. 2006; Everest et al. 2011), which mostly rely on VRQ/VRQ sheep. ARQ sheep and experimentally infected goats revealed (as far as known) similar distribution but delayed dynamics. In ARR sheep PrP^{Sc} is mainly confined to the CNS. *Dotted arrows* indicate possible but not yet clarified routes of dissemination. *LN* lymphonodus, *GALT* gut-associated lymphoid tissue, *GIT* gastrointestinal tract, *PP* Peyer's patches, *ENS* enteric nervous system, *ANS* autonomous nervous system, *PNS* peripheral nervous system, *CMGC* celiac and mesenteric ganglion complex, *IL* Ileum, *Duod* duodenum

plateau level around 6 months (Andreoletti et al. 2000). At this time, after one-third of the incubation period, infectivity is found first time in blood with increasing tendency until to the clinical stage (Houston et al. 2008). The enteric nervous system (ENS) of the duodenum and the ileum are the first parts of the peripheral nervous system, which become affected after 5 months (van Keulen et al. 2000). The exact route of infection is not understood completely yet, especially whether a prion

replication in the GALT is necessary for further neuroinvasion. In this regard, sheep with more resistant genotypes fail to accumulate PrP^{Sc} in the LRS. For example, sheep of the VRQ/ARR genotype have no or only low amounts of PrP^{Sc} in the lymphoid tissues but develop scrapie albeit only after longer incubation periods (Bossers et al. 1996; van Keulen et al. 1996). Thus, a direct infection via subepithelial nerve endings or an indirect infection via infected Peyer's patches and submucosal plexus of the ENS are conceivable (Jeffrey et al. 2006; van Keulen et al. 2008). With the progression of the disease starting at 14 months, PrP^{Sc} spreads within the ENS in all directions and other parts of the small intestine and at later stages (21–26 months) even the oesophagus, forestomach, large intestine and rectum become involved (van Keulen et al. 2000). Along parasympathetic and/or sympathetic nerve fibres, prions ascend after 10 months via the celiac and mesenteric ganglion complex to the spinal cord and/or brainstem (van Keulen et al. 2000). From these sites in the CNS, a further ascending and descending spread of PrPSc takes place (van Keulen et al. 2008).

There is also evidence for the hematogenous spread of the scrapie agent demonstrated by the transmission of the disease via blood and blood particles (Houston et al. 2008; Dassanayake et al. 2015) as well as by the early detection of PrP^{Sc} in the Circumventricular Organ of the CNS, an area without a blood–brain barrier (Siso et al. 2009).

Between 7 and 10 months of age, PrP^{Sc} can be demonstrated first time in the brainstem and spinal cord of young VRQ sheep (Andreoletti et al. 2000; Jeffrey et al. 2001; van Keulen et al. 2002). At 13 months of age, PrP^{Sc} is eventually identified in skeletal muscle (Andreoletti et al. 2004) and after 20–30 months in the liver of naturally infected sheep (Everest et al. 2011).

Most of the aforementioned data were obtained for VRQ/VRQ animals, which are considered to be most susceptible and having a comparatively fast dissemination dynamic. Only limited data are available for sheep of other genotypes (Jeffrey et al. 2001; Lacroux et al. 2008). However, these data indicate that the topology and the timing of the PrP^{Sc} dissemination in ARQ/ARQ and ARQ/VRQ sheep are quite similar, apart from a slightly delayed dynamic in ARQ carriers (EFSA 2010). There are only a few reports on classical scrapie in heterozygous ARR sheep, perhaps due to the lower susceptibility of animals carrying this genotype. In such cases, PrP^{Sc} is mainly confined to the CNS (van Keulen et al. 1996; Greenlee et al. 2014).

The dissemination dynamics of classical scrapie in goats is well documented but relies mostly on experimentally challenged wild-type goats. The spread of PrP^{Sc} during the prion ascension seems to be quite similar to classical scrapie in sheep (EFSA 2009; González et al. 2009, 2010a, b; Niedermeyer et al. 2016). However, a French study shows that the time course may be prolonged as compared to scrapie in sheep. In goat kids infected around birth, PrP^{Sc} was detectable in the GALT not before 4 months of age, peripheral lymphoid tissues turned PrP^{Sc} positive after 6 months of age and the CNS showed the first PrP^{Sc} accumulations at 18 months of age. In skeletal muscle, PrP^{Sc} was not detected before 21 months of age (EFSA 2010).

However, it should be noted that there is a high diversity of classical scrapie strains in sheep and goats. Their interaction with the particular host genotypes may result in different dissemination dynamics. Therefore, the tissue distribution

described above cannot be considered as definitive (EFSA 2010). For example, several ARQ/VRQ and ARQ/ARQ sheep and some goats affected with classical scrapie were reported with few, if any detectable PrP^{Sc} in the LRS (Jeffrey et al. 2002; Ligios et al. 2006; Konold et al. 2007a, b; González et al. 2009; Niedermeyer et al. 2016). Additionally, results from experiments of scrapie-infected I142M goats revealed that the dissemination of the TSE agent in peripheral tissues is delayed as compared to wild-type goats (EFSA 2010).

The limited data concerning the tissue distribution of atypical scrapie indicate that detectable amounts of PrP^{Sc} seem to be confined to the CNS (Benestad et al. 2003, 2008; Simmons et al. 2007; Vidal et al. 2008). However, in mouse bioassays, infectivity was shown in the absence of any detectable PrP^{Sc} in peripheral tissues including the LRS (Andreoletti et al. 2011; Simmons et al. 2011).

26.6 Clinical Signs

Clinical signs are quite variable in different breeds, flocks, regions and countries and are influenced by genotype, agent strain and stage of the disease (for a detailed review see Parry 1983; Ulvund 2007, 2008).

The clinical phase mostly progresses slowly over several weeks and months, but acute onsets and durations up to 1 year with intermittent remission of the signs are also seen. Recumbent or sudden deaths of animals were recorded (Parry 1983; Clark et al. 1994; Capucchio et al. 2001; Healy et al. 2003; Humphrey et al. 2004).

Deficits in the disease recognition by shepherds/veterinarians, the subtle onset, the variability of signs as well as the slow clinical progression of the disease are reasons why the disease often remains unidentified. Isolation of animals from the flock is often the first clinical sign. More specific symptoms at the early stage are central nervous system deficits and loss of wool caused by pruritus. Affected animals may appear normal but stimulated by stress (i.e., sudden noise, excessive movement and handling) tremor becomes obvious. At later stages, the animal may even fall down into a convulsive state (Hörnlimann et al. 2007; Ulvund 2007). Clinical signs of scrapie fall into five different categories (Ulvund 2008):

- General signs: Depression, wool loss, regurgitation and cardiac arrhythmia.
- Changes in behaviour: Head tremor, altered mental status, nibble response (reflex), teeth grinding, altered head carriage, hyperresponsive, anxious, apprehensive, salivation, aggressiveness and reluctance to be milked.
- Changes in sensitivity: Pruritus, “cannibalism”, allotriophagia and biting.
- Changes in locomotion: Hind limb ataxia, dysmetria, abnormal posture, hind limb weakness and circling.
- Other signs: Weight loss, labial oedema, visual impairment, brief epileptiform attacks and hypogalactia.

Not all symptoms are always present, but usually at least more than one is noticeable (Hörnlimann et al. 2007). Moreover, a nervous form may dominate in one

flock, while the pruritic form prevails in another (Ulvund 2008). In general, head tremor, nibble response, hyperresponsiveness, salivation, pruritus and weight loss are the most often reported symptoms in different flocks and countries (Healy et al. 2003; Capucchio et al. 2001; Ulvund 2007; Vargas et al. 2005). The final stages are characterised by massive weight losses often despite of unchanged appetite and recumbency due to severe ataxia (Hörnlimann et al. 2007; Ulvund 2007).

Data on clinical signs in classical scrapie infected goats are rare and most authors refer to symptoms as described for sheep. Disease durations from 1 up to 3 months are described (Capucchio et al. 1998; Foster et al. 2001; Konold et al. 2007b). The most frequent signs described are weight loss despite of remaining appetite, ataxia and progression to recumbency and pruritus. Behavioural changes include apathy, nervousness or aggressiveness. Less frequently found symptoms are sometimes confined to single animals and include lateralisation of neurological signs such as circling, biting, ptyalism, hyperaesthesia, dribbling/regurgitation, visual impairment, difficulties with milk and tremor (Brotherston et al. 1968; Hourrigan et al. 1969; Harcourt and Anderson 1974; Wood et al. 1992a, b; Capucchio et al. 1998; Foster et al. 2001; Konold et al. 2007a, b).

Only a few reports are describing clinical signs of atypical scrapie in sheep and goats. This could be interpreted as if there was a less pronounced clinical phase. However, since normally only singleton animals are affected, they are recognised not quite well by veterinary professionals (Benestad et al. 2008). The overall clinical signs of atypical scrapie are ataxia and weight loss and behavioural changes such as nervousness and anxiety. Circling movements of the sheep may also occur. The tremor was hardly seen and—with the exception of two British cases—alopecia due to pruritus did not occur. Animals die unexpectedly or after a very acute progression phase. One goat was described as having blindness, stiff gait and apathy (Benestad et al. 2003; Gavier-Widen et al. 2004; Onnasch et al. 2004; Epstein et al. 2005; Nentwig et al. 2007; Simmons et al. 2007; Dagleish et al. 2008).

None of the clinical signs described above, in combination or alone, are pathognomonic for scrapie. Therefore, the clinical diagnosis must always be confirmed by laboratory investigations (Ulvund 2007, 2008).

26.7 Diagnosis of Scrapie

The diagnosis of TSEs is discussed separately in Chap. 35. Nevertheless, the most important facts are summarised here.

The TSE surveillance in small ruminants is based on rapid tests using brainstem material. To diagnose atypical scrapie, samples of the cerebellum have to be included as well (TSE EURL, a technical handbook for National Reference Laboratories in the EU). All samples of active surveillance with a reactive result in one of the approved rapid tests must be retested in the national reference laboratory using one of the OIE-approved confirmatory methods (Matthews et al. 2004). Clinical suspect animals (passive surveillance) may be directly examined by such methods. These

are histopathology, immunohistochemistry (IHC), electron microscopy and scrapie-associated fibrils (SAF) immunoblot. For practical reasons, mainly the IHC and SAF immunoblot are of relevance today.

26.7.1 Discriminatory Immunoblot

According to the EU legislation (January 2005, EC regulation 36/2005), all confirmed classical scrapie cases in small ruminants should be examined by discriminatory testing to reveal BSE infections in sheep and goats. These include discriminatory immunoblots (following defined immunoblot protocols, see TSE EURL, a technical handbook for National Reference Laboratories in the EUs) and, in equivocal cases, mouse bioassays (for strain typing). Size differences of proteinase K (PK)-treated, non-glycosylated PrP^{Sc} can be shown by high-resolution sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) followed by immunoblotting. Moreover, monoclonal antibodies binding to an epitope located on the ragged N-terminal end of PK-cleaved PrP^{Sc} are useful tools in discriminating classical scrapie from ovine BSE. One of these antibodies is, for example, mab P4, whose N-terminal epitope remains detectable after PK digestion of scrapie PrP^{Sc}, in contrast to BSE PrP^{Sc}, from which this epitope is trimmed off by this enzyme. Antibodies that recognise an epitope in the core region of PrP^{Sc}, mab L42 for example, detect scrapie as well as BSE PrP^{Sc} after PK digestion because this treatment has no influence on epitopes of the protein's core region (Figs. 26.2 and 26.3).

In the last years, several biochemical strain typing techniques were developed, which utilise these differences in the PK cleavage site of PrP^{Sc} (Stack et al. 2002; Lezmi et al. 2004; Nonno et al. 2003; Thuring et al. 2004; Gretzschel et al. 2005, for details see TSE EURL, a technical handbook for National Reference Laboratories in the EU). In Germany, the so-called FLI test is applied (Gretzschel et al. 2005), which is a biochemical BSE/scrapie typing strategy that utilises the differences in

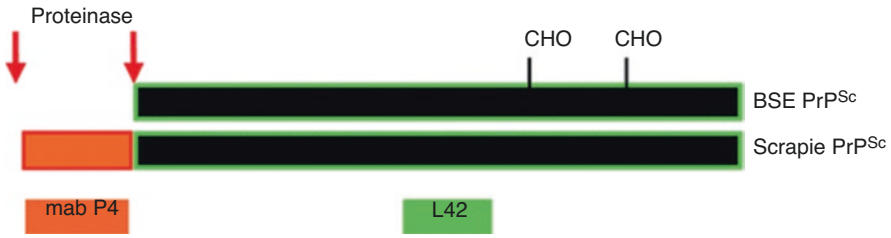

Fig. 26.2 Lack of detection of ovine and bovine BSE PrP^{Sc} by mab P4. As the PK cleavage sites vary between BSE and scrapie, mab P4 can be used to discriminate between these two TSE types. While BSE-related PrP^{Sc} is trimmed approximately to the amino acid 100 and the P4 epitope is therefore destroyed, the trimming of scrapie-related PrP^{Sc} stops 10–15 amino acid positions further N terminally. Therefore, the P4 epitope remains intact and the PK-digested PrP^{Sc} is easily detected by the antibody

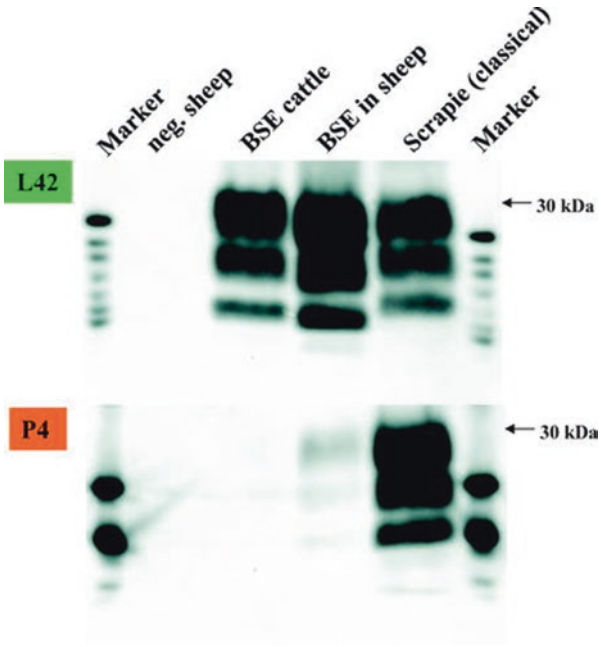

Fig. 26.3 Comparison of electrophoretic profiles and antibody labelling of PrP^{Sc} after proteinase K digestion, PTA precipitation and immunoblotting using mab L42 or mab P4. Both blots are loaded with the same quantities of precipitated PrP^{Sc} of each sample

the glycosylation and PK cleavage site of PK treated and immunoblotted ovine BSE and scrapie PrP^{Sc}. Detection antibodies are mabs L42 and P4.

According to the discriminatory testing PrP^{Sc} in a sample will be judged BSE-like, if the sample conforms to the following three biochemical attributes: (1) the diglycosylated band is the dominating moiety (FLI test: the glycoform ratio for the diglycosylated form is above 50%); (2) Certain N-terminal antibodies fail to detect PrP^{Sc} (FLI test: the antibody binding ratio P4/L42 has a lower value than 0.4) and (3) low molecular weight of the unglycosylated band (FLI test: the molecular mass is by >0.5 kDa lower than that of the internal scrapie standard). Deviations in one or more characteristics exclude BSE in the isolate concerned since only the complete characteristics define the BSE agent.

26.7.2 Histopathology

Gross lesions are not visible and the histomorphological alterations are confined to the central nervous system. The first description of typical scrapie lesions dates back to the nineteenth century (Besnoit and Morel 1898). Scrapie is a neurodegenerative disease with vacuolation of the grey matter as a hallmark, often accompanied by

astrocytosis but without signs of inflammation. Neuronal loss is present, but significant cell losses are not evident on routine examination (Jeffrey and Gonzalez 2004; Wells et al. 2007). The development of clinical signs is not necessarily reflected by the severity of the pathology changes (Jeffrey and Gonzalez 2004, 2007).

Lesions are usually bilaterally symmetrical (Fraser 1993), especially at the brainstem at the level of the obex (Fig. 26.4), and the dorsal motor nucleus of the vagus nerve is the most commonly affected site (Wood et al. 1997). However, a considerable variation in the neuroanatomical distribution of the spongiform lesions is obvious, especially in more rostral areas of the brain. The formation of lesions depends not only on the prion strain but also on the genotype of the host, breed and presumably also other individual factors (Ligios et al. 2002; Begara-McGorum et al. 2002). Additionally, the magnitude of vacuolation is influenced by the age at the onset of clinical disease (Ligios et al. 2002).

In classical scrapie vacuolation is detectable in the neuronal perikarya and in the neuropil but can be rare in some naturally occurring and experimental scrapie cases (Zlotnik 1960; Dickinson 1976; Fraser 1976; Chaplin et al. 1998; Begara-McGorum et al. 2002). These membrane-bound vacuoles are found within the neuronal perikarya as single or multiple vacuoles distending the cell body and/or within processes leading to the typical spongiform appearance in the grey matter neuropil (Jeffrey et al. 1995; Jeffrey and Gonzalez 2004). The proportion of perikaryonal to neuropil vacuolation differs in respect of the disease and agent strain. In murine scrapie models, dendrites are most frequently affected, neuronal perikarya, axons and axon terminals to a lesser extent (for a detailed review see Jeffrey et al. 1995). Additional findings might be other signs of neuronal degeneration like

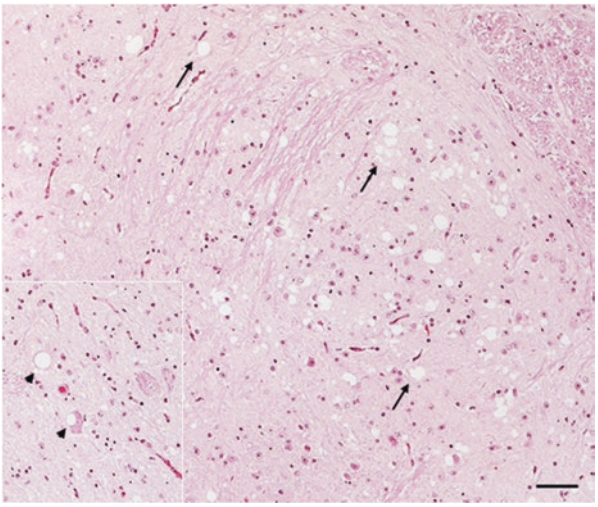

Fig. 26.4 Scrapie-infected goat with clear signs of spongiform encephalopathy in the brainstem at the level of the obex. Vacuolation is detectable in the neuronal perikarya (*arrowheads*) and in the neuropil (arrows), H & E staining, bar 50 µm

chromatolysis, neuronophagia and dark shrunken neurons. Astrocytosis is also an inconsistent finding seen in some scrapie cases (Wood et al. 1997; Jeffrey and Gonzalez 2004; Wells et al. 2007).

In atypical scrapie, the vacuolation is most prominent in the molecular layer of the cerebellar cortex, neocortex hippocampus, basal nuclei and nucleus accumbens. The brainstem is, in contrast to classical scrapie, affected to a much lesser degree and no lesions are observed at the level of the obex (Benestad et al. 2003; Moore et al. 2008). Intraneuronal vacuolation is not (Moore et al. 2008) or only infrequently seen (Benestad et al. 2003).

26.7.3 Immunohistochemistry

The second hallmark of TSEs is the accumulation of PrP^{Sc} in the brain, which precedes morphological alterations (DeArmond 1993; Jeffrey et al. 2000). Previous studies (van Keulen et al. 2000) with classical scrapie demonstrated that the brainstem at the level of the obex, in particular the dorsal motor nucleus of the vagus nerve, is the first area in the CNS to become affected in advance of any morphological alterations. With the progression of the disease, the PrP^{Sc} accumulation becomes more widespread and spreads in ascending and descending directions to finally involve at clinical endpoint the entire neuraxis.

It is possible to differentiate several morphological types of PrP^{Sc} accumulation (Table 26.2). These PrP^{Sc} profiles provide strain and source-specific information on the cell types, which sustain the infection (cellular tropism) and the cellular processing of PrP^{Sc}. Not all these types and patterns are found in all scrapie cases. Furthermore, in immunohistochemistry (IHC), a differentiation between ovine/caprine BSE and scrapie is possible by using the immunoreactivity of antibodies recognising different epitopes of PrP^{Sc} (epitope mapping) (for a detailed review see Jeffrey and Gonzalez 2007). This method relies on the different protease cleavage sites for PrP^{Sc} in different cell types (the same principle as shown in Fig. 26.2).

Atypical scrapie cases are characterised by a distinctly different PrP^{Sc} distribution pattern as compared to classical scrapie. The brainstem at the level of the obex is only inconstantly involved. In contrast to classical scrapie, a PrP^{Sc} accumulation at the DMNV was never seen (Nentwig et al. 2007; Benestad et al. 2008). PrP^{Sc} accumulations found at the obex are mainly confined to the spinal tract nucleus of the trigeminal nerve with primary involvement of the white matter, formatio reticularis, ventrolateral solitary tract and ambiguous nucleus (for a detailed review see Benestad et al. 2008). The most pronounced immunostaining is usually detectable in the cerebellar (Fig. 26.5) and cerebral cortices (Benestad et al. 2008) as well as in the substantia nigra, thalamus and basal nuclei (Moore et al. 2008). However, cases without any cerebellar accumulation were also described (Nentwig et al. 2007). PrP^{Sc} accumulations are generally mild to moderate, and only a few morphological types (including fine granular, aggregates, plaque-like, linear and perineuronal) can

Table 26.2 Morphological types of PrP^{Sc} accumulation (Jeffrey and Gonzalez 2007)

Intracellular	Intraneuronal, intramicroglial and intra-astrocytic	
Extracellular/cell membrane-associated	Neuropil associated	Linear
		Fine punctuate
		Coarse particulate
		Coalescing
		Perineuronal
	Glial cell associated	Stellate
		Perivacuolar
		Subpial
		Subependymal
		Perivascular
	Ependymal cell associated	Supraependymal
	Endothelial cell associated	Vascular plaques

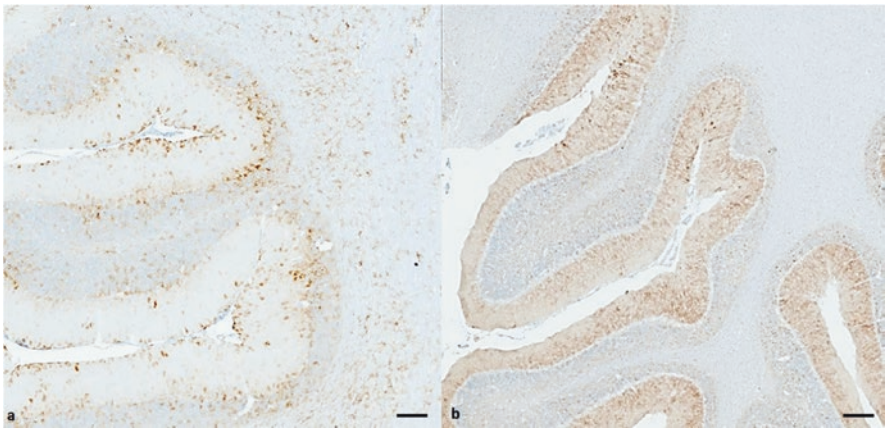

Fig. 26.5 Distinct differences in the PrP^{Sc} distribution pattern in the cerebellar cortices of sheep infected with (**a**) classical scrapie and (**b**) atypical scrapie, immunohistochemistry mab L42, bar 100 µm

be seen. An intraneuronal deposition staining has never been reported (Benestad et al. 2008; Moore et al. 2008).

26.8 Scrapie Agent Strains

It is important to recognise that classical scrapie can occur in different phenotypes in hosts of the same species or genotype, and this characteristic could be explained by the evolution of new prion strains (EFSA 2017; Nonno et al. 2019). The first reports on the existence of different prion strains date back to the 1960s (Fraser and

Dickinson 1968, 1973) and three classes of scrapie strains were defined in the UK based on the phenotypes after passage in inbred wild-type mice (Bruce and Dickinson 1987; Westaway et al. 1987).

Prion strain properties depend on the specific conformation of PrP^{Sc}. Firstly, they are defined by their phenotypic characteristics in the natural host, including clinical signs, brain lesion profile, immunohistochemical and biochemical (e.g. protease sensitivity, molecular migration on Western blot, glycosylation patterns) pattern of PrP^{Sc}. Even more important are the disease properties in experimental rodent models, in particular attack rate and incubation period as well as lesion and PrP^{Sc} profiles in the mouse brain and biochemical characteristics of prions (EFSA 2015).

The process of prion strain evolution is still unknown, but the impact of selective pressure, that is, host genetic or environmental factors could induce an alteration of the propagation process, and only a "new" PrP^{Sc} is able to cross a transmission barrier, adapt and emerge. Another hypothesis states that selective pressure results in a selection of the most suitable PrP^{Sc} conformer already present in a given isolate, which is a mixture of different PrP^{Sc} conformers (Collinge and Clarke 2007; Li et al. 2010; Beringue et al. 2007). In any case, different replication environments most probably play an important role in determining the biological properties of scrapie isolates and their further evolution. Thus, the full characterisation of isolates today includes, besides bank voles, the use of transgenic mouse models, overexpressing the prion protein of animals and man. That way the influence of different hosts on the biological properties of the original isolate is determined (EFSA 2014, 2015).

Up to now, strain typing studies have identified several prion strains responsible for classical scrapie in sheep and goats (EFSA 2015, 2017; Nonno et al. 2019; Beringue et al. 2008; Marín-Moreno et al. 2021), but the exact number is unknown and most classical scrapie field isolates contain sub-strains in different proportions (Nonno et al. 2019). Evidently, some isolates are not completely stable and their biological properties can shift on transmission, even affecting the ability of certain strains to cross the species barrier (EFSA 2015), as shown for some classical scrapie isolates in humanised transgenic mice (Cassard et al. 2014). These divergences might be the reason for the high diversity of classical scrapie strains and could be the basis for the emergence of new prion strains.

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Chapter 27
Research Models for Studying Chronic Wasting Disease

Julianna Sun and Glenn Telling

Abstract Chronic wasting disease (CWD) is a burgeoning epidemic prion disease of cervids. While its origins are mysterious, the disease was first described in North America in captive mule deer and was subsequently identified in free-ranging, as well as captive Rocky Mountain elk, white-tailed deer, moose, and most recently reindeer and red deer. Chronic wasting disease (CWD) is the only recognized prion disease in wild as well as captive animals. In addition to its expanding host range, the disease continues to spread to new geographic areas including South Korea and Northern Europe. The unparalleled efficiency of CWD prion transmission, combined with high deer densities in certain areas of North America, complicates strategies for controlling CWD and calls into question the potential for spread to new species. The appearance of variant Creutzfeldt–Jakob disease (vCJD) following human exposure to bovine spongiform encephalopathy (BSE), and the demonstration of CWD prions in a variety of materials consumed by humans, place the human species barrier to CWD at the forefront of public health concerns. Since North American hunters harvest thousands of deer and elk each year, and cervid products are widely used in traditional Asian medical practices, it is likely that humans are exposed to CWD prions. Here, we describe aspects of CWD pathogenesis and epidemiology, review recent progress in the development of model systems in which to study the basic biology of CWD, and, in doing so, outline some of the remaining uncertainties and challenges surrounding this enigmatic prion disease.

Keywords BSE · Cell culture models · Chronic wasting disease · CWD · CWD prions · Prion diseases

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27.1 Epidemiology

First identified in the late 1960s in a northern Colorado research facility as a fatal wasting syndrome of mule deer (*Odocoileus hemionus hemionus*), CWD was confirmed to be a prion disease by histopathological assessment (Williams and Young 1980, 1992). A retrospective study also revealed CWD infection of mule deer and black-tailed deer (*Odocoileus hemonius columbianus*) residents at the Toronto Zoo between 1973 and 2003 (Dube et al. 2006). CWD was also identified in mule deer in a research facility in Wyoming and in captive Rocky Mountain elk (*Cervus elaphus nelsoni*) in both the Colorado and Wyoming facilities. Thereafter, the disease was described in free-ranging mule deer and elk in southeastern Wyoming and northeastern Colorado (Williams and Young 1980, 1982, 1992). Surveillance and modeling studies indicated that CWD occurred endemically among free-ranging deer and elk in a contiguous area in northeastern Colorado, southeastern Wyoming, and western Nebraska, and that CWD was most likely present in free-ranging cervids in this "endemic region" several decades prior to its eventual recognition (Miller et al. 2000). Additionally, CWD has occurred in wild and captive North American moose (*Alces alces shirasi*) (Kreeger et al. 2006; Baeten et al. 2007) in the endemic region. Most recently, CWD cases have been reported on a red deer farm in Quebec, Canada (Gagnier et al. 2020).

The prevalence of CWD varies across North America but can be as high as 30% in some areas (Williams 2005). Based on hunter-harvested animal surveillance, the prevalence of CWD in the endemic area from 1996 through 1999 was estimated at approximately 5% in mule deer, 2% in white-tailed deer, and < 1% in elk (Spraker et al. 1997). Surveys conducted by the Colorado Division of Wildlife from June 2006 to June 2009 continue to demonstrate the wide distribution of CWD in Colorado. Summaries of harvest survey data varied from <1 to 14.3% among mule deer, <1 to 2.4% among elk, and < 1% among moose (http://wildlife.state.co.us/NR/rdonlyres/763F5731-F895-4D52-9F27-2B8D5BE91175/0/CO_CWDreport_06082.pdf).

Wildlife management efforts to contain or eradicate CWD in Colorado have proven unsuccessful (Conner et al. 2007). Originally thought to be limited in the wild to the endemic area, in 2002 CWD emerged in free-ranging populations of white-tailed deer (*Odocoileus virginianus*) east of the Mississippi (Joly et al. 2003). At the time of writing, CWD has been recognized in wild and/or farm-raised cervids from at least 29 North American states and, in addition to its aforementioned detection in Ontario, the Canadian provinces of Saskatchewan and Alberta. While identification of CWD-affected animals in areas previously thought to be free of infection may be partly related to increased surveillance, the spread of the disease by natural migration, and translocation of infected cervids by humans, almost certainly plays a role in the emergence of disease. The latter mechanism is exemplified by outbreaks occurring in South Korea as a result of importation of sub-clinically infected animals (Sohn et al. 2002; Kim et al. 2005). However, although most US states and Canadian provinces have introduced CWD surveillance programs, they range from

targeted surveillance in some states, to mandatory testing of all animals suspected of dying of CWD in others. Complicating the issue, diagnosis can only be unequivocally made following post-mortem analysis of central nervous system (CNS) materials, and current evaluations almost certainly underestimate the true prevalence of disease.

In 2016, CWD was detected in a free-ranging reindeer (*Rangifer tarandus tarandus*) in Norway, marking the index case in Europe and in this species (Benestad et al. 2016). Subsequent testing revealed evidence of CWD in additional reindeer, European moose (*Alces alces alces*) (Pirisinu et al. 2018), and red deer (Vikoren et al. 2019) in Norway. Following this discovery, mandatory surveillance in European Union states with moose and reindeer populations lead to detection of CWD in two moose in Finland and four moose in Sweden (Agren et al. 2021). To date, ~ 20 reindeer, 11 moose, and two red deer have been found to be CWD-positive in Norway.

27.2 Pathogenesis

Signs in clinically affected deer and elk include weight loss, behavioral alterations, apparent ruminal atony, and salivary defluxion in late-stage disease. Clinical features include gradual loss of body condition, resulting in emaciation (hence the term wasting disease, and behavioral changes which include generalized depression, and loss of fear of humans (Williams 2003). At later stages, affected animals may display polydipsia and polyuria, sialorrhea, and generalized incoordination. The clinical course in captive animals is slowly progressive, and after diagnosis, most animals survive for a few weeks up to 3–4 months. Like other prion diseases, pathognomonic lesions are confined to the CNS and consist of intraneuronal vacuolation, neuropil spongiosis, astrocytic hypertrophy, and hyperplasia (Williams and Young 1993). CWD in North America is characterized by extensive CNS and lymphoid tissue deposition of PrP^{Sc}, the latter being detectable early in the disease (Sigurdson et al. 1999; Fox et al. 2006). Pathogenesis seems to vary between deer and elk with less PrP^{Sc} deposition in the lymphoid tissues of elk compared to deer (Race et al. 2007). Also, florid amyloid plaques feature in the neuropathology of diseased deer (Liberski et al. 2001). Other tissues and bodily fluids of deer and elk in which PrP^{Sc} or infectivity has been detected include skeletal muscle (Angers et al. 2006), pancreas (Sigurdson et al. 2001; Fox et al. 2006), adrenal gland (Sigurdson et al. 2001; Fox et al. 2006), and cardiac muscle (Jewell et al. 2006). CWD prions have been detected in saliva and blood by bioassay (Mathiason et al. 2006) and in urine by protein misfolding cuclic amplification (PMCA) (Haley et al. 2009a, b) and bioassay (Haley et al. 2009a, b) suggesting a role for these body fluids in transmission and dissemination. Fecal material from subclinical deer also harbors infectivity (Haley et al. 2009a, b; Tamguney et al. 2009). CWD prions have also been detected in fetal tissue from white-tail deer by PMCA (Bravo-Risi et al. 2021; Nalls et al. 2021).

27.3 Transmission

While CWD is experimentally transmissible after intracerebral inoculation of mule deer with incubation periods of up to 2 years (Williams and Young 1992), limited transmission studies indicated that CWD developed ~25% more rapidly in orally challenged elk than deer (16 months for mule deer and 12 months for elk) (Williams 2003). Maximum incubation periods in naturally affected animals are not known, but most natural cases occur in animals 3–7 years old, with the majority of animals probably developing CWD within 3 years of infection (Miller et al. 1998).

In the wild, the highly efficient transmission of CWD appears unparalleled among prion diseases (Williams and Young 1980; Miller et al. 2000; Miller and Williams 2003). The remarkably contagious nature of CWD has been documented in a captive mule deer population wherein 90% of the mule deer present for more than 2 years ultimately developed disease (Williams and Young 1980). Although the natural route of CWD transmission is not precisely known, lateral transmission (Williams and Miller 2002) by ingestion of forage or water contaminated by secretions, excretions, or other sources, for example, CWD-infected carcasses (Miller et al. 2004), has long been thought the most plausible natural route. The presence of CWD prions in saliva, blood, urine, and feces (Mathiason et al. 2006; Haley et al. 2009a, b; Tamguney et al. 2009; Haley et al. 2011) is consistent with the mechanism of contagious lateral transmission. While less common, vertical transmission from mother to offspring has been demonstrated in Muntjac deer (Nalls et al. 2021). The detection of CWD prions in elk antler velvet by bioassay, and the annual shedding of this material, raises the possibility that it may also play a role in CWD transmission (Angers et al. 2009). Pertinent to this issue is the well-known persistence of prions in the environment, a feature that is linked to their unusual resistance to degradation. Coupled with this, prions bound to soil particles remain infectious after oral consumption (Saunders et al. 2012).

In addition to its increased geographic spread, the known host range of CWD is also expanding. Since 2002, CWD has emerged in free-ranging populations of white-tailed deer (Joly et al. 2003). CWD in North America has occurred in the wild (Baeten et al. 2007) and captive moose (*Alces alces shirasi*) (Kreeger et al. 2006), and has been experimentally transmitted to European red deer (*Cervus elaphus elaphus*) (Martin et al. 2009), and muntjac deer (*Muntiacus reevesi*) (Nalls et al. 2011). While brain material from CWD-infected white-tailed deer and elk produced disease in four of 13 intracerebrally inoculated fallow deer (*Dama dama*) (Hamir et al. 2008), the same species appeared resistant when co-housed in paddocks with CWD-affected mule deer (Rhyan et al. 2011). With the discovery of CWD in Europe, affected wild species have expanded to include reindeer, European elk, and European red deer (*Cervus elaphus elaphus*).

Whether the natural host range of CWD extends beyond the family Cervidae is currently unclear. However, the remarkably high rate of CWD prion transmission brings into question the risk posed to livestock from developing a novel CWD-related prion disease via shared grazing of CWD-contaminated rangeland. This

issue has been indirectly addressed by transmitting CWD to Tg mice expressing ovine or bovine PrP, thus far with negative outcomes (Tamguney et al. 2006). Wild-type mice are generally resistant to CWD infection (Browning et al. 2004). Experimental transmission to other species has had mixed results. Studies by Dr. Richard Marsh (University of Wisconsin) in the mid-80s demonstrated that the CWD agent transmitted poorly to hamsters, ferrets, and mink (personal communications with Dr. Jason Bartz and colleagues, Creighton University) (Bartz et al. 1998; Marsh et al. 2005; Sigurdson et al. 2008). After multiple passages, domestic cats succumb to feline-adapted CWD (Mathiason et al. 2013). Oronasal transmission from mule deer to Suffolk sheep resulted in subclinical disease in one of seven inoculated sheep (Cassmann et al. 2021). Predators and other animals may be naturally exposed to CWD prions as well. CWD prions remain infectious after passage through the digestive tracts of coyotes (Nichols et al. 2015), crows (VerCauteren et al. 2012), and earthworms (Pritzkow et al. 2021a, b). Passage through the digestive tract of mountain lions appears to decrease detectable CWD by >96% (Baune et al. 2021).

The identification and characterization of distinct CWD strains, and the influence of PrP primary structure on their stabilities, is of importance when considering the potential for inter-species transmission. The appearance of variant Creutzfeldt–Jakob disease (vCJD) following human exposure to bovine spongiform encephalopathy (BSE) (Bruce et al. 1997; Hill et al. 1997) place the human species barrier to other animal prion diseases, particularly CWD, at the forefront of public health concerns. Since North American hunters harvest thousands of deer and elk each year, the demonstration of CWD prions in skeletal muscle and fat of deer (Angers et al. 2006; Race et al. 2009a, b), makes it likely that humans consume CWD prions. The substantial market for elk antler velvet in traditional Asian medicine also warrants concern (Angers et al. 2009). Estimates of the zoonotic potential of CWD are currently mixed. Surveillance currently shows no evidence of CWD transmission to humans (Belay et al. 2004; Mawhinney et al. 2006). While initial cell-free conversion studies suggested that the ability of CWD prions to transform human PrP^{C} into protease-resistant PrP was low (Raymond et al. 2000), subsequent results showed that cervid PrP^{Sc} induced the conversion of human PrP^{C} by protein misfolding cyclic amplification, following CWD prion strain stabilization by successive passages in vitro or in vivo (Barria et al. 2011). These results have implications for the human species barrier to CWD and underscore the role of strain adaptation on interspecies transmission barriers. Additional studies using transgenic (Tg) mice expressing human PrP^{C} showed that CWD failed to induce disease following intracerebral CWD infection (Kong et al. 2005; Tamguney et al. 2006; Sandberg et al. 2010). However, CWD transmission was reported in nonhuman primates through the intracerebral inoculation of squirrel monkeys (*Saimiri sciureus*) (Marsh et al. 2005; Race et al. 2009a, b). Systematically addressing the zoonotic potential, as well as the tissue distributions of the newly recognized CWD1 and CWD2 strains (Angers et al. 2010) in infected deer and elk, would appear to remain a high priority.

27.4 Effects of Polymorphic Variation in Cervid Prion Protein Genes on Disease Susceptibility

As demonstrated in other species in which prion diseases occur naturally, susceptibility to CWD is highly dependent on polymorphic variation in deer and elk *PRNP*. In mule deer, polymorphism at codon 225 encoding serine (S) or phenylalanine (F) influences CWD susceptibility, the 225F allele being protective. The occurrence of CWD was 30-fold higher in deer homozygous for serine at position 225 (225SS) than in heterozygous (225SF) animals; the frequency of 225SF and 225FF genotypes in CWD-negative deer was 9.3%, but only 0.3% in CWD-positive deer (Jewell et al. 2005). Polymorphisms at codons 95 [glutamine (Q) or histidine (H)] (Johnson et al. 2003), 96 [glycine (G) or serine (S)] (Raymond et al. 2000; Johnson et al. 2003), and 116 [alanine (A) or glycine (G) (Heaton et al. 2003) in white-tailed deer have been reported. While all major genotypes were found in deer with CWD, the Q95, G96, and A116 allele (QGA) was more frequently found in CWD-affected deer than the QSA allele (Johnson et al. 2003; O'Rourke et al. 2004). Orally infected H95 white-tailed deer presented with less peripheral PrP^{Sc} accumulation compared to Q95, while S96 does not reduce peripheral PrP^{Sc} accumulation but does delay disease onset (Otero et al. 2019). Additionally, PMCA with 96S substrate results in low conversion of CWD (Otero et al. 2021).

The elk *PRNP* coding sequence is also polymorphic at codon 132 encoding either methionine (M) or leucine (L) (Schatzl et al. 1997; O'Rourke et al. 1998). This position is equivalent to human *PRNP* codon 129. Studies of free-ranging and captive elk with CWD (O'Rourke et al. 1999), as well as oral transmission experiments (Hamir et al. 2006; O'Rourke et al. 2007), indicate that the 132 L allele protects against CWD.

27.5 Transgenic Mouse Models

While CWD is transmissible after intracerebral inoculation of mule deer with incubation periods of up to 2 years (Williams and Young 1992), the expense of housing cervids under prion-free conditions for long periods and the highly communicable nature of CWD present significant challenges for using deer as experimental hosts (Mathiason et al. 2006). Transmission of CWD to other species had mixed results. The resistance of mice (Browning et al. 2004) and the inefficient transmission of CWD to ferrets (Bartz et al. 1998) are examples of species barriers to CWD prions. The discovery that the primary structure of PrP was an important determinant of interspecies prion transmission (Scott et al. 1989; Prusiner et al. 1990) paved the way for the development of a variety of facile Tg mouse models in which to study the biology of mammalian prion diseases (Buschmann et al. 2000; Castilla et al. 2003; Crozet et al. 2001; Scott et al. 1989, 1997; Telling et al. 1994, 1995; Vilotte et al. 2001; Windl et al. 2005). Based on this concept, several Tg mouse lines

expressing either elk or deer PrP have been produced in which the species barrier to CWD has been eliminated. Prototype Tg mice expressing deer PrP designated Tg(CerPrP)1536$^{+/-}$ (Browning et al. 2004), recapitulated the cardinal neuropathological, clinical and biochemical features of CWD, an observation subsequently confirmed in comparable Tg mouse models expressing deer or elk PrP (Kong et al. 2005; LaFauci et al. 2006; Tamguney et al. 2006; Meade-White et al. 2007; Angers et al. 2009). The generation of CWD-susceptible Tg mice and the development of PMCA-based approaches for amplifying CWD infectivity using PrP expressed in the CNS of those mice (Green et al. 2008a, b; Meyerett et al. 2008) have provided crucial information about the biology of CWD and cervid prions. For example, Tg approaches in combination with cell-free prion amplification were shown to maintain CWD prion strain properties and to provide a means of generating novel cervid prion strains (Kurt et al. 2007, 2009; Green et al. 2008a, b; Meyerett et al. 2008). These approaches have also facilitated our understanding of the mechanism of CWD transmission among deer and elk (Mathiason et al. 2006; Haley et al. 2009a, b; Tamguney et al. 2009) and have been essential for assessing the potential risk of human exposure to CWD prions (Angers et al. 2006, 2009; Race et al. 2009a, b). The availability of CWD-susceptible Tg mouse models also provided a means of quantifying CWD infectivity by end-point titration (Angers et al. 2009). Such studies provided information about the sensitivity of Tg(CerPrP) mice to CWD as well as an accurate assessment of CWD titers. However, this is a time consuming and expensive proposition involving ascertainment of the dilution point at which only half the inoculated animals in a group develop clinical symptoms (the ID50). To date, only two CWD prion samples have been assessed by this method.

While Tg(CerPrP) mice provided initial data on CWD in a mouse bioassay, drawbacks include PrP^C transgene overexpression and uncontrolled position effects which may lead to artifacts in relation to strain differentiation. Additionally, the Tg(CerPrP) mice fail to fully recapitulate lymphotropic aspects of natural CWD infection as prions do not replicate in the periphery of these mice (Bian et al. 2019). To address this, a gene-targeted approach was taken in which the coding sequence of mouse *Prnp* was replaced by homologous recombination in embryonic stem cells with the coding sequence of cervid *PRNP* (Bian et al. 2019). These mice accurately express wild-type levels of cervid PrP, and CWD prions are detectable in lymphoid tissue of infected mice indicating peripheral replication (Bian et al. 2019). Transgenic mouse modeling also provided a means of assessing the role of PrP gene polymorphisms and species-specific amino acid differences on CWD pathogenesis. Mice expressing either E226 or Q226 were created to address the sole amino acid difference between elk and deer. Inoculation of these mice reveals distinct strain propagation through each mouse line (Bian et al. 2019).

To more fully address the influence of the elk 132 polymorphism, the transmissibility of CWD prions was assessed in Tg mice expressing cervid PrP^C with L or M at residue 132 (Green et al. 2008a, b). While transgenic mice expressing CerPrP-L132 afforded partial resistance to CWD, SSBP/1 sheep scrapie prions transmitted efficiently to Tg mice expressing CerPrP-L132, suggesting that the elk 132 polymorphism controls prion susceptibility at the level of prion strain selection. The

contrasting ability of CWD and SSBP/1 prions to overcome the inhibitory effects of the CerPrPL132 allele is reminiscent of studies describing the effects of the human codon 129 methionine M/V polymorphism on vCJD/BSE prion propagation in transgenic mice, which concluded that human PrP V129 severely restricts propagation of the BSE prion strain (Wadsworth et al. 2004). Resistance to CWD was also reported in transgenic mice expressing serine at residue 96 (Meade-White et al. 2007).

27.6 Other Transgenic Models

While Tg mouse models of CWD have advanced in the field, one drawback is the long time to disease onset in mice, which can surpass 500 days post-inoculation. To combat this, transgenic *Drosophila melanogaster* has been created, which express various mammalian PrP sequences and are susceptible to prion infection of the same host species (Thackray et al. 2014, 2021; Bujdoso et al. 2022). Similarly, *Caenorhabditis elegans* were made to express the E200K PrP mutation seen in inherited CJD cases (Bizat et al. 2021). Expression of this mutation induces specific behavioral patterns consistent with neurodegeneration and could serve as a high throughput screening process for drug compounds to treat prion diseases. Utilizing both the *Drosophila* and *C. elegans* model systems for CWD could compliment the mouse bioassay and allow for faster, less cumbersome bioassessment.

27.7 CWD Strains

Although original studies in Tg mice (Browning et al. 2004), and subsequent work (LaFauci et al. 2006) raised the possibility of CWD strain variation, the limited number of isolates and the lack of detailed strain analyses in those studies meant that this hypothesis remained speculative. Subsequent studies supported the feasibility of using Tg(CerPrP)1536$^{+/-}$ mice for characterizing naturally occurring CWD strains, CWD prions generated by protein misfolding cyclic amplification (PMCA), and novel cervid prions (Green et al. 2008a, b). Comparative studies of CWD in Tg mice expressing deer, and elk PrP (Angers et al. 2009) also identified residue 226, the sole primary structural difference between deer and elk PrP, as a major determinant of CWD pathogenesis, and supported the different clinical and pathological properties of CWD in these species.

To address whether different CWD strains occur in various geographic locations or in different cervid species, bioassays in Tg mice were used to analyze CWD in a large collection of captive and wild mule deer, white-tailed deer and elk from various geographic locations in North America (Angers et al. 2010). These findings provided substantial evidence for two prevalent CWD prion strains, referred to as CWD1 and CWD2, with different clinical and neuropathological properties.

Remarkably, primary transmissions of CWD prions from elk produced either CWD1 or CWD2 profiles, while transmission of deer inocula favored the production of mixed intra-study incubation times and CWD1 and CWD2 neuropathologies. These findings indicate that elk may be infected with *either* CWD1 *or* CWD2, while deer brains tend to harbor CWD1/CWD2 strain mixtures.

The different primary structures of deer and elk at residue 226 provide a framework for understanding these differences in strain profiles of deer and elk. Because of the role played by residue 226, the description of a lysine polymorphism at this position in deer (Johnson et al. 2006), and its possible role on strain stability may be significant. It is unknown whether CWD1 and CWD2 interfere or act synergistically, or whether their co-existence contributes to the unparalleled efficiency of CWD transmission. Interestingly, transmission results reported in previous studies suggested that cervid brain inocula might be composed of strain mixtures (Tamguney et al. 2006). Additional previous studies also support the existence of multiple CWD strains. CWD has also been transmitted, albeit with varying efficiency, to Tg mice expressing mouse PrP (Sigurdson et al. 2006; Tamguney et al. 2006). In the former study, a single mule deer isolate produced disease in all inoculated Tga20 mice. On successive passages, incubation times dropped to ~160 days. In the second study, one elk isolate from a total of eight deer and elk CWD isolates induced disease in 75% of inoculated Tg4053 mice. It is worth noting that the distribution of lesions in both studies appeared to resemble the CWD1 pattern. Low-efficiency CWD prion transmission was also recorded in hamsters and Tg mice expressing Syrian hamster PrP (Raymond et al. 2007). In that study, during serial passage of mule deer CWD, fast and slow incubation time strains with different patterns of brain pathology and PrP^{Sc} deposition were also isolated.

The discovery of CWD in Norway, Sweden, and Finland raises questions about the emergence of new CWD strains in these regions. Studies in bank voles reveal different incubation times, neuropathology, and deposition between Norwegian and North American CWD strains (Nonno et al. 2020). Bioassays in gene-targeted mice also reveal stark differences in the transmission properties of Norwegian reindeer, Norwegian moose, and North American CWD (Bian et al. 2021). Gene-targeted mice expressing cervid PrP with Q at residue 226 (GtQ) are susceptible to intracerebral inoculation with either Norwegian reindeer or Norwegian moose CWD prions. Gene-targeted mice expressing cervid PrP with E at residue 226 (GtE) are also susceptible to intracerebral inoculation with Norwegian reindeer CWD. However, Norwegian moose CWD prions do not readily transmit to GtE mice. Inoculation with Norwegian reindeer results in equivalent incubation times in GtQ and GtE mice. Additionally, immunoblotting reveals a smaller PK-resistant core, diffuse immunohistology, and higher conformational stability of Norwegian moose CWD compared to North American CWD. The distinct strain properties of new CWD cases in Nordic countries raise concerns about the risks posed to other species. To address this in vitro, Norwegian CWD prions were used as a seed to convert various mammalian PrPs by PMCA demonstrating that Norwegian reindeer and red deer have the potential for spillover to other species (Pritzkow et al. 2021a, b).

27.8 Cell Culture Models for Studying CWD Prions

Unlike most animal viruses, which can be propagated and titrated in cultured cells, bioassay in susceptible animals has been the only means for assaying prion infectivity. Many unsuccessful attempts to infect by in vitro challenge were reported, but not until the persistent infection of neuroblastoma N2a cells with mouse-adapted scrapie prions did this field expand (Butler et al. 1988). Weissmann and colleagues subsequently derived highly susceptible N2A subclones to develop a novel quantitative in vitro assay for prion infectivity, namely the scrapie cell assay (SCA) (Klohn et al. 2003), which is about as sensitive as the mouse bioassay, 10 times faster and more than 100-fold less expensive. Using these assays, it is now possible to quantify titers of the widely-used mouse-adapted scrapie prion isolate developed at the Rocky Mountain Laboratories, referred to as RML, at levels about as sensitive as those determined by endpoint titration in mice. Single PrP^{Sc}-positive cells can be visualized by an Elispot system.

The SCA represented a substantial technical development for analyses of prion diseases, equivalent in importance to the creation of plaque assays of animal viruses. However, the assay was limited to the detection of mouse prion infectivity. Based on observations showing that rabbit kidney epithelial (RK13) cells engineered to express sheep PrP were capable of propagating scrapie prions (Vilette et al. 2001), cloned RK13 cells expressing elk PrP were developed. A highly susceptible clone-producing disease-specific cervid PrP^{Sc} ($CerPrP^{Sc}$), referred to as Elk-21^{+}, was isolated in which CWD infection was continually maintained for >100 passages (Bian et al. 2010). Inoculation of CWD-susceptible Tg(CerPrP-E226)5037 mice with prions from Elk-21^{+} cells resulted in disease transmission with clinical and neuropathological features identical to CWD. Sustained treatment of Elk-21^{+} cells with dextran sulfate 500 (DS-500) resulted in the clearance of $CerPrP^{Sc}$ which did not re-emerge after >40 passages. These cells are referred to as Elk-21^{-}. Elk-21^{-} cells were used to develop a novel cell-based assay for CWD prion quantification, analogous to the SCA, as a facile alternative to in vivo CWD prion quantification, referred to as the cervid prion cell assay (CPCA). Detection and quantification of cervid prions, including naturally occurring CWD prions and experimentally adapted cervid prion strains, was made possible using the CPCA.

In the standard CPCA, CWD prion-susceptible Elk-21^{-} cells in wells of 96-well plates are exposed to serial dilutions of the prion-containing sample for 4 days, grown to confluence, split at a ratio of 1:8, grown to confluence once more, and split similarly once more. When the cells have reached confluence after the second split, 20,000 cells, are filtered onto membranes of Elispot plates, and the proportion of cells containing protease-resistant $CerPrP^{Sc}$ is identified by an enzyme-linked immunosorbent assay (ELISA) using automated counting equipment (Elispot). The inclusion of RK13 cells stably transfected with an empty vector showed that positive spots detected after three splits were the result of newly generated $CerPrP^{Sc}$. While $CerPrP^{Sc}$ purification as described for other CWD cell culture systems (Raymond et al. 2006) was not a prerequisite for sustained cellular infection, expression of retroviral Gag facilitated prion susceptibility, and cell cloning was also critical.

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Part VIII
Yeast Prions

Chapter 28
Introduction to Yeast and Fungal Prions

Reed B. Wickner and Herman K. Edskes

Abstract "Prion" now means "infectious protein," not requiring an accompanying nucleic acid for transmission to a new individual. In 1994, we found that the long-known cytoplasmic genes [URE3] and [PSI+] were actually prions of Ure2p and Sup35p, respectively. These, and a variety of yeast and fungal prions found since then, are based on self-propagating amyloids, but one prion based on a self-activating protease showed that not all infectious proteins are amyloids. The importance of chaperones and other cellular components in prion propagation and generation, including the discovery of cellular anti-prion systems—some with homologs or at least analogs in mammals, have enriched the prion field. The folded in-register parallel architecture of yeast prion amyloids can explain how a single protein can faithfully propagate any of several structurally different prion variants/strains. Prion variants/strains based on phenotype intensity, propagation stability, sensitivity to anti-prion systems, propagon number, pathogenicity, and other properties have been recognized.

Keywords Ure2p · Sup35p · Rnq1p · HET-s · [PSI+] · [URE3] · [PIN+] · [Het-s] · Chaperones · Prion variants

Abbreviations

Gene names

URE	ureidosuccinate
SUP	nonsense-suppressor
RNQ	rich in N and Q (aspartate and glutamine)
HET	heterokaryon incompatibility
PIN	[PSI+] – inducibility

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28.1 Mysterious Non-chromosomal Genetic Elements in Yeast

The non-chromosomal genetic elements [PSI+] and [URE3] were discovered in the 1960s and 1970s due to the pioneering work of Brian Cox (1965) and Francois Lacroute (1971) and their coworkers. [PSI+] enhances weak nonsense-suppressor tRNAs or can even be a nonsense-suppressor on its own, allowing the growth of cells with a premature translation termination mutation in an essential gene (Cox 1965; Liebman et al. 1975). In combination with a strong nonsense-suppressor tRNA mutation, [PSI+] is lethal, as one would expect from an excessive read-through of normal termination codons (Cox 1971). [PSI+] segregated 4+:0 in meiosis (compared to 2+:2− for a chromosomal mutation) and was transferred by cytoplasmic mixing, behavior typical of a non-chromosomal genetic element, but was not yet identified with a replicating DNA plasmid or RNA virus (Cox et al. 1988).

The [URE3] non-chromosomal genetic element was found in studies involving uracil biosynthesis that led to control of nitrogen source utilization (Lacroute 1971; Drillien et al. 1973). In the first step of uracil biosynthesis, aspartate is condensed with carbamyl phosphate to form ureidosuccinic acid (USA), a reaction catalyzed by aspartate transcarbamylase (*URA2*). On media with a rich nitrogen source, such as ammonia, yeast will not take up the USA to feed a *ura2* mutant. However, cells growing on a poor nitrogen source, such as proline, or *ure2* mutants, can do so (Aigle and Lacroute 1975). One dominant "mutant" able to take up the USA on an ammonia-containing medium showed non-chromosomal segregation in meiosis (like [PSI+] above) and was designated [URE3] (Lacroute 1971).

28.2 Discovery of Yeast Prions and the Three Genetic Criteria

These cytoplasmic genes were long unexplained, but careful studies by Michel Aigle, with Lacroute, showed that the [URE3] cytoplasmic element required the chromosomal *URE2* gene for its propagation (Aigle and Lacroute 1975). Both *ure2* mutants and strains carrying the [URE3] genetic element have the *same* phenotype (Aigle and Lacroute 1975). In contrast, chromosomal *mak* mutants unable to propagate the killer factor (M dsRNA) have the *opposite* phenotype (non-killer) of strains carrying M dsRNA (killer), and *pet* mutants unable to propagate the mitochondrial DNA have the *opposite* phenotype (glycerol negative) of cells carrying mitDNA (glycerol positive). This was the first clue that led us to suggest that [URE3] and [PSI+] were prions (Wickner 1994) (Fig. 28.1). Nucleic acid replicons (viruses and plasmids) depend for their propagation on chromosomal genes, but the general pattern is that a mutant in the chromosomal gene has a phenotype opposite to that of cells carrying the replicon. If a prion produces a phenotype as a result of deficiency of the normal form, then the presence of the prion should give the same phenotype as mutation of the gene for the normal form (Wickner 1994). But a continuous

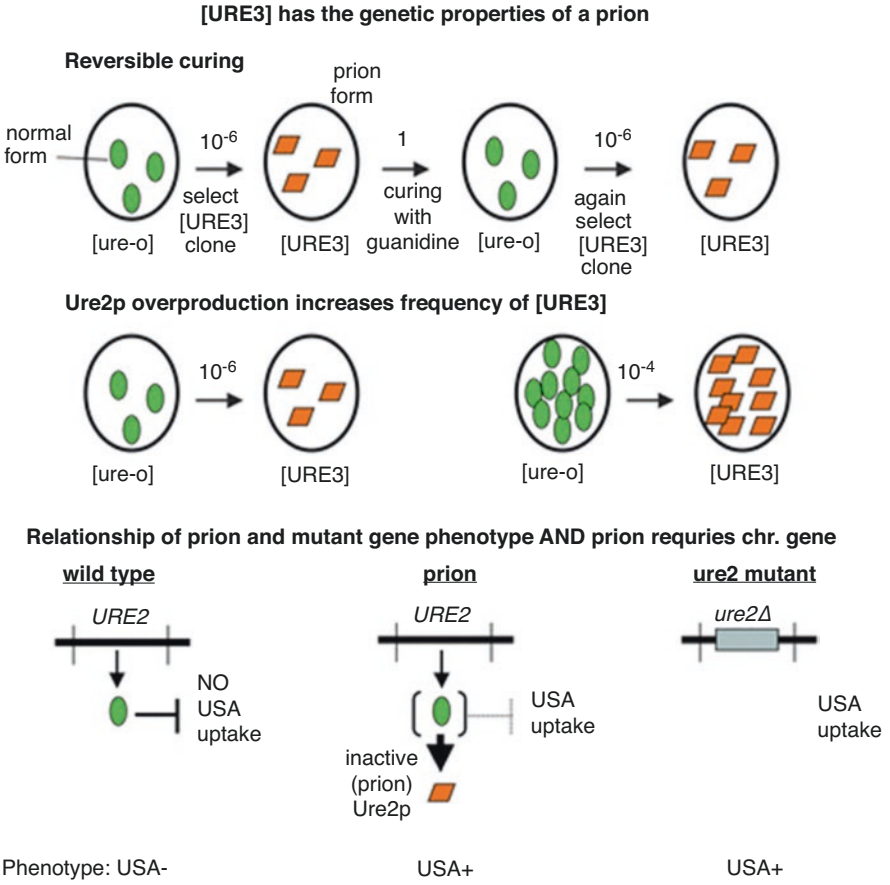

Fig. 28.1 Three genetic criteria for identification of prions in yeast and fungi. (Wickner 1994)

supply of the normal form is necessary for the continued propagation of the prion. Thus we inferred that [URE3] must be a prion of the Ure2 protein. The same relation had just been reported by Cox for [PSI+] and *sup35* (Cox 1993; Doel et al. 1994), from which we inferred that [PSI+] was a prion of Sup35p (Wickner 1994).

Two other tests for a prion are shown in (Fig. 28.1). Curing a nucleic acid replicon leaves a cell that cannot re-acquire the element without its introduction from outside or from another cell. In contrast, prion curing should be reversible. The protein is still being made and could undergo the prion change (rarely) to produce prion-containing cells from those previously cured (Wickner 1994). We showed this to be true for [URE3] (Wickner 1994) as Lund and Cox had previously found for [PSI+] (Lund and Cox 1981), supporting our view that both were prions.

Overproducing a chromosomally encoded protein required for the propagation of a plasmid or virus will not induce the appearance of either replicon. But overproduction of a protein able to form a prion should increase the frequency of prion

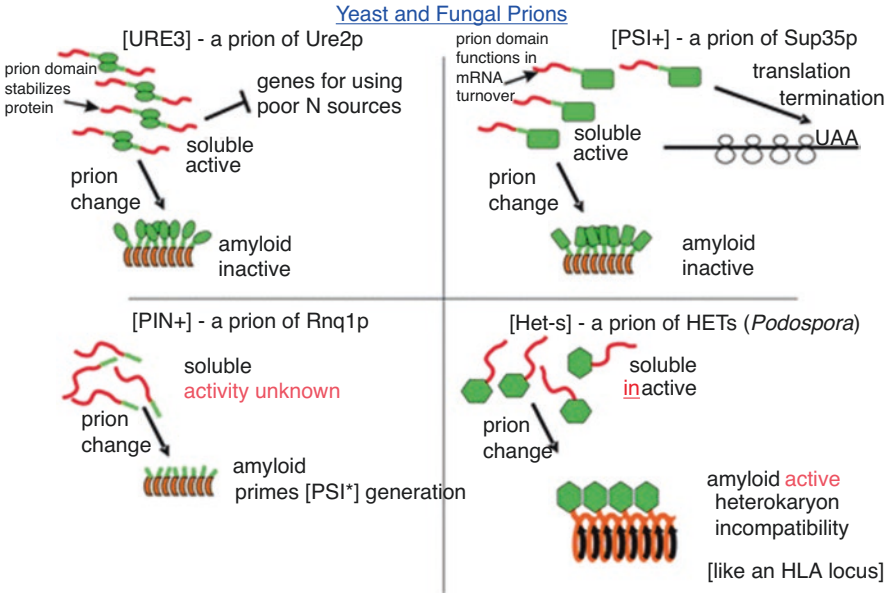

Fig. 28.2 The four most-studied prions of yeast and fungi

formation. With more molecules of the protein present, the chances that a prion conversion will happen should increase, whatever the mechanism of prion formation. We showed that the overproduction of Ure2p increased the frequency of [URE3] arising by ~100-fold (Wickner 1994), and Chernoff reported a similar result for the overproduction of Sup35p inducing [PSI+] appearance (Chernoff et al. 1993). We inferred that [URE3] is a prion of Ure2p, and [PSI+] is a prion of Sup35p (Wickner 1994) (Figs. 28.1 and 28.2).

28.3 The [Het-s] Prion of *Podospora anserina*

When two colonies of a filamentous fungus grow toward each other, they fuse cellular processes to form, in effect, a single syncytium (a heterokaryon), allowing the exchange of nutrients. However, this fusion process is limited to closely related strains, a limitation enforced by testing the identity of about a dozen polymorphic loci scattered about the genome. The nonidentity of alleles at even a single one of these loci produces the death of the first fusing cellular processes and a barrier to further fusions, a process called heterokaryon incompatibility (Saupe 2000).

One of these loci is called *het-s* with alleles *het-s* and *het-S*. The proper incompatibility between *het-s* and *het-S* strains is only observed if the *het-s* cells have a non-chromosomal gene, [Het-s] (Rizet 1952), shown to be a prion of the HET-s protein (product of the *het-s* allele) (Coustou et al. 1997) (Fig. 28.2). The HET-s prion amyloid recruits the HET-S protein, activating its membrane association and

pore-forming ability (Mathur et al. 2012). [Het-s] has proven to be a very important system for many aspects of prion studies [reviewed by Saupe (2011)], and has led to an understanding of the mechanism of action of certain NOD-like receptors in signal transduction (Loquet and Saupe 2017).

28.4 The [PIN+] Prion (Rarely) Seeds Other Prions

Although overproduction of Sup35p induced [PSI+] appearance (Chernoff et al. 1993), it was noted that in some strains, there was no such effect (Derkatch et al. 1997). Crossing strains in which overproduction of Sup35p induced [PSI+] generation with those in which it did not, resulted in all meiotic segregants showing the induction, evidence of a non-chromosomal genetic element, that was named [PIN+], for [PSI]-inducibility (Derkatch et al. 1997). Using the genetic criteria above, it was shown that [PIN+] was a prion of Rnq1p (Derkatch et al. 2001), a protein-rich in N and Q residues that had been shown to carry out a self-propagating aggregation (Sondheimer and Lindquist 2000).

In the course of showing that [PIN+] is a prion of Rnq1p, it was found that overexpression of any of a variety of QN-rich proteins had a [PIN+]-like effect, stimulating prion formation by Sup35p (Derkatch et al. 2001; Osherovich and Weissman 2001). In fact, several proteins detected in this screen were later found to form prions themselves, including Swi1p ([SWI+]) and Cyc8p ([OCT+]) (Du et al. 2008; Patel et al. 2009), and a similar screen identified the [MOD+] prion (Suzuki et al. 2012). The [MOT3+] prion was found in a survey of aggregation-prone Q/N-rich domains (Alberti et al. 2009).

The induction of [PSI+], [URE3], and other prions by transient prion protein overproduction increases prion appearance frequency from extremely rare to rare, often a 100-fold or 1000-fold increase (Wickner 1994; Chernoff et al. 1993; Kochneva-Pervukhova et al. 1998). Another general screen for prions was carried out looking for phenotypes persisting after transient overproduction of each yeast gene (Chakrabortee et al. 2016). Surprisingly, there were 46 proteins whose transient overexpression produced phenotypes in all cells, with the phenotype change persisting for >100 generations after the overexpression was ended. This is in marked contrast to the rare appearance of the previously described prions. One of these, [SMAUG+], a prion of Vtn1p, has been studied in some detail (Chakravarty et al. 2020).

28.5 [BETA], an Enzyme-Based Prion

Most yeast prions are self-propagating amyloids, but one is simply an enzyme that, under certain circumstances, is necessary for the activation of its own inactive precursor protein (Roberts and Wickner 2003). Vacuolar protease B is made as an

inactive precursor that is normally activated by vacuolar protease A (Jones 1991). In the absence of protease A, the protease B can inefficiently activate its own precursor, but on the usual medium, this activation cascade quickly dies out (Zubenko et al. 1982). However, because protease B expression is glucose-repressed, the growth of cells in glycerol medium results in the self-activation being indefinitely sustained. The active enzyme then acts like a prion (called [BETA]), showing that prions (infectious proteins) need not be amyloids (Roberts and Wickner 2003).

28.6 Amyloids as the Basis of Most Yeast Prions

Restricted domains of Ure2p (Masison and Wickner 1995; Masison et al. 1997) and Sup35p (Ter-Avanesyan et al. 1994) are sufficient for propagation of the [URE3] and [PSI+] prions. These prion domains are the N-terminal Q/N-rich parts of the respective molecules, although the prion domains of HET-s and Rnq1p are C-terminal (Balguerie et al. 2003; Vitrenko et al. 2007), and the HET-s prion domain is not Q/N rich.

Amyloid formation by prion domains (King et al. 1997; Taylor et al. 1999) and full-length prion proteins (Taylor et al. 1999; Glover et al. 1997), along with protease resistance of Ure2p in extracts of [URE3] strains (Masison and Wickner 1995) and aggregation of Sup35p in [PSI+] strains (Patino et al. 1996; Paushkin et al. 1996) first suggested that amyloid was the basis of [URE3] and [PSI+]. The [Het-s] system was the first in which prion infection by amyloid formed in vitro from recombinant protein was achieved (Maddelein et al. 2002). The key to this experiment was that the amyloid form of HET-s was infectious, but the soluble form or a nonspecific aggregate was not. Since the overexpression of prion proteins dramatically increases the frequency of prion induction, it was critical to show that one was not simply increasing the supply of the prion protein in the transfected cells. Similar results were shown for [PSI+] (King and Diaz-Avalos 2004; Tanaka et al. 2004) and later for [URE3] (Brachmann et al. 2005), [PIN+] (Patel and Liebman 2007), and [OCT] (Patel et al. 2009).

As will be discussed in another chapter, the amyloids of the prion domains of Ure2p, Sup35p, and Rnq1p are in-register parallel beta sheets, multiply folded along the long axis of the fiber. This architecture can explain the ability of proteins to template any of several different structures, based on different locations of the folds and/or different extents of the beta-sheet (see Wickner et al., Chap. 29).

Although the designation “prion domain” might suggest that part of the protein is selected in evolution for prion-forming ability, the prion domains of Ure2p and Sup35p have clear non-prion functions that account for their persistence. The Ure2p prion domain is important for the stability of the protein against degradation (Shewmaker et al. 2007), while the Sup35p prion domain is required for the protein’s role in mRNA turnover and other non-prion functions (Hosoda et al. 2003; Li et al. 2014; Franzmann et al. 2018).

28.7 Chaperones and Other Cellular Factors Affecting Prion Propagation

The finding that overproduction or deficiency of the disaggregating chaperone Hsp104 resulted in the loss of [PSI+] (Chernoff and Ono 1992; Chernoff et al. 1995) began a series of studies in which a host of chaperones were found to intimately affect the generation and propagation of yeast prions [reviewed in (Reidy and Masison 2011)]. Hsp104 works with Hsp70s and Hsp40s in renaturing proteins (Glover and Lindquist 1998), and, indeed, cytoplasmic Hsp70s are needed for yeast prion propagation (Jung et al. 2000), and Hsp40s also affect yeast prions (Moriyama et al. 2000; Sondheimer et al. 2001; Higurashi et al. 2008; Reidy et al. 2014; Troisi et al. 2015; Berger et al. 2020).

The primary means by which Hsp104–Hsp70–Hsp40 help prions propagate is by removing one Sup35 molecule from the filament, thus splitting the amyloid filaments, and producing two filaments where there was one (Paushkin et al. 1996; Ness et al. 2002; Kryndushkin et al. 2003; Tessarz et al. 2008) (Fig. 28.3). This constitutes prion replication, a process which must keep up with cell division if the prion is to be maintained. However, the mechanism by which overexpression of Hsp104 cures [PSI+] is unclear (Reidy and Masison 2010; Ness et al. 2017; Park et al. 2014; Helsen and Glover 2012; Winkler et al. 2012).

The Hsp70 family includes four soluble cytoplasmic members, Ssa1–Ssa4 and the two ribosome-associated chaperones, Ssb1 and Ssb2. The highly homologous Ssa's show surprising specificity for promoting or inhibiting the [PSI+] and [URE3] prions (Schwimmer and Masison 2002; Sharma and Masison 2008).

Hsp90s (Hsp82 and Hsc82 in *S. cerevisiae*) and their co-chaperones have several effects on yeast prions, none of which are fully understood. The curing of [PSI+] by overproduced Hsp104 is blocked by radicicol, an Hsp90 inhibitor, or by mutations in the Sti1 co-chaperone (Reidy and Masison 2010; Moosavi et al. 2010). In a study of the [PIN+] prion, it was found that deletions of *HSC82, AHA1, CPR6, CPR7, SBA1*, or *TAH1,* each encoding Hsp90 or a co-chaperone, change the [PIN+] variant so that it produces much higher or much lower [PSI+]-induction frequency than the original variant (Lancaster et al. 2013). The effect of the mutation was variant dependent, and the change persisted when the [PIN+] was transferred from the mutant to the wild-type. Hsp90 has a C-terminal MEEVD sequence that interacts with several co-chaperones. A mutation of this sequence was found to destabilize [URE3], as did a *cpr7*Δ mutation (Kumar et al. 2015). Cpr7p, at near stoichiometric concentrations, enhanced the rate of fibril formation by Ure2p, but whether this is the mechanism of support of [URE3] in vivo is not yet clear (Kumar et al. 2015).

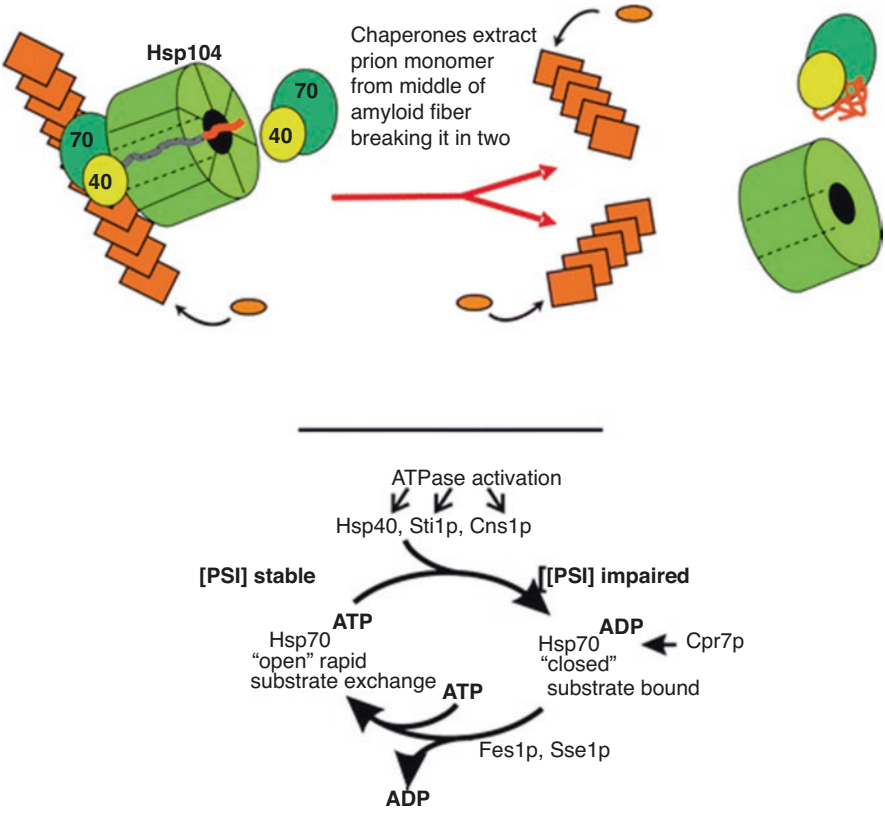

Fig. 28.3 Chaperones cleave prion amyloid filaments, generating new seeds (*Top*). Hsp104, Hsp70s, and Hsp40s extract a monomer from the middle of the filament, thus producing two filaments [reviewed by Reidy and Masison (2011)]. Chaperones (Liu et al. 2010; Reidy and Masison 2011) and Btn2p (Kryndushkin et al. 2008) may also function in prion segregation (not shown). Hsp40s, co-chaperones, and nucleotide exchange factors regulate the role of Hsp70s in prion propagation [reviewed by Sharma and Masison (2009)] (*Bottom*)

28.8 Prion Variants and the Species Barrier

A striking characteristic of nearly all prions (the exceptions are interesting) is the ability of a single protein sequence to stably propagate any of an array of prion "strains" or "variants." Different prion variants are distinguished in mammals by the incubation period, the regions of the brain affected, and the disease signs [reviewed by (Bruce and Fraser 1991; Vorberg 2019)]. In yeast, prion variants (Derkatch et al. 1996; Schlumpberger et al. 2001; Bradley et al. 2002) differ in the intensity of the prion phenotype, the stability of the prion, the response to overproduction or deficiency of various chaperones (Borchsenius et al. 2006; Kushnirov et al. 2000), the ability to cross interspecies barriers determined by prion protein sequence (Edskes et al. 2009) or intraspecies barriers (Bateman and Wickner 2012) (Fig. 28.4),

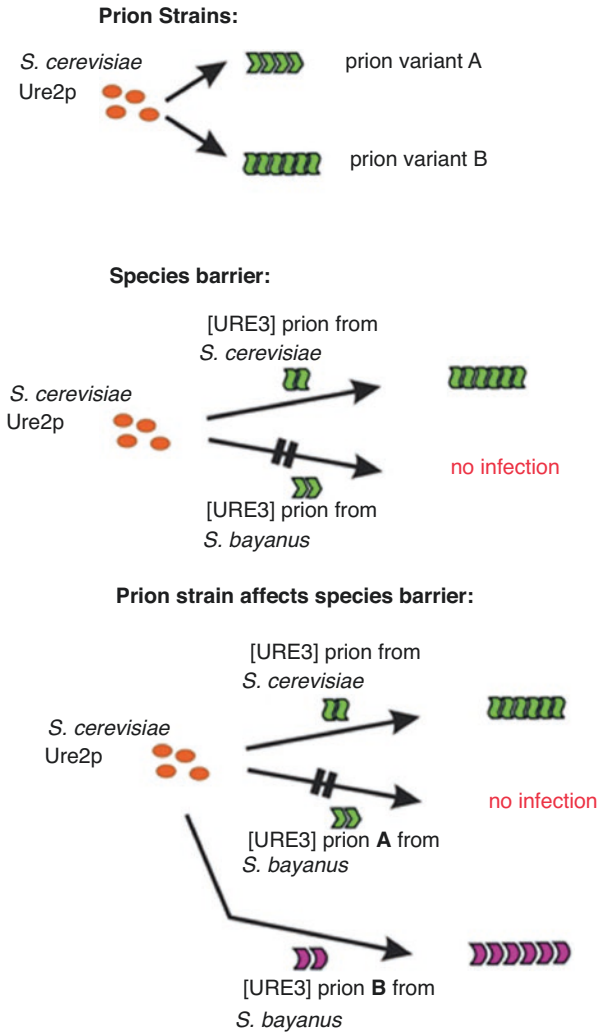

Fig. 28.4 Prion variants and the species barrier. As in animal systems, the facility of prion transmission across a species barrier depends on the prion variant. (Edskes et al. 2009; Bateman and Wickner 2012, 2013; Chen et al. 2010)

whether the prion is lethal or pathogenic or mild (McGlinchey et al. 2011) and sensitivity to anti-prion systems (Wickner et al. 2014, 2017; Gorkovskiy et al. 2017; Son and Wickner 2018, 2020) (see Wickner et al., Chap. 29).

Prion variants are clearly due to faithfully propagated differences in amyloid structure (e.g., (King and Diaz-Avalos 2004; Tanaka et al. 2004; Gorkovskiy et al. 2014; Dergalev et al. 2019)), but for no prion variant is the detailed structure yet known. However, as detailed in the next chapter (Chap. 29), yeast prions have a folded in-register parallel architecture that suggests a detailed mechanism of variant

information propagation. An important subject of future studies will be elucidation of the detailed nature of prion variant differences, and the study of how they produce different pathologies.

The [Het-s] prion of *Podospora anserina* does not show biological variants/strains (Saupe 2011), a finding correlated with the very uniform structure of the amyloid formed in vitro by its prion domain (Wasmer et al. 2008). Because [Het-s] is a functional prion, it is expected that evolution has favored a single structure that carries out that function.

While prion variants are relatively stably propagated, both mutation and segregation have been demonstrated, even under non-selective conditions (Bateman and Wickner 2013). Sup35p consists of an N-terminal prion domain (N, residues 1-123), a middle-charged domain (M, residues 124-253), and the C-terminal domain responsible for translation termination (C, 254-685). Wild yeast strains are polymorphic in their Sup35 sequences, particularly in the prion domain and the M domain (Bateman and Wickner 2012; Jensen et al. 2001; Resende et al. 2003). These sequences form three rough groups (laboratory, Δ19, and E9), each able to form [PSI+] prions, but with barriers to transmission of [PSI+] between strains in different groups (Bateman and Wickner 2012). The strength of the barrier and its specificity depends quite strongly on the prion variant used and can vary between no apparent barrier and a nearly complete barrier (Bateman and Wickner 2012). A particular [PSI+] variant in a yeast strain with the Sup35 sequence of laboratory strains showed limited transmission to isogenic Δ19 or E9 hosts. However, extensive non-selective growth of the reference [PSI+] strain produced subclones with varying transmission phenotypes, indicating that there was segregation of a mixture of prion variants (Bateman and Wickner 2013). Further extensive growth of each of these apparently purified variants again led to the appearance of the same mixed array of variants from any of the single variants (Bateman and Wickner 2013). This indicates that prion variant "mutation" and segregation are occurring, even under non-selective conditions. These findings provide evidence for the hypothesized "prion cloud" notion earlier suggested for both yeast and mammalian prions (Tanaka et al. 2005; Collinge and Clarke 2007), based largely on experiments in which the 'mutation' may have been induced by a selective condition.

28.9 Perspective

The yeast and fungal prion field has blossomed, becoming important for the overall understanding of prions and amyloid diseases in general. Yeast's traditional lead in genetic studies is being complemented with biochemical, cell biological, and structural studies to produce a variety of insights important for all prion systems.

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Chapter 29
Yeast Prions Are Folded, In-Register Parallel Amyloids Subject to Multiple Anti-prion Systems

Reed B. Wickner, Herman K. Edskes, Moonil Son, Songsong Wu, and Madaleine Niznikiewicz

Abstract Most yeast prions are self-propagating amyloids of normally non-amyloid proteins. The prion domains of Ure2p, Sup35p, and Rnq1p each form highly infectious folded in-register parallel β-sheet amyloids. This architecture can explain perhaps the most mysterious prion phenomenon: the stable propagation of any of several prion variants ("strains") by a single amino acid sequence. We have thus proposed a detailed model for the mechanism of templating of protein conformation by amyloid filaments. The yeast prions [URE3] and [PSI+] are diseases of yeast, with variants differing in the degree to which they deter cell growth or viability, but even the most mild forms are rare in wild strains. Cells defend against prion infection by inhibiting prion formation, curing most prions soon after they arise, largely blocking prion infection through mating ("the intraspecies barrier"), and limiting the toxicity of those prions that are not prevented by the first three mechanisms.

Keywords Lethal prions · Solid-state NMR · Templating of protein conformation · Prion structure · Anti-prion systems · Upf proteins · Ribosome-associated chaperones · Btn2 · Cur1 · Hsp104 · Lug1 · Sis1

It is now well established in both mammalian and yeast systems [see Chap. 28] that a single prion-forming protein can support the faithful propagation of any of several (perhaps many) different prion "strains" or "variants" (Derkatch et al. 1996; Bruce 1993). It is also clear that different prion variants are based on different amyloid conformations (Bessen and Marsh 1994; Toyama et al. 2007; Dergalev et al. 2019). This means that having assumed a particular amyloid conformation, a prion protein

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can instruct a new molecule joining the end of the amyloid filament to assume the same conformation as those already in the filament. How does this work? This is the central mystery of the prion phenomenon. Our proposed mechanism (Wickner et al. 2007, 2008a, 2015) based on our demonstration of the in-register parallel architecture of yeast prion proteins (Shewmaker et al. 2006; Baxa et al. 2007; Wickner et al. 2008b; Gorkovskiy et al. 2014) appears to be the only candidate explanation.

A second leading issue is the biological role of yeast prions. The [Het-s] prion of *Podospora anserina* is necessary for a normal physiological function of this organism, heterokaryon incompatibility (Coustou et al. 1997; Saupe 2011). This led us to state that this was the first functional prion (Wickner 1997). Because the yeast prion variants usually studied are relatively benign, it was suggested that yeast prions actually helped the host (Eaglestone et al. 1999). Claims of an advantage of [PSI+] or [URE3] (True and Lindquist 2000) have not been reproducible (Namy et al. 2008), and we will review the evidence that these prions are, in fact, diseases of yeast.

29.1 Shuffled Prion Domains of Sup35p or Ure2p Can Still Be Prions

To determine if there were specific sequences in the Ure2p or Sup35p prion domains that were needed for prion formation, we randomly shuffled these domains and tested five shuffled sequences for prion formation. Surprisingly, we found that each of the five shuffled sequences of each prion domain could form prions (Ross et al. 2004, 2005a, b), showing that, for at least these prion domains, the sequence was not critical and that prion formation depended more on amino acid content. The degree to which different residue types contribute to prion formation has been further examined as well (Toombs et al. 2010, 2011). Because the Sup35p prion domain, in common with the mammalian prion protein PrP, has oligopeptide repeat sequences, many authors have proposed that these sequences are important. Indeed, deletion or further duplication of these repeats do indeed affect prion propagation and generation (Liu and Lindquist 1999; Shkundina et al. 2006), but such manipulations also affect the length and composition of the prion domain. Shuffling the whole prion domain sequences (lacking repeats) (Ross et al. 2004, 2005a) or shuffling just the repeats (Toombs et al. 2011) does not impair the generation or propagation of prions implying that the repeats are not critical. It is possible that the repeats are significant for the mRNA turnover role of the Sup35p prion domain (Hoshino et al. 1999; Hosoda et al. 2003; Funakoshi et al. 2007) (see below).

That prion-forming ability was impervious to shuffling the amino acid sequence, combined with the requirement for near sequence identity between donor and recipient for transmission of a prion, also implying that the prion structure must be an in-register parallel sheet (Ross et al. 2005b). The well-known sequence dependence of prion *propagation*, the "species barrier," seemed to be at odds with our finding that prion formation did not require any specific sequence. However, the sequence

specificity for propagation simply means that there are specific interactions between amino acid side chains in the process of molecules adding to the end of an amyloid filament. If these specific interactions are complementary interactions, like the A–T and G–C interactions of DNA strands, shuffling the sequence would surely destroy the complementarity. However, if the specific interactions were between identical amino acid residues, then shuffling the sequence would still allow the same interactions, but they would occur in a different order. We thus predicted that the Ure2p and Sup35p prion domains would have an in-register parallel structure in their infectious amyloids (Ross et al. 2005b). As we describe in the following section, we verified this inference over the next few years.

29.2 Solid-State NMR Shows In-Register Parallel Architecture of Yeast Prion Amyloids

Meredith and coworkers were the first to demonstrate an in-register parallel amyloid structure (a peptide fragment of Abeta in this case) and used a solid-state NMR approach (Benzinger et al. 1998). Using singly carbonyl ^{13}C-labeled peptides, they showed a uniform ~5 Å distance between the labeled atoms, essentially the distance between strands in a beta-sheet. Because the molecules were singly labeled in each case, this could only be explained by an in-register parallel structure (Benzinger et al. 1998). Indeed, detailed studies have shown that the full-length Abeta amyloid has this architecture (Antzutkin et al. 2000; Balbach et al. 2002).

We have used a similar approach, but because the yeast prion domains are too long to synthesize, we used molecules labeled with a single carbonyl-^{13}C amino acid, at each of the (usually several) sites it occurs in the sequence. We found that the nearest neighbor labeled amino acid was generally about 5 Å away (Shewmaker et al. 2006; Baxa et al. 2007; Wickner et al. 2008b; Gorkovskiy et al. 2014). Because there were several labeled residues in each molecule, it was critical to show that the nearest neighbor labeled atom was indeed in another molecule. This was done by diluting labeled molecules with unlabeled molecules and showing that the nearest neighbor distance was increased to the extent predicted based on the degree of dilution. Confirmation of the in-register parallel structure of the Ure2p prion domain has come from mass per length measurements (Diaz-Avalos et al. 2005; Baxa et al. 2003; Chen et al. 2009) and electron spin resonance studies (Ngo et al. 2011, 2012), and locations of some folds are suggested by NMR and electron spin resonance experiments (Gorkovskiy et al. 2014; Wang et al. 2020). A recent cryoEM study of highly infectious PrP^{Sc} has shown the folded in-register parallel β-sheet architecture for this prion (Kraus et al. 2021).

Amyloids of the Ure2 or Sup35 prion domains made for these NMR experiments generally produced a mixture of prion variants on transformation into yeast (King and Diaz-Avalos 2004; Tanaka et al. 2004; Brachmann et al. 2005). Correspondingly, two-dimensional ^{13}C–^{13}C solid-state NMR experiments show broad peaks indicative of microheterogeneity of sample conformations (Shewmaker et al. 2006; Baxa et al.

2007; Wickner et al. 2008b). Growing Sup35NM filaments at 4 °C or 37 °C produces amyloid that on infection in yeast gives [PSI+] variants that are homogeneous with respect to phenotype strength, but may be mixed by other criteria, such as transmissibility across intraspecies barriers (Tanaka et al. 2004; Bateman and Wickner 2013). Interestingly, hydrogen–deuterium exchange showed different extents of the slow-exchange regions in these preparations (Toyama et al. 2007). We found that each of these variant amyloid preparations showed the in-register parallel architecture (Shewmaker et al. 2009). However, it is not clear that these amyloid preparations are homogeneous since the H–D exchange does not show single-exponential kinetics (Toyama et al. 2007).

Electron micrographs of amyloid formed from Ure2p or Sup35p prion domains show diameters of roughly 5 and 12 nm, respectively (Glover et al. 1997; Taylor et al. 1999). However, if the structures were single unfolded beta-sheets, they would be about 23 and 40 nm wide. Thus, the sheets must be folded along the long axis of the filaments. We suggest that prion variants may differ in the location of these folds (Wickner et al. 2007, 2015).

29.3 In-Register Parallel Architecture Explains Protein Templating of Conformation

A model of the in-register parallel structure is shown in Fig. 29.1. There is a line of each amino acid residue along the long axis of the filaments. What holds the molecules in-register in the yeast prion amyloid structure? The main chain hydrogen bonds between the amide H and the amide carbonyl of the peptide bond are the primary beta-sheet hydrogen bonds between molecules and are oriented along the long axis of the filament but are not sequence specific. It is interactions between the amino acid side chains that must be maintaining the structure in-register. If aligned, glutamine side chains can form hydrogen bonds as first suggested by Perutz for Huntingtin (Perutz et al. 1994). Aligned asparagine side chains can form a similar line of hydrogen bonds as can serines or threonines. Alignment of hydrophobic residues will likewise be favored by hydrophobic interactions of their side chains. Only charged residues will not want to be aligned because it brings identical charges close together, but charged residues are strongly underrepresented in the yeast prion domains.

At least for Sup35p amyloid filaments, elongation occurs by the addition of monomers to the ends of the filament (Collins et al. 2004). The prion domain of at least the native Ure2p is unstructured (Pierce et al. 2005). Formation of these amyloids is a change from unstructured to parallel in-register beta-sheet, with the sheet folded length-wise at specific sites (Fig. 29.1). We proposed that the same side chain—side chain bonds that hold the molecules in the filament in register direct the molecule joining the end of the filament to assume the same conformation as the molecules already in the filament, and that different prion variants have the folds of the sheet in different locations (Wickner et al. 2007, 2015) (Fig. 29.2). Assuming

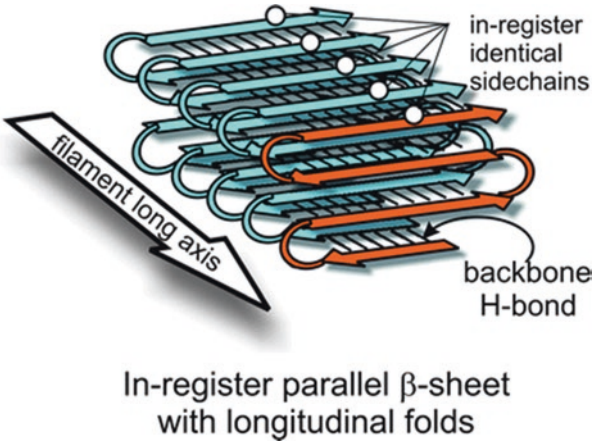

Fig. 29.1 In-register parallel beta-sheet architecture of the yeast prion amyloids [modified from Shewmaker et al. (2006)]. The side chains of a given residue form a line along the long axis of the filament. It is favorable interactions among such identical aligned side chains that keep the chains in-register. Electron microscopic measurements of filaments imply that the sheets must be folded along the long axis of the filaments as shown here

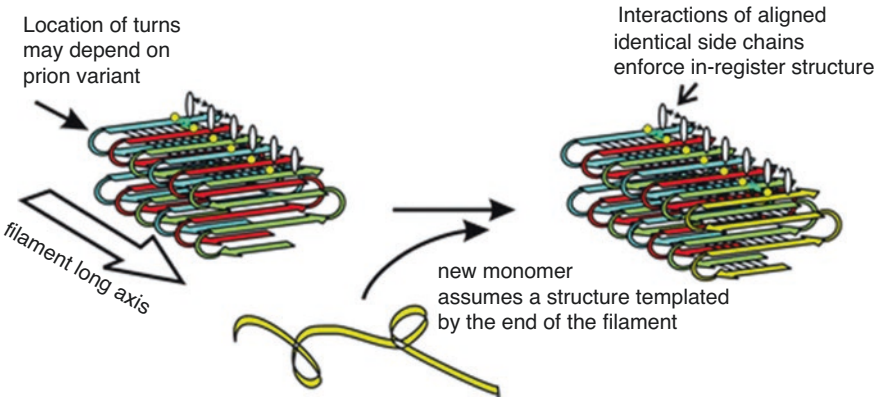

Fig. 29.2 The in-register parallel beta-sheet architecture suggests that prion variants differ in the location of the folds of the sheet, and implies a mechanism by which conformation can be inherited (Wickner et al. 2007, 2015). The same favorable interactions among identical side chains that keep the structure in-register direct a monomer joining the end of the filament to assume the same conformation as molecules already in the filaments. Thus, the protein templates its own conformation in the same way that a DNA molecule templates its sequence

that the folds are more protease sensitive than the β-sheet regions, the studies of Dergalev et al. indicate that different variants indeed have the folds in different locations (Dergalev et al. 2019). Thus, just as DNA templates sequence, a protein can template conformation. Different protein conformations (=different prion variants/strains) can be faithfully propagated, and so proteins can act as genes.

29.4 Biology of Yeast Prions

Because de novo generation of prions in yeast, as in mammals, is a stochastic process, it is not well suited as an adaptive measure and is likely to be simply an infectious disease. Ure2p is a regulator of nitrogen catabolism, turning off the genes encoding transporters and enzymes needed for the assimilation of poor nitrogen sources when the cells have available a good nitrogen source (Cooper 2002). When Ure2p is converted to amyloid in [URE3] cells, it loses its flexibility and is locked in the off position. Sup35p is a subunit of the translation termination factor, and it seems unlikely that cells will regulate translation at the termination step. Moreover, inefficient translation termination must produce a read-through of many or most mRNAs, resulting in a wide variety of pathologic proteins.

29.4.1 [Het-s]: Benefit and Detriment

[Het-s], a prion of the filamentous fungus *P. anserina*, is necessary for heterokaryon incompatibility, a normal process in which the fungus recognizes self/nonself, and avoids fusing with colonies not very closely related (Coustou et al. 1997; Saupe 2011). Restricting mating to closely related strains reduces the risk of becoming infected with deleterious viruses or senescence plasmids (Debets et al. 2012). The *het-s* locus has alleles *het-s* and *het-S*, differing at 13 amino acid residues in the 289 residue protein and found at about equal frequency in wild strains (Dalstra et al. 2003). Only *het-s* cells can have the prion form, and when *het-s* [Het-s] cells fuse with *het-S* cells, the fused cells die and build a barrier to further fusions. We suggested that this was the first prion to have a function for the host, rather than being a disease (Wickner 1997). However, the [Het-s] prion also is involved in a meiotic drive phenomenon (much like the t locus of mice or segregation distorter in *Drosophila*), where an allele of a gene promotes its inheritance, not by benefiting the organism, but by cheating on meiosis, killing germ cells with other alleles. When female *het-s* [Het-s] cells mate with male *het-S* cells, most meiotic segregants with the *het-S* allele are killed (Dalstra et al. 2003). Thus, [Het-s] might be viewed as a disease of *Podospora* and heterokaryon incompatibility, a secondary phenomenon.

29.4.2 Proposed Benefits of Yeast Prions

Yeast prions were first suggested to benefit their hosts when it was reported that [PSI+] had a general protective effect against heat or elevated ethanol concentrations (Eaglestone et al. 1999). A subsequent report explored a large array of conditions in several isogenic pairs of [PSI+] and [psi-] strains and failed to reproduce the

reported protective effects against heat or ethanol; in fact, there was no condition under which [PSI+] was protective in all cases (True and Lindquist 2000). In most strains under most conditions, if there was a difference, [psi-] proved to be healthier than [PSI+]. Nonetheless, the authors proposed that [PSI+] was helping yeast evolve by, in some cases, protecting cells from adverse conditions (True and Lindquist 2000). If the phenotypes produced by yeast prions were to aid evolution, they would have to be beneficial, at least occasionally. Thus, it is particularly damaging to the evolvability model for yeast prions that, using the same strains, the favorable phenotypes reported earlier were not reproducible (Namy et al. 2008). Later claims of [PSI+] advantage (Halfmann et al. 2012) were also not reproducible with the same strains (Wickner et al. 2015). In addition, none of these experiments included the majority of highly toxic prion variants of [PSI+] (see below; ref. (McGlinchey et al. 2011)).

It has been reported that under certain stress conditions, the frequency of [PSI+] arising increases, and this is interpreted as an adaptive response (Tyedmers et al. 2008). However, the authors could not detect this effect with the normal Sup35 protein sequence and only found it with an artificial construct that converts to [PSI+] with dramatically higher frequency. In addition, the authors reported that of four of the six conditions producing more frequent [PSI+], acquisition of the prion was detrimental to the cells. These results actually argue against the "prion as evolvability factor" model. It is also argued that prion-forming ability is conserved across evolution, but we will see (in the following section) that this is not the case, with some close homologs of prion proteins of *Saccharomyces cerevisiae* being unable to form prions.

29.4.3 Evidence That Yeast Prions Are Diseases

Although it is impossible to test all possible conditions or to know what conditions represent what portion of the yeast natural habitat, there is a way to take a sort of integral over all conditions. The infectivity of yeast prions means that even if they are a net detriment to yeast, they may be found at some frequency in the wild. For example, the uniformly fatal chronic wasting disease of deer and elk is found to infect ~10% of animals in Wyoming and Colorado (Williams 2005). An infectious element that was beneficial to its host would spread rapidly because the effect on the host and infectivity would be working in the same direction instead of in opposition. Thus, an infectious element that is not found in the wild must be detrimental to its host unless it is geographically limited (on an island) or newly arisen. We surveyed 70 wild strains and found each of the known parasitic nucleic acid replicons, including the L-A and L-BC dsRNA viruses, the 20S and 23S single-stranded RNA replicons, and the two micron DNA plasmid. However, neither [PSI+] nor [URE3] was present in any of the wild strains (Nakayashiki et al. 2005). This indicates that the overall effect of these prions is detrimental. In more limited surveys, other groups also found [PSI+] absent from wild strains (Chernoff et al. 2000; Resende et al.

2003). A much larger survey found that ~1% of wild strains carried [PSI+] (Halfmann et al. 2012), statistically consistent with the earlier smaller surveys. We did, however, find the [PIN+] prion at a frequency comparable to that of the parasitic nucleic acid replicons. Population genetic arguments indicate that the mildest variants of each of these three prions places a >1% detriment on the growth or survival of the host strains in the wild (Kelly et al. 2012).

The partial conservation of the sequence of the prion domains of Ure2p and Sup35p has been advanced as an argument that prion formation must be a benefit to yeast (Harrison et al. 2007). However, we have shown that prion formation is not determined by the prion domain sequence for either Ure2p or Sup35p (Ross et al. 2004, 2005a). Rather it is the amino acid composition that is critical (Toombs et al. 2010, 2011). The sequence conservation probably reflects the normal non-prion functions of the prion domains. The Sup35p prion domain is necessary for the general mRNA turnover system, linking translation termination to the mRNA decay process by interactions with the polyA binding protein and the polyA RNAses (Hoshino et al. 1999; Hosoda et al. 2003; Kobayashi et al. 2004). The Sup35 prion domain is also involved in cytoskeleton-associated translation (Li et al. 2014) and phase separation under stress conditions (Franzmann et al. 2018). The Ure2p prion domain stabilizes the protein against decay in vivo (Shewmaker et al. 2007). Thus, the presence of these domains across evolution and their conservation of sequence probably reflect the importance of these functions and do not provide evidence for the value of the prions to the host.

Although several homologs of Sup35p and Ure2p have been shown capable of being prions (Chernoff et al. 2000; Kushnirov et al. 2000a, b; Santoso et al. 2000), there are notable exceptions. The Ure2p's of *Saccharomyces castellii* (Edskes et al. 2009), *Candida glabrata* (Edskes et al. 2011), and *Kluyveromyces lactis* (Safadi et al. 2011) are apparently unable to form prions. *Candida glabrata* is closely related to *S. cerevisiae*, and cannot form a [URE3] prion, but *C. albicans*, which is farther away, forms a [URE3] with properties similar to those of [URE3] of *S. cerevisiae* (Edskes et al. 2011). The ability to form the [PSI+] prion is likewise scattered among yeast and fungal strains, not conserved (Edskes et al. 2014).

Yeast cells (like other cells) react to a variety of stresses by inducing the production of heat shock proteins. Yeast induces both Hsp104 and Hsp70s on infection with the [URE3] and/or [PSI+] prions, indicating that the yeast cell's view of prion infection is unfavorable (Jung et al. 2000; Schwimmer and Masison 2002).

The prion domains of Sup35p and Ure2p change far more rapidly in evolution than do the non-prion parts of the same molecules (Kushnirov et al. 1990, 2000a; Chernoff et al. 2000; Santoso et al. 2000; Edskes and Wickner 2002; Baudin-Baillieu et al. 2003). Many of these changes produce barriers to transmission, species barriers that prevent the spread of the prions among the inter-mating *Saccharomyces* species (Edskes et al. 2009; Chen et al. 2007). In analogy with the protection afforded by the 129 M/V PrP polymorphism in humans, it is likely that these mutations were selected to protect cells against infection by a prion from a more common Sup35p or Ure2p allele.

In spite of this array of data that [PSI+] and [URE3] are detrimental to yeast (Table 29.1), these prions are frequently cited in reviews as functional/beneficial amyloids, probably because cells carrying the usually studied yeast prion variants do not seem particularly sick in the laboratory. If there were a [PSI+] variant that adsorbed all of the cell's Sup35p in the filaments, the cells would be dead because Sup35p is essential. To isolate such a "suiψdal [PSI+]," we prepared a strain with a normal full-length chromosomal *SUP35* carrying a counter-selectable plasmid with a doxycycline-repressed *SUP35C* gene, lacking the prion domain. The plasmid-encoded *Sup35C* cannot be incorporated into the amyloid filaments because it lacks the prion domain, and its expression was adjusted so that it was sufficient by itself to keep cells alive, but so low that cells would be Ade^+ because of increased read-through of the *ade1–14* nonsense mutation. [PSI+] clones were then tested for growth on the medium that required loss of the plasmid carrying *SUP35C*. Eight percent of [PSI+] isolates were totally unable to grow after plasmid loss, and 46% grew extremely slowly (McGlinchey et al. 2011). The existence of "suiψdal" and sick [PSI+] show that maintaining the ability to become [PSI+] does not come without a severe price (Table 29.1).

Ure2p is not essential to yeast, and in many strains, *ure2*Δ does not even slow growth. However, we found that frequently [URE3] isolates grew extremely slowly, forming only tiny colonies on rich media (McGlinchey et al. 2011). These prion variants are not slowing growth by producing a deficiency of Ure2p, since deletion of the *URE2* gene in this background is harmless. The prion must be having some toxic effect on the cell, perhaps adsorbing some essential component or interacting in a detrimental way with some other cellular component. Further work will be required to understand the nature of these toxic actions.

Table 29.1 Evidence that [PSI+] and [URE3] prions are diseases

[PSI+] and [URE3] are rare in wild strains	Halfmann et al. (2012), Nakayashiki et al. (2005), Chernoff et al. (2000), and Resende et al. (2003)
Prion domains of Sup35p and Ure2p have important non-prion functions	Hoshino et al. (1999), Hosoda et al. (2003), Li et al. (2014), Franzmann et al. (2018), and Shewmaker et al. (2007)
Cells mount a stress reaction when infected with [PSI+] or [URE3]	Jung et al. (2000) and Schwimmer and Masison (2002)
Prion domains change more rapidly than non-prion domains, producing prion transmission barriers	Edskes et al. (2009) and Chen et al. (2007)
Lethal and extremely toxic prion variants of [PSI+] and [URE3] are common	McGlinchey et al. (2011)
Prion-forming ability is not well conserved even among close relatives of *S. cerevisiae* Sup35p and Ure2p	Edskes et al. (2009, 2011, 2014), and Safadi et al. (2011)

29.5 Anti-prion Systems That Cure Prions in Normal Cells

The known harmful effects of prions [URE3] and [PSI+] suggest that cells must have mechanisms to cure prions as they arise. While there are many ways of curing yeast prions by the overproduction or underproduction (or inhibition) of one or another component, it is when a component expressed at *normal* level cures a yeast prion that we refer to it as an anti-prion protein. In fact, there are now six such systems, as well as cellular components that reduce the frequency of prion formation or limit the toxicity of prions (Table 29.2). In addition, sequence difference between prion proteins of different yeast strains largely block prion transmission (the intra-species barrier; see Chap. 28.8). Such species differences are likely selected because they protect the cell from acquiring a prion by infection.

Table 29.2 Anti-prion systems in *S. cerevisiae*

Anti-prion protein	Prion affected	Prion appearance	Mechanism	Ref.
Btn2	[URE3]	5 × ↑	Sequestering amyloid filaments producing asymmetric segregation of prion and curing	Kryndushkin et al. (2008) and Wickner et al. (2014)
Cur1	[URE3]	5 × ↑	Curing prion by an unknown mechanism	Kryndushkin et al. (2008) and Wickner et al. (2014)
Hsp104	[PSI+], [URE3]	13 × ↑	Curing prion, mechanism controversial; essential for the propagation of amyloid prions	Gorkovskiy et al. (2017)
Upf1,2,3	[PSI+]	15 × ↑	Nonsense-mediated decay components forming a complex with Sup35 and curing	Son and Wickner (2018)
Ssb1/2 Ssz1 Zuo1	[PSI+]	15 × ↑	Ribosome-associated chaperones facilitating the proper folding of nascent proteins	Chernoff et al. (1999) and Son and Wickner (2020)
Swi14	[PSI+]	2 × ↑	Pyrophosphatase acting on 5-diphospho-inositolhexakisphosphate (5-PP IP5). Mechanism unknown	Wickner et al. (2017) and Steidle et al. (2016)
Sis1	[PSI+]	NA	Essential protein. Prevents prion lethality by avoiding total depletion of Sup35 monomers by the amyloid formation	Kirkland et al. (2011) and Kumar et al. (2021)
Lug1	[URE3]	NA	F-box containing E3 ubiquitin ligase (targets unknown). Prevents prion lethality by an unknown mechanism	Edskes et al. (2018)
Prion domain mutations	[URE3], [PSI+]	NA	Prion domain mutations partially block the transmission of prion by mating	See text

29.5.1 Btn2 Sequesters Prion Amyloids Promoting Loss by Segregation

Batten's disease is a heritable neuronal ceroid-lipofuscinosis, in which this material accumulates in lysosomes. The causative mutations are in the human *CLN3,* closely homologous to the yeast *BTN1* and capable of complementing *btn1* mutants, correcting their excess vacuole acidification defect (Pearce et al. 1999). *BTN2* was first identified as a gene whose transcription was induced in *btn1* mutants and shown to be involved in endosome-Golgi protein sorting as a v-SNARE binding protein (Chattopadhyay et al. 2003; Kama et al. 2007).

BTN2 was then detected, along with its paralog, *CUR1,* in a screen for genes whose overexpression cures [URE3] (Kryndushkin et al. 2008). In the course of curing [URE3], Btn2-RFP colocalizes with the Ure2-GFP-labeled amyloid filaments which have been collected at one site in the cell (Kryndushkin et al. 2008). It was suggested that the overproduced Btn2 cures by sequestering the many prion filaments in one place so that cell division often produces one progeny cell without any filaments, and thus cured of the prion (Kryndushkin et al. 2008). Btn2 and Cur1 also cure an unrelated artificial prion, and Btn2 sequesters several non-prion non-amyloid aggregates as well (Kryndushkin et al. 2012; Malinovska et al. 2012).

To determine whether Btn2 or Cur1 act in *normal cells at normal levels*, a series of [URE3] variants were isolated in a *btn2*Δ *cur1*Δ strain, and each was mated with a wild type. Remarkably, over 90% of the variants were cured in the diploids (but not in *btn2*Δ *cur1*Δ/*btn2*Δ *cur1*Δ diploids), suggesting that most [URE3] variants arising in a wild-type strain are cured as they arise (Wickner et al. 2014). The frequency of spontaneous [URE3] generation in *btn2*Δ *cur1*Δ strains is ~5 times that in a wild type, and it is specifically variants with low seed number that are cured by normal levels of Btn2 and Cur1, consistent with this interpretation (Wickner et al. 2014). This approach became a pattern that was repeated with other anti-prion proteins (Hsp104, ribosome-associated chaperones, Upf proteins, and Siw14) and other prions.

There is apparently no relation between the endosome and Golgi protein sorting function of Btn2 and its role in sequestering prion or other aggregates. None of the six genes shown to be necessary for Btn2's protein sorting role (Kama et al. 2011) were found to be necessary for its prion-curing role (Bezsonov et al. 2021), although Btn3 binds Btn2 and inhibits both roles (Kanneganti et al. 2011).

29.5.2 Cur1

Unlike Btn2, Cur1 does not co-localize with Ure2p amyloid filaments in the course of curing and is located in the nucleus (Kryndushkin et al. 2008). Unlike its effects on [URE3], Cur1 strengthens the nonsense suppression (termination read-through) phenotype in [PSI+] strains (but not in [psi−] strains)(Barbitoff et al. 2017), and

only a rare [PSI+] variant is cured by overproduction of Cur1 (Bezsonov et al. 2021). As discussed elsewhere, the mechanism of action of Cur1 is not yet clear (Wickner et al. 2021).

29.5.3 Proteasomes, Btn2, and Cur1

Both Btn2 and Cur1 are controlled by proteasome activity. A *ubr2* mutation elevates proteasome activity by relieving degradation of the Rnp4 transcription factor that promotes the transcription of genes encoding proteasome components. Mutations in genes for 60S ribosomal subunit proteins also stimulate proteasome activity. Both prevent the curing of [URE3] by overproduction of Btn2 or Cur1 (Bezsonov et al. 2021). Impairing proteasome activity by mutation of the sole non-essential core subunit or by deletion of genes for proteasome-assembly factors results in dramatic elevation of Btn2 and Cur1 levels and consequent curing of [URE3] (Edskes et al. 2021). It is suggested that Btn2 and Cur1 act as a backup for cleaning up the debris when the proteasome is overwhelmed by some stress condition. The automatic failure to degrade Btn2 and Cur1 dramatically increases their levels and activities.

29.5.4 Hsp104 at Normal Levels Cures Many [PSI+] Prions

As discussed in Chap. 28, Hsp104 has two activities: filament scission (with Hsp70s and Hsp40s) as part of prion propagation, and, on Hsp104 overproduction, curing of the [PSI+] prion (Chernoff and Ono 1992; Chernoff et al. 1995). Mutants in the Hsp104 N-terminal domain impair the second activity without loss of the first (Hung and Masison 2006). However, even at normal expression levels of Hsp104, [PSI+] variants arise in the N-terminal mutants (e.g. *hsp104-T160M*) that are cured by restoration of normal Hsp104 (Gorkovskiy et al. 2017). The 13-fold elevation of [PSI+] arising in *hsp104-T160M* strains includes an increased frequency of generation of variants which are stable in wild-type cells.

Hsp104 overproduction also weakly cures [URE3] (Kryndushkin et al. 2008; Matveenko et al. 2018) and some variants of [PIN+] (Huang et al. 2021). There are also occasional [PSI+] variants that are more stable in the wild type than in *hsp104-T160M* strains (Gorkovskiy et al. 2017; Huang et al. 2021).

29.5.5 Ribosome-Associated Chaperones and [PSI+]

Ssb1/2 and Ssz1 (both Hsp70s) and Zuo1 (Hsp40) insure the proper folding of nascent proteins emerging from the ribosome (Nelson et al. 1992; Pfund et al. 1998; Zhang et al. 2020). Mutants lacking any of these proteins have an elevated

frequency of [PSI+] prion generation (Chernoff et al. 1999; Amor et al. 2015; Kiktev et al. 2015), including the production of many [PSI+] variants that are cured by the replacement of normal levels of the wild-type protein (Son and Wickner 2020). The known activity of these chaperones is expected to affect misfolding of all proteins, but, perhaps surprisingly, no effect on [URE3] was observed (Son and Wickner 2020).

29.5.6 Nonsense-Mediated Decay Proteins (Upf) Have Anti-prion Activity by Complexing with the Prion Protein

An mRNA with a premature stop codon is degraded more rapidly than normal in a process, called nonsense-mediated mRNA decay, and carried out by Upf1, Upf2, and Upf3 (He and Jacobson 2015). The Upf proteins are located on the ribosome and associate normally with Sup35p, a subunit of the translation termination factor. In a general screen for anti-prion components, Upf proteins were detected (Son and Wickner 2018). It was shown that the anti-prion activity of Upf proteins does not correlate well with their nonsense-mediated decay activity, but the former does correlate well with the complex formation with Sup35p (Son and Wickner 2018). Indeed, Upf1 associates in vitro (Czaplinski et al. 1998) and in vivo (Son and Wickner 2018), and Upf1 directly inhibits amyloid formation by Sup35p (but not Ure2p) (Son and Wickner 2018). One would expect that the propagation of any prion will be in competition with the normal interactions that the prion protein has with other cellular components.

29.5.7 Siw14 and Inositol Polyphosphates' Role in [PSI+] Propagation

In the screen for anti-prion factors, *siw14*Δ strains were found to produce [PSI+] variants curable by restoring normal levels of Siw14 (Wickner et al. 2017). Siw14 is a pyrophosphatase specific for inositol 5-pyrophosphates, and strains deficient in this enzyme have ~6-fold elevated levels of the substrate, 5-diphosphoinositol pentakisphosphate (5PP-IP5) (Steidle et al. 2016). Finding [PSI+] variants that required elevated levels of 5PP-IP5 suggested that other [PSI+] variants might require this or related compounds, but not as much. In fact, most [PSI+] variants require some amount of either 5PP-IP5, 5PP-IP4 or IP6 (Wickner et al. 2017). None of the 15 [URE3] variants tested required any of the inositol polyphosphates. The inositol pyro/polyphosphates are signaling molecules, but it is not yet clear how their effects on [PSI+] are mediated.

29.5.8 *Sis1 and Lug1 Limit Toxicity of [PSI+] and [URE3], Respectively*

Although most variants of these prions are highly toxic (see above), the variants used for most lab studies show little or no obvious growth-slowing effects. In studying the role of the Hsp40 family member Sis1p in prion propagation, it was found that the N-terminal portion of Sis1p, including only the J-domain and the GF-rich region, was sufficient to maintain cell growth in the absence of the prion, but made a normally mild [PSI+] variant be lethal (Kirkland et al. 2011). This Sis1-JGF does not lose the [PSI+] prion, but cells cannot grow. Detailed studies of this effect showed that in this *sis1-JGF* strain, nearly all of the Sup35p is sequestered by the filaments and there is not sufficient translation termination activity to support growth (Kumar et al. 2021).

Using saturation transposon mutagenesis, Edskes et al. designed a screen for cellular components preventing the toxicity of [URE3] using saturation transposon mutagenesis and selecting genes readily mutable if the cells were [ure-o], but not if they had [URE3] (Edskes et al. 2018). *LUG1* (for lets [URE3] grow) gave the most dramatic results in the screen although a number of chaperones not known to be needed for [URE3] propagation were also counter-selected for insertions. Lug1 is an F-box protein, an E3 ubiquitin ligase that is the substrate-specifying subunit of a cullin-containing ubiquitin ligase complex (Seol et al. 2001). However, the substrates determined by Lug1 are not yet known.

29.6 Perspective

Because it forms a single amyloid structure—corresponding to a single prion variant—the HET-s amyloid structure has been solved in a series of elegant studies (Ritter et al. 2005; Siemer et al. 2006; Wasmer et al. 2008). [Het-s] was evolved to be a prion, and so forms only a single amyloid structure with the selected properties. It will be necessary to develop a method to obtain substantial amounts of yeast prion amyloid in a single conformation in order to obtain more detailed structural information. The in-register parallel architecture represents what is common among the structures, but the material used in these studies has, unavoidably, represented a mixture of structures. Future work on the biology of yeast prions will include studies of the mechanisms by which they produce harm to the cells, mechanisms that go beyond the mechanism known since the first studies of yeast prions of simple depletion of active prion protein by conversion to the prion form.

The recent uncovering of an array of anti-prion systems has changed the picture of these molecular diseases. Instead of a single prion generation event producing inevitable detrimental infection, it is clear that most such events are prevented, that most of the prions that are generated are immediately cured, that the cells block many potential infecting variants by prion protein polymorphisms, and that the

pathogenicity of some yeast prion variants is limited by cellular systems. It is hoped that analogous systems in humans can be found and manipulated to enable the treatment of these diseases. Nearly all of the human pathogenic amyloids have the same folded in-register parallel β-sheet structure as the yeast prion amyloids. We infer that this makes yeast prions excellent models for human diseases.

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Part IX
Diagnosis and Human Prion Surveillance

Chapter 30
Real-Time Quaking-Induced Conversion (QuIC) Assays for the Detection and Diagnosis of Human Prion Diseases

Christina D. Orrù, Onyekachi Isiofia, Andrew G. Hughson, and Byron Caughey

Abstract The seeding activity of prions has been exploited for the development of ultrasensitive assays for prion diseases. Among the more practical are Real-Time Quaking Induced Conversion (RT-QuIC) assays that use recombinant prion protein as a substrate for prion-seeded conversion into amyloid fibrils, shaking rather than sonication, and fluorescence detection in multi-well plates. International testing and validation of RT-QuIC in the *antemortem* diagnosis of sporadic Creutzfeldt–Jakob disease (sCJD) using cerebrospinal fluid have led to the inclusion of RT-QuIC findings in official diagnostic criteria. Diagnostic applications of RT-QuIC to additional human biospecimens such as olfactory mucosa brushings and skin have also shown promise. While RT-QuIC assays are now providing more practical prion detection in humans, animals, biomaterials, and the environment, further improvements in practicality, quantitative precision, and range of applications would be helpful. In this chapter, we focus primarily on applications of RT-QuIC to human prion disease diagnostics.

Keywords Amyloid seeding assay · Prion protein · Real-time QulC · sCJD · Transmissible spongiform encephalopathies · TSE

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30.1 Introduction

Prions are infectious forms of prion protein (PrP) known generically as PrP^{Sc} (PrP-scrapie) or, when protease-resistant, PrP^{Res}. Ultrasensitive detection of prions may help to prevent prion diseases, or transmissible spongiform encephalopathies (TSEs), by allowing the detection of potential sources of infection. Prion detection can also facilitate early and accurate diagnosis in infected humans and animals. It has long been known that PrP^{Sc} can induce or seed the conversion of its normal precursor, PrP^{C} (cellular PrP), or PrP^{Sen} (protease-sensitive PrP) into forms that, like PrP^{Sc} itself, are higher in beta-sheet content, multimeric, and more protease-resistant (McKinley et al. 1983; Caughey et al. 1991, 2009; Safar et al. 1993; Pan et al. 1993; Kocisko et al. 1994; Kraus et al. 2021; Hoyt et al. 2022). The development of a sustainable in vitro PrP^{Sc} formation system called protein misfolding cyclic amplification (PMCA) by Soto and colleagues allowed extraordinarily sensitive detection of prions (Castilla et al. 2006; Saborio et al. 2001; Saa et al. 2006; Chen et al. 2010). In this reaction, test samples are mixed with PrP^{C}-containing brain homogenates from uninfected animals and subjected to cycles of intermittent sonication and rest. Prion seeding activity within the test sample induces the conversion of PrP^{C} in the brain homogenate to PrP^{Res}, which may ultimately be detected by proteinase K treatment (to eliminate unconverted PrP^{C}) and immunoblotting. With serial PMCA (sPMCA) reactions, as little as ~1 attogram (10^{-18} g) of PrP^{Sc} could be detected within ~3 weeks (Saa et al. 2006), amounting to amplifications of more than a billion-fold. Indeed, such starting quantities of PrP^{Res} are far smaller than those required to cause TSE disease by inoculation into animals. Further applications of PMCA have allowed the detection of prions in numerous tissues and sample types (Castilla et al. 2006; Jones et al. 2011). Although classical PMCA has been invaluable as a research tool, certain features limit its practical applicability for routine screening and diagnostics. Notably, (1) brain homogenates are neither ideal nor abundant sources of PrP^{C} substrate; (2) sonication can be difficult to control and standardize; (3) the use of individual reaction tubes and immunoblotting is labor-intensive and ill-suited to automation; (4) the overall time required for optimal sensitivity is prohibitive for many routine surveillance, screening, or diagnostic applications; and (5) the massively amplified products of the assays are themselves infectious and, therefore, biohazardous.

Efforts to improve on these practical limitations of PMCA led ultimately to prion seeding activity-based assays that use bacterially expressed recombinant PrP^{Sen} ($rPrP^{Sen}$) as substrate (Atarashi et al. 2007, 2008), multi-well plate formats with thioflavin T (ThT) fluorescence readouts (e.g., Colby et al. 2007; Atarashi et al. 2011; Wilham et al. 2010), shaking rather than sonication (Atarashi et al. 2008), and markedly reduced overall assay times (for recent reviews, see Saijo et al. 2019; Ferreira and Caughey 2020; Vascellari et al. 2022). In this chapter, we will highlight recent advances in RT-QuIC, focusing primarily on applications to human prion disease.

30.2 RT-QuIC

In RT-QuIC assays, a small volume (usually 1–20 μl) of the test sample is combined with a reaction mix containing an appropriate $rPrP^{Sen}$ substrate and ThT in a plate with 96 or 384 wells. The plate is then subjected to cycles of shaking and rest in a temperature-controlled fluorescence plate reader. The ThT fluorescence is monitored periodically or in "real-time" for any increases due to fibrillization of the $rPrP^{Sen}$ substrate. Typically, multiple replicate reactions are seeded with aliquots of each sample and the fluorescence from replicate wells is monitored over time (Fig. 30.1). In the past 10 years, RT-QuIC assays have been developed for most types of prions, including those of human sporadic, acquired, and genetic prion diseases, as well as animal strains such as chronic wasting disease, and classical and atypical forms of BSE and scrapie (e.g., Wilham et al. 2010; Elder et al. 2013; Henderson et al. 2013; Haley et al. 2013, 2018, 2020; Orru et al. 2015a, b; Henderson et al. 2015; Masujin et al. 2016; Ferreira et al. 2021). A variety of biospecimens have been used successfully, including brain, cerebrospinal fluid (CSF) (Atarashi et al. 2011; Wilham et al. 2010), and peripheral tissues such as nasal lavages (Bessen et al. 2010) and brushings (Bongianni et al. 2017; Orru et al. 2014), skin (Orru et al. 2017; Wang et al. 2019a; Mammana et al. 2020), lymphoid tissue (Haley et al. 2014; Favole et al. 2021), placenta (Luk et al. 2021), feces (John et al. 2013; Cheng et al. 2016a) and muscle (Shi et al. 2013; Orru et al. 2018; Honda et al. 2021; Li et al. 2021). This assay format has proven to be versatile and robust such that dozens of laboratories worldwide currently use it for research, diagnostic, and surveillance purposes (Cramm et al. 2016; McGuire et al. 2016; Orru et al. 2020).

30.3 RT-QuIC for Human Prion Disease Diagnostics

Over the past decade, human Creutzfeldt–Jakob disease (CJD) diagnosis has been improved markedly by RT-QuIC testing [reviewed in Vascellari et al. 2022; Zanusso et al. 2016; Hermann et al. 2021; Cazzaniga et al. 2021]. Otherwise, *antemortem* CJD diagnosis can be problematic, relying on a battery of tests such as clinical signs, EEG, MRI, and assays for CSF markers such as the 14-3-3 and tau proteins that are not fully specific for prion disease (Zanusso et al. 2016; Hermann et al. 2021). Atarashi and colleagues first demonstrated in analyses of CSF samples from living sporadic CJD (sCJD) and non-CJD patients that their original RT-QuIC assay had >80% sensitivity and 100% specificity in identifying CSF specimens from CJD patients (Atarashi et al. 2011). Initial RT-QuIC assays could detect up to 10^{10}-fold dilutions of sporadic CJD (sCJD) brain tissue containing ~1 fg of PrP^{Res} while maintaining specificity with respect to a variety of non-CJD brain tissue controls (Atarashi et al. 2011; Peden et al. 2012). Consistent with the initial Atarashi et al. results, McGuire et al. achieved 91% sensitivity and 98% specificity in the analysis of a large panel of antemortem CSF specimens from human sCJD cases and

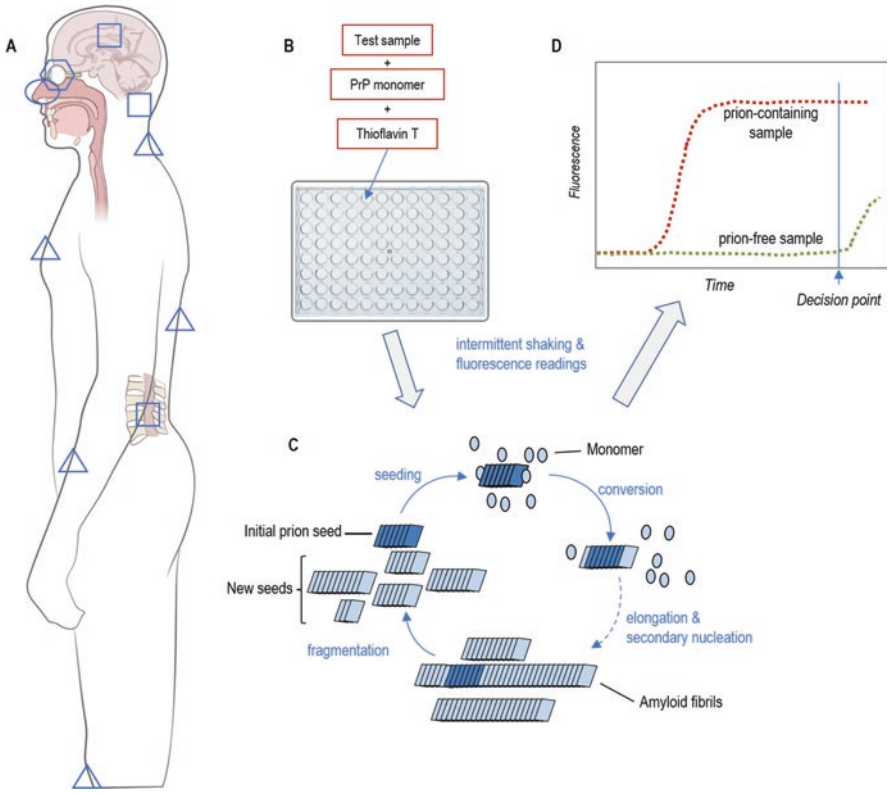

Fig. 30.1 RT-QuIC assay schematic. (**a**) Human biospecimens that can be tested by RT-QuIC (*squares* indicate CNS-associated tissues such as brain and cerebrospinal fluid; *triangles* indicate skin at different locations on the body; *hexagon* indicates various eye tissue components; and *oval* indicates olfactory mucosa (OM) samples). (**b**) Main components of the RT-QuIC assay. (**c**) Diagram of the RT-QuIC prion seed amplification process. (**d**) Representation of RT-QuIC results from prion-contaminated and prion-free samples. *Decision point* refers to the reaction time at which binary calls of positive versus negative reactions should usually be made, that is, before reactions seeded with appropriated matched prion-free negative control specimens begin to show increases in fluorescence due to spontaneous (unseeded) nucleation and fibrillization of the rPrP substrate

controls (McGuire et al. 2012). Analysis of another large panel of CSFs by Lattanzio et al. showed an overall sCJD diagnostic sensitivity of 82% and a specificity of 99% but noted poorer sensitivity for the rarer PrP^{Sc} type 2 sCJD subtypes (Lattanzio et al. 2017). Orru and colleagues developed a faster and more sensitive "second generation" RT-QuIC assay for CSF that showed, in a side-by-side comparison, substantially improved diagnostic sensitivity while maintaining the same high specificity (Orru et al. 2015c; Groveman et al. 2017). Extensive further studies and two international ring trials have now shown a high degree of concordance in the analyses of sCJD CSF with various RT-QuIC assay permutations (Bongianni et al. 2017;

McGuire et al. 2016; Orru et al. 2020; Hermann et al. 2021; Rhoads et al. 2020; Foutz et al. 2017; Franceschini et al. 2017).

Alternative biospecimens have also shown promise for sCJD diagnosis by RT-QuIC including olfactory mucosa (Bongianni et al. 2017; Orru et al. 2014), skin (Orru et al. 2017; Wang et al. 2019a; Mammana et al. 2020; Honda et al. 2021; Ding et al. 2021; Xiao et al. 2021), and skeletal muscle (Honda et al. 2021). Testing of an initial panel of sCJD olfactory mucosa and CSFs from the same sCJD patients yielded a combined 100% diagnostic sensitivity (Bongianni et al. 2017). An international ring trial again indicated high reproducibility between five laboratories (Orru et al. 2020). Similarly, high diagnostic accuracies have been reported when RT-QuIC testing was applied to skin samples from sCJD patients (Orru et al. 2017; Mammana et al. 2020; Xiao et al. 2021). The evidence of CJD prions in the skin has raised concerns about skin as a source of infection (Orru et al. 2017; Nihat and Mead 2018; Starling 2018); however, to our knowledge, the transmission of CJD between humans by casual contact has not been demonstrated.

A further permutation of RT- QuIC assay employs a bank vole $rPrP^{Sen}$ substrate, allowing it to detect prion seeding activity associated with multiple genetic prion diseases associated with mutations of the human prion protein (PRNP) gene. At least 28 different prion strains can be detected using bank vole RT-QuIC. Bank vole and other $rPrP^{Sen}$ substrates can sometimes give strain-specific amplification kinetics and/or conversion products, allowing discrimination between sporadic and variant CJD (Orru et al. 2015a) or classical versus atypical bovine and sheep prion strains (Orru et al. 2015b).

However, in the case of genetic prion disorders, the detection of prion seeds in CSF can be more challenging. While the RT-QuIC can efficiently detect E200K associated prions, for example, the sensitivity for Fatal familial insomnia (FFI) and several types of Gerstmann-Sträussler-Scheinker (GSS) has been lower (Franceschini et al. 2017; Wang et al. 2019b; Mok et al. 2021).

30.4 RT-QuIC for vCJD

Variant CJD (vCJD) is thought to have originated from human consumption of BSE-tainted meats (Hill et al. 1997). On average, this acquired form of prion disease affects younger individuals relative to those with sCJD and has in a few cases been transmitted via blood transfusion (Llewelyn et al. 2004; Herzog et al. 2004). RT-QuIC detection of human brain-derived vCJD seeding activity has been reported both using bank vole (Orru et al. 2015a) and chimeric hamster-sheep $rPrP^{Sen}$ (Orru et al. 2011). Whereas RT-QuIC can be highly sensitive in detecting vCJD seeds in brain tissue, only one study has reported detection in CSF, with positive results from only one of the two cases tested, both of whom were sampled *post-mortem* (Mok et al. 2021).

30.5 Quantitation by RT-QuIC

Approaches to measuring prion seeding activity by RT-QuIC include end-point dilution titrations and comparisons of lag phases. With appropriately matched types of samples and reaction conditions, lag phases tend to be inversely proportional to seed concentration within individual experiments, especially at higher seed concentrations (Wilham et al. 2010; Henderson et al. 2015; Shi et al. 2013; Peden et al. 2012). Although lag phases are simpler to measure than end-point dilutions, they can be variable between experiments and influenced by factors other than seed concentration such as differences in sample matrix components. This can be a particular problem at the low concentrations found in many diagnostic specimens such as CSF for which lag phase variance can be more marked. In such situations, the end-point dilution approach can provide a more accurate assessment of seed concentrations. End-point dilution data are typically processed using the Spearman-Kärber algorithm to obtain estimates of the sample dilution giving positive reactions in 50% of replicate reactions, that is, the 50% seeding dose or SD_{50} (Wilham et al. 2010). Back calculations then establish the SD_{50}s per unit of the original specimen. Multiple applications of end-point dilution RT-QuIC to 4 independent scrapie-infected hamster brain tissue homogenates demonstrated consistent SD_{50} per g brain determinations of 10^{11}–10^{12}, which were ~ 1 log higher than the 50% lethal dose (LD_{50}) per g measured by end-point dilution bioassay in corresponding hamster bioassays (Wilham et al. 2010).

End-point dilution RT-QuIC analyses also allowed the first in vitro measurements of seeding activities in the CSF (~10^5 SD_{50}/ml) and nasal lavages (10^3–10^6 SD_{50}/ml) from prion-infected hamsters (Bessen et al. 2010). Such substantial levels of seeding activity, and infectivity (Bessen et al. 2010), in nasal fluids is consistent with the detection of PrP^{Res} in the olfactory neuroepithelia of hamsters (Bessen et al. 2010) and humans (Zanusso et al. 2003).

We have used end-point dilution RT-QuIC to assess the time course of CSF prion seeding activity accumulation in the hamster model (Orru et al. 2012). After intracerebral inoculation, seeding activity appeared in CSF within a day and then decreased for several days, presumably due to clearance of the inoculum. Soon thereafter seeding activity climbed ~100-fold before plateauing at ~30 days, prior to the onset of clinical signs at 60 days. In contrast, after intratongue inoculations, a model of peripheral inoculation route, seeding activity was first detected in CSF near the onset of the clinical phase at ~85 days. This time point was well after seeds had accumulated too much higher concentrations in the brain tissue. These results raise the possibility that, for TSE infections originating in peripheral sites, there may be insufficient accumulation of seeding activity in the CSF to allow preclinical detection with our current RT-QuIC assay.

30.6 Combining Prion Capture and RT-QuIC

Sample components such as lipids (Hoover et al. 2017), proteins (Green 2019), and red or white blood cell contaminations (Cramm et al. 2016; Foutz et al. 2017) have been identified as inhibitors of RT-QuIC assays. With such samples, especially those with low prion titers, it can be helpful to isolate and concentrate PrP^{Sc} seeds from such inhibitors. One approach has been to incorporate a front-end PrP^{Sc}-selective immunoprecipitation step using the 15B3 monoclonal antibody in what we have called enhanced (e) QuIC (Orru et al. 2011). Another useful approach to capturing prions for RT-QuIC analysis is iron oxide magnetic extraction (IOME) (Denkers et al. 2016).

When applied to the detection of human CJD brain homogenate spiked into human plasma samples, eQuIC was several orders of magnitude more sensitive than the application of RT-QuIC alone using comparable brain homogenate dilutions in a non-plasma buffer. Up to 10^{14}-fold dilutions of vCJD brain homogenates in 0.5 ml plasma, containing ~1 ag of PrP^{Res} were detected (Orru et al. 2011). This is ~10,000-fold more sensitive than previously reported assays for dilutions of vCJD brain homogenate (Edgeworth et al. 2011) and is similar to the sensitivity limit for hamster PrP^{Res} using serial PMCA (Saa et al. 2006). In subsequent tests, we have found that the eQuIC sensitivity varies when similar CJD brain homogenate spikes are diluted in different normal human plasma lots (unpublished data). Nonetheless, sensitivities in the 1–100 ag range have also been achieved in eQuIC assays of sheep, hamster and murine scrapie PrP^{Res} diluted into sheep, human, and murine blood plasma, respectively (C.D. Orrù, A. Hughson, and S. Vascellari, unpublished data; (Vascellari et al. 2012)). eQuIC also has readily detected prion seeding activity naturally present in the blood of scrapie-infected hamsters (Orru et al. 2011), as indicated by the discrimination of plasma samples from infected and uninfected controls. IOME has aided the detection of endogenous prions not only in blood fractions but also in other challenging specimens such as saliva, urine, feces, and skin punches (Ferreira et al. 2021; Henderson et al. 2017; Davenport et al. 2018a; Davenport et al. 2018b). Unfortunately, from a practical perspective, we and others have encountered inconsistencies in the prion capture capabilities of once commercially available antibody 15B3 and iron oxide bead preparations. Further work will be required to understand and minimize such inconsistencies in future applications.

30.7 Technical Considerations

Ongoing studies with RT-QuIC assays continue to expand applications and better define the influences of various reaction conditions and parameters (e.g., Cramm et al. 2016; Orru et al. 2016; Metrick et al. 2019; Hwang et al. 2021). For example, a study of the long-term reproducibility of the RT-QuIC (Orru et al. 2016) revealed that despite the use of a variety of batches of materials over time, results can remain

remarkably consistent. However, we and many other laboratories have encountered occasional technical difficulties in even our routine assays. The sources of such problems have not always been clear and deserve further study. Many factors are influential. For example, increases in the reaction temperature or shaking speeds may accelerate the reaction's kinetics without compromising specificity. On the other hand, elongation of shaking periods may reduce prion-seeded reaction times, and continuous shaking can induce false-positive reactions (Orru et al. 2016). Even small variations in the amount of sodium dodecyl sulfate (SDS) present in the reaction may markedly impact the assays (e.g., Vascellari et al. 2012; Orru et al. 2016). Systematic studies have investigated how the ionic composition of the reaction mixture affects a wide variety of RT-QuIC assays (Metrick et al. 2019; Hwang et al. 2021). Such analyses have revealed that specific experimental conditions further improved the RT-QuIC sensitivity, for example, in detecting sCJD seeds in human olfactory mucosal brushings (Metrick et al. 2019).

We would also like to address some additional issues that often arise when RT-QuIC assays are initiated in new laboratories. Most important is the generation of suitable $rPrP^{Sen}$, which can be costly and requires particular care. The main goal, obviously, is to generate $rPrP^{Sen}$ which can be readily seeded by TSE-infected materials but does not spontaneously nucleate and fibrillize in the presence of comparable seed-negative specimens. The more that sensitivity is pushed for example by increasing overall reaction times, the more that spontaneous fibrillization becomes an issue. We assume that there may be multiple methods for preparing appropriate $rPrP^{Sen}$ substrates, but clearly, some types of preparations have not worked well. This problem may often be due to the presence of preformed seeds or nuclei in the $rPrP^{Sen}$ preparations. Strict adherence to the protocols that we and many other groups have published can minimize such problems. Spontaneous fibrillization can also be greatly reduced by optimizing reaction temperature, shaking, SDS, and salt concentrations for specific seed and substrate combinations. We are often asked whether the expensive nickel-NTA beads that are used for $rPrP^{Sen}$ purification can be reused. In our limited experience, it seems that the beads might be reused, albeit with reduced yields, and increased danger of build-up of spontaneously arising seeds on the beads that might slough off into the $rPrP^{Sen}$ preparation. As noted above, a second possible cost-cutting measure is the incubation and shaking of plates in temperature-controlled, programmable plate shakers rather than in much more sophisticated shaking fluorescence plate readers. In this case, frequent fluorescence readings during the course of the reaction would not be practical because they would require repeated interruptions of the shaking cycle to move the plate into a fluorescence plate reader. However, frequent measurements would not be necessary for most routine applications with well-characterized kinetics in which a single reading at the end of the overall reaction should suffice for simple discrimination of positive and negative results (Orru et al. 2016; Cheng et al. 2016b; Kaelber et al. 2019).

30.8 Biosafety Issues

As RT-QuIC is increasingly used in diagnostic settings, biosafety concerns have been raised about handling human biospecimens such as CSF and nasal (OM) brushings, as well as the amplification of prion seeding activity in such samples by as much as a trillion-fold. Raymond et al. approached this question by inoculating humanized transgenic mice with CSF and OM brushings from sCJD patients (Raymond et al. 2020). Whereas 10^2- to 10^5-fold dilutions of sCJD patients' brain tissue caused prion disease in 47/48 inoculated mice, the maximum acutely tolerable doses of insoluble OM pellets only caused evidence of prion disease in 4 out of 28 mice. This indicated that infectivity in these samples is detectable, but at levels that are orders of magnitude lower than in brain tissue. No clinical disease was observed in mice inoculated with *antemortem* CSF samples collected by lumbar puncture. Importantly, no evidence of clinical disease was observed in mice inoculated with sCJD-seeded RT-QuIC assay products. This is likely due to the fact that RT-QuIC seed amplification reaction does not fully recapitulate the refolding of PrP molecules as it occurs in the brain. Notable evidence of this is the much smaller proteinase K-resistant core of RT-QuIC product relative to ex vivo PrP^{Res} (Wilham et al. 2010). Such differences in the size of the core have been noted between highly infectious ex vivo prions and much less (if at all) infectious synthetic PrP amyloids (Kraus et al. 2021; Hoyt et al. 2022).

30.9 Conclusions

Considerable progress has been made in the development of more practical assays for TSE prions. RT-QuIC assays have made important contributions toward improving *antemortem* diagnosis of sCJD in humans. Further work with RT-QuIC assays is needed to fully understand their diagnostic and prognostic implications for diverse human and animal prion diseases. Of particular importance will be improvements in the early detection of incipient prion pathogenesis in carriers of prion disease-linked PRNP mutations. Hopefully monitoring such carriers with prion seed amplification assays such as RT-QuIC will eventually help to optimize times for implementation of treatments as they may become available. Efforts should also be made to extend and simplify RT-QuIC applications to additional types of diagnostic and environmental samples, and to increase the precision of quantitating prion seed concentrations in such samples. Although simple binary determinations of the presence or absence of prion seeds has proven useful diagnostically, an ability to monitor prion seeding activities in individuals over time may be valuable. Further miniaturization of prion seed amplification assays should reduce costs and increase both throughput and quantitative precision by increasing the number of replicate reactions that can be performed in routine practice. Ultimately, the availability of multiple sufficiently sensitive tests for prion detection and TSE diagnostics would be desirable. Diagnoses

of prion disease have dire implications for both individuals and society, emphasizing the need for accurate and practical primary tests as well as complementary confirmatory tests.

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Chapter 31
Protein Misfolding Cyclic Amplification

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Abstract Prion diseases are caused by a conformational conversion of the cellular prion protein (PrP^{C}) to a pathological conformer (PrP^{Sc}). The "prion-only" hypothesis suggests that PrP^{Sc} is the infectious agent that propagates the disease acting as a template for the conversion of PrP^{C}. In 2001, we developed a novel technique, called protein misfolding cyclic amplification (PMCA), which mimics in vitro this pathological process in an accelerated way. Thereby, a minimal amount of PrP^{Sc} can be amplified several million folds, providing an important tool for the diagnosis and investigation of prion biology, and the molecular mechanism of prion conversion. PMCA also offers a great platform for the study and amplification of the protein misfolding process associated with other neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Here we are updating this previously published chapter to incorporate recent advances.

Keywords Prion diseases · Transmissible spongiform encephalopathies · Protein misfolding cyclic amplification · PMCA · Prion transmission · Prion decontamination procedures

31.1 PMCA: A Great Tool to Study Prion Biology

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of fatal disorders that affect both humans and animals. Prions are the proteinaceous infectious agents that are responsible for TSEs. Prions replicate through a

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nucleation-dependent process which is characterized by a long and silent incubation period followed by a rapid clinical phase. Thereby, a minute quantity of the pathological prion protein (PrP^{Sc}) works as a template to induce the conformational conversion of the cellular prion protein (PrP^{C}) to the pathogenic isoform (Prusiner 1998).

In 2001, we described an efficient technique to reproduce prion replication in the test tube in an accelerated manner, which is called protein misfolding cyclic amplification (PMCA) (Saborio et al. 2001). PMCA consists of cycles of incubation and fragmentation of a sample containing small amounts of PrP^{Sc} in the presence of an excess of PrP^{C}. During the incubation step, PrP^{Sc} aggregates grow through recruitment and conversion of PrP^{C} molecules. The following fragmentation phase, which can be done by any mechanical force such as sonication or shaking, is responsible for fragmenting these polymers to create new PrP^{Sc} seeds, which can induce further conversion of the cellular prion protein (Saborio et al. 2001; Soto et al. 2002). This method allows the exponential amplification of PrP^{Sc} in a PCR-like manner and can begin the reaction with the equivalent to a single molecule of PrP^{Sc}, which after amplification can give rise to billions of PrP^{Sc} molecules (Saá et al. 2006a). The principle of PMCA is schematically illustrated in Fig. 31.1.

In the following years, PMCA was improved through automation and the development of serial PMCA (sPMCA) (Fig. 31.2). Thereby, an aliquot of a PMCA sample, already subjected to many cycles of incubation and fragmentation, was diluted into fresh uninfected brain homogenate and subsequently exposed to further PMCA cycles. In this way, minute amounts of PrP^{Sc} can be detected through autocatalytic in vitro amplification, while the original inoculum is continuously diluted (Bieschke et al. 2004; Castilla et al. 2005a). An additional improvement was the addition of Teflon beads, which increase the efficiency and reproducibility of prion amplification (Gonzalez-Montalban et al. 2011).

Further experiments showed that the in vitro-generated prions were fully infectious when injected into wild-type animals (Castilla et al. 2005a; Moudjou et al. 2013). They caused a similar disease with analog biochemical, biological, and structural properties observed in animals injected with brain-derived PrP^{Sc} (Castilla et al. 2005a, 2008b; Weber et al. 2007; Cali et al. 2019; Bistaffa et al. 2021). Studies of the components required to sustain PMCA amplification demonstrated the importance of cellular molecules and cofactors (e.g., nucleic acids, metals, and lipids) for efficient prion amplification (Lucassen et al. 2003; Deleault et al. 2003, 2007; Abid et al. 2010; Makarava et al. 2017; Vanni et al. 2017). Variations in both composition and availability of such cofactors during prion propagation, as well as modifications of the environment of prion replication, can significantly modulate prion strain properties sometimes leading to the onset of significantly different forms of PrP^{Sc} (Fernández-Borges et al. 2018; Makarava et al. 2018). The changes in prion strain properties represent a key issue in the field of prion therapeutic since the emergence of drug-resistant PrP^{Sc} has been reported and this probably explains the failures observed in various treatments trials (Ghaemmaghami et al. 2009; Berry et al. 2013; Burke et al. 2020; Chen and Dong 2021). Moreover, some indications suggest that the drug itself might have a strain-dependent efficacy (Oelschlegel and Weissmann 2013; Bian et al. 2014; Berry et al. 2015). Interestingly, extensive PMCA cycling

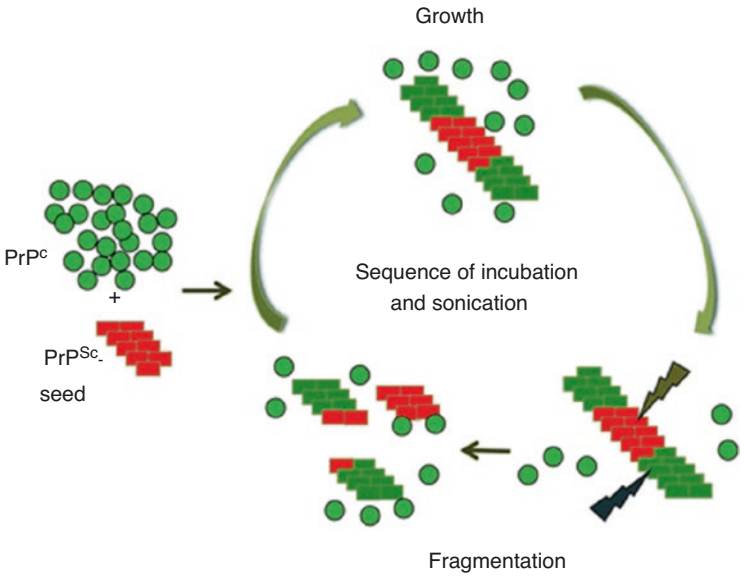

Fig. 31.1 Schematic representation of the PMCA principle. PMCA offers the chance to amplify minute quantities of PrP^{Sc} to a detectable level. In a cyclic manner consisting of two phases (incubation and sonication), PrP^{Sc} seeds from a sample are amplified at the expense of an excess of PrP^{C}. During the incubation phase, polymers of PrP^{Sc} grow by incorporation of PrP^{C}. In the following sonication phase, the large polymers are fragmented to generate multiple smaller PrP^{Sc} seeds for further prion replication

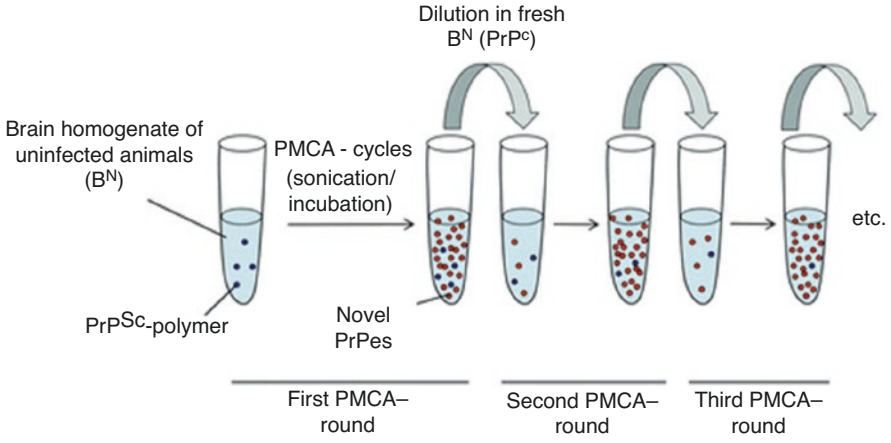

Fig. 31.2 Schematic design of serial PMCA. An aliquot of a PMCA sample, previously exposed to several PMCA cycles of incubation and sonication, is diluted in fresh brain homogenate and exposed to further PMCA cycles. Through sequences of serial PMCA rounds, the inoculum will be infinitely diluted and, in this way, prions can be maintained replicating indefinitely in vitro

allows the de novo formation of infectious prions mimicking the sporadic appearance of the disease (Deleault et al. 2007; Barria et al. 2009). In some of these cases (e.g. using hamster and cervid PrP^C), the prions produced through de novo creation in the test tube produced a new disease phenotype with unique clinical, neuropathological, and biochemical characteristics, never seen in nature (Barria et al. 2009; Chianini et al. 2012; Meyerett-Reid et al. 2017).

An important development was the use of bacterially expressed recombinant prion protein (rPrP) as a substrate for PMCA (Atarashi et al. 2007). Wang and coworkers demonstrated that infectious prions can be generated from rPrP in the presence of synthetic lipids together with total RNA from normal mouse liver. When injected into wild-type mice, they caused a prion disease with similar incubation periods compared to naturally occurring prions. This study strongly supported the protein-only hypothesis (Wang et al. 2010). Recombinant PrP could also be labeled to perform structural studies of the prion protein.

The ability of PMCA to mimic the process of prion conversion in vitro provides great opportunities to analyze many aspects of prion biology, including (1) the biochemical mechanism of prion conversion and replication, (2) the species barrier and prion strain phenomena, (3) the potential role of cellular cofactors in PrP^C to PrP^{Sc} conversion, (4) the sensitive detection of prions for an early diagnosis of patients silently incubating the disease, (5) the evaluation of methods to remove and decontaminate prions, (6) the identification of prions in biological and environmental samples, and (7) the discovery and development of novel drugs to halt the prion conversion process.

31.2 PMCA Applications to Understand the Mechanism of Prion Transmission, Species Barrier, and Strain Phenomena

The unorthodox nature of infectious prions makes it very difficult to understand the unique properties of this novel class of protein-based infectious agents. Among the intriguing features of prions are interspecies prion transmission and prion strain diversity. Interspecies transmission is a process not well understood and limited by the so-called "species barrier" that corresponds to the ability of prions coming from one species to infect only a limited number of other species (Hill and Collinge 2004). This phenomenon is manifested as an incomplete attack rate and prolongation of the time to develop the disease in animals injected with infectious material from another species. The molecular basis of this event is not clear but convincing evidences indicate that the sequence of PrP controls this process; however, the degree of the species barrier cannot be measured only by comparing the sequence of the proteins (Moore et al. 2005). The best way to investigate the species barrier is by infectivity experiments using animal models of the disease. However, these studies are costly and time-consuming because it is necessary to wait for several months

or even years until the animals develop the clinical symptoms. Furthermore, the assessment of the species barrier for prion transmission to humans is compromised by the use of transgenic animal models expressing human PrP^{C}. PMCA can provide an in vitro alternative for studying the species barrier by combining PrP^{Sc} and PrP^{C} from different sources in distinct quantities. In this way, it is possible to quantitatively evaluate the efficiency of the conversion. Several studies confirmed that PMCA exhibits species specificity that faithfully reflects the same transmission barrier observed in animals (Meyerett et al. 2008; Green et al. 2008; Castilla et al. 2008a).

Transmission of sporadic Creutzfeldt–Jakob disease (sCJD) between humans occurred through neurosurgical procedures as a consequence of using inappropriate techniques to sterilize instruments or devices that had been in contact with the brain tissue of sCJD-infected individuals. Treatment with human-derived pituitary growth hormones or cornea or dura mater transplants, derived from infected recipients, also efficiently transmitted the disease (Brown et al. 2000). Conversely to variant CJD (vCJD), numerous studies have shown no evidence of human-to-human transmission of sCJD through the transfusion of blood or plasma, or the administration of plasma-derived therapeutic products (Operskalski and Mosley 1995). Prions can also be transmitted from animals to humans. Epidemiological evidence suggests that among the animal TSEs, only BSE has been transmitted to humans through the consumption of contaminated beef products, generating the variant form of CJD (Will et al. 1996). The zoonotic potential of BSE has been assessed by PMCA using the brains of transgenic mice expressing human PrP as substrates of amplification. The results well recapitulated the species barrier observed in animals (Raymond et al. 1997; Levavasseur et al. 2014).

Another concern is CWD, a disorder affecting mule deer and elk (Sigurdson and Aguzzi 2007) with high incidence in North America that has recently appeared in Europe (Benestad et al. 2016; Mysterud et al. 2019). CWD is highly transmissible within deer and elk populations. The mechanism of transmission is not well understood, but evidence supports the possibility that the disease is spread through direct animal-to-animal contact or as a result of indirect exposure to prions in the environment (e.g., in contaminated food and water sources) (Pritzkow et al., 2021a). Whether the American and European CWD strains are linked with each other or no is still under investigation (Pirisinu et al. 2018; Nonno et al. 2020). Our recent studies using PMCA have provided evidence for European CWD prions to be a different strain from their North American counterparts (Pritzkow et al., 2022; Bian et al., 2021). Transmission of CWD to humans cannot be excluded at this moment and transmissibility studies have been performed in many species to predict the spreading of the disease (e.g., as a consequence of the consumption of CWD-infected meat) (Sigurdson and Aguzzi 2007; Sandberg et al. 2010; Wadsworth et al. 2021). We showed that cervid PrP^{Sc} can induce the conversion of human PrP^{C}, but only after the CWD prion strain has been stabilized by successive passages in vitro or in vivo (Barria et al. 2011). Interestingly, the newly generated human PrP^{Sc} exhibits a distinct biochemical pattern that differs from any of the currently known forms of human PrP^{Sc}. These findings imply that CWD prions have the potential to infect humans and that this ability depends on CWD strain adaptation. Interestingly, our

recent studies have shown that CWD potential transmission into humans is different depending on the species of the animals and geographical location (Pritzkow et al., 2022). Indeed, PMCA studies suggested that North American CWD is more zoonotic than European CWD (Pritzkow et al. 2022). Among the North American CWD, white-tailed deer PrP^{Sc} strains are more zoonotic than mule-deer and elk prions (Barria et al. 2018b; Escobar et al. 2020; Pritzkow et al. 2022).

An intriguing feature of prions that has been often used against the prion hypothesis is the existence of prion strains (Soto 2011). Nearly all TSEs are known to exhibit various strains characterized by different incubation periods, clinical features, and neuropathology (Morales et al. 2007). In traditional infectious diseases, different strains generally arise from mutations or polymorphisms in the genetic makeup of the infectious agent. To reconcile the infectious agent composed exclusively of a protein with the strain phenomenon, it has been proposed that PrP^{Sc} obtained from different prion strains has slightly different conformation or aggregation states that can faithfully replicate at the expense of the host PrP^{C} (Bessen et al. 1995; Telling et al. 1996; Safar et al. 1998). Various reports have shown that PMCA allows the faithful replication of prion strains in many different species of prions, indicating that all the elements required for strain determination are enciphered in the folding of PrP^{Sc} (Castilla et al. 2008b; Jones et al. 2009; Shikiya and Bartz 2011; Cali et al. 2019).

31.3 PMCA Applications in Prion Detection and Diagnosis

Clinical diagnosis of definite CJD can only be made postmortem by histological analysis of spongiform changes and accumulation of PrP^{Sc} in the brain (Soto 2004). It is important to develop an objective and sensitive test which has the potential to identify infected individuals at presymptomatic stages of the disease.

To date, PrP^{Sc} represents the main component of the infectious agent and is the only disease-specific marker for CJD (Prusiner 1998; Brown et al. 2001; Soto 2004). It is abundant in the brain at late stage of the disease, while minute amounts are present in peripheral tissues and biological fluids, such as lymphoid organs, cerebrospinal fluid (CSF), urine, and blood (Aguzzi 2000; Brown et al. 2001; Wadsworth et al. 2001; Gonzalez-Romero et al. 2008). The latter two fluids represent the best candidates for routine noninvasive diagnosis of sCJD and vCJD (Soto 2004). In this regard, we and others have shown that PMCA enabled the detection of PrP^{Sc} in samples of blood and/or urine from prion-infected hamsters, mice, sheep, and cervids, sometimes even at presymptomatic phases of the disease (Castilla et al. 2005b; Saá et al. 2006b; Gonzalez-Romero et al. 2008; Thorne and Terry 2008; Haley et al. 2009; Tattum et al. 2010; Kramm et al. 2017). In 2014, we demonstrated that PMCA could detect PrP^{Sc} in the urine of patients with vCJD with high sensitivity and specificity (Moda et al. 2014). The appearance of vCJD in subjects who received blood from asymptomatic vCJD patients (Llewelyn et al. 2004; Peden et al. 2004, 2005; Hewitt et al. 2006; Clarke et al. 2007; Gillies et al. 2009; Davidson et al. 2014; Seed

et al. 2018) confirmed that undetectable levels of PrP^{Sc} could be present in the blood of individuals silently incubating vCJD who may never develop clinical symptoms but remaining asymptomatic carriers able to transmit the disease to other individuals (Bishop et al. 2006). These findings prompted us and other researchers to optimize PMCA for the analysis of blood samples collected from vCJD patients. In this regard, Lacroux and colleagues showed that PMCA can detect PrP^{Sc} in the blood of macaques intravenously inoculated with vCJD in their preclinical disease stage. In addition, they detected prions in 3/4 blood samples of patients with vCJD (Lacroux et al. 2014). In 2016, we demonstrated that the PMCA could detect PrP^{Sc} in the blood of 14 vCJD patients with 100% sensitivity and specificity (Concha-Marambio et al. 2016). In the same year, Bougard et al. found that the blood of two asymptomatic vCJD patients (collected 16 and 31 months before the clinical onset of the disease) contained PrP^{Sc} detectable by PMCA (Bougard et al. 2016). Finally, we showed that PMCA enables PrP^{Sc} detection in the blood of macaques as early as 2 months after their peripheral infection with vCJD (Concha-Marambio et al. 2020). The presence of PrP^{Sc} in the blood could be a problem for public health, especially for individuals who routinely rely on the blood supply and blood therapies. Through PMCA, PrP^{Sc} was detected also in the CSF (Barria et al. 2018a; Bougard et al. 2018) and various peripheral tissues (Douet et al. 2017) of vCJD patients thus supporting its wide peripheral distribution in the body.

In contrast to vCJD, PMCA has not yet been successfully optimized to detect high-efficiency PrP^{Sc} associated with sCJD. This has limited the application of the technology to the diagnosis of vCJD. Thanks to the discovery of new PMCA substrates (Watts et al. 2014), initial studies showing the possibility to amplify PrP^{Sc}, although with limited efficiency, from the brain or other peripheral tissues (including the CSF and the olfactory mucosa) of selected subtypes of sCJD, have been published (Redaelli et al. 2017; Bélondrade et al. 2021). We have recently modified the PMCA conditions to reach highly sensitive and specific detection of prions in all forms of sCJD and, indeed, we have been able to detect PrP^{Sc} in the urine of these patients (Pritzkow and Soto, unpublished observations).

These findings suggest that PMCA enables efficient, specific, and rapid detection of prions in a variety of samples, offering a high promise for developing a noninvasive early diagnosis of prion diseases. Serial PMCA has also been used for the detection of different forms of animal prion diseases, including scrapie in hamster, mice and sheep; BSE in cattle; and CWD in cervids. In particular, PMCA allowed the detection of PrP^{Sc} in the brain of presymptomatic hamsters, enabling a clear identification of infected animals as early as 2 weeks after inoculation (Soto et al. 2005). We demonstrated as well the presence of PrP^{Sc} in an experimentally infected cow 32 months postinoculation, that did not show clinical signs and was negative by standard western blot analysis (Soto et al. 2005). Subsequently, we reported that PMCA enables highly sensitive detection of prions in blood samples from experimental scrapie at the symptomatic and presymptomatic stages of the disease (Castilla et al. 2005b; Saa et al. 2006). More recently, PMCA has been optimized for highly efficient detection of CWD prions in the CSF of elk (Nichols et al. 2012),

as well as blood of symptomatic and asymptomatic white-tailed deer (Kramm et al. 2017, 2020).

Early diagnosis is very important for improving therapeutic perspectives, as treatment should start in an early stage, before the appearance of clinical signs and the occurrence of irreversible brain alterations. In addition, it should be possible to screen blood banks, reduce the iatrogenic transmission of the disease, and identify populations at risk (Soto 2004).

31.4 PMCA Applications in the Development of Drugs and Prion Decontamination Procedures

One of the best targets for TSE therapy is the inhibition and reversal of PrP^{C} to PrP^{Sc} conversion. In drug development, it is crucial to have a relevant and robust in vitro assay to screen compounds for activity before testing them in more time-consuming and expensive in vivo assays. PMCA represents a convenient biochemical tool to identify and evaluate the activity of drug candidates for TSE treatment because it mimics in vitro the central pathogenic process of the disease (Moda et al. 2019). Inhibitors and promoters could be tested quickly in different contexts using human, bovine, or cervids prions. The simplicity of the method and the relatively rapid outcome are important features of this type of study. Moreover, the fact that PMCA can be applied to prion conversion in different species provides the opportunity to validate the use in humans of drugs that have been evaluated in experimental animal models of the disease.

In a similar way, the efficacy of devices and procedures to remove infectious prions from biological or environmental samples can be investigated in a rapid and efficient way using PMCA. The fact that PMCA enables to detect quantities of prions several orders of magnitude smaller than infectivity bioassay makes PMCA more effective in studying prion removal procedures. Particularly useful for this type of application is the development of the quantitative PMCA technology, which in addition to detect prions, also permits estimating the concentration of PrP^{Sc} present in the sample (Chen et al. 2010). Various articles have been published using PMCA to evaluate prion inactivation and removal from biological and environmental samples using diverse procedures (Morales et al. 2008; Pritzkow et al. 2011; Saunders et al. 2011; Ding et al. 2012; Belongrade et al. 2016, 2020).

In addition, PMCA has been used to detect prions in a variety of natural and man-made surfaces including stainless steel, plastic, glass, wood, rocks, etc. (Pritzkow et al. 2018), supporting its application on evaluating prion contamination in reusable materials. Finally, PMCA has been also used to detect prion infection in biological materials, such as plants, small invertebrates, and cells (Pritzkow et al. 2015, 2021b; Lyon et al. 2019), which may have application to quality control biological materials for human administration.

31.5 Expanding PMCA Beyond Prion Diseases

As prion diseases, most of the neurodegenerative disorders (e.g., Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Parkinson's disease (PD), multiple system atrophy (MSA), Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD)) are thought to be caused by the brain accumulation of misfolded protein aggregates (Soto 2003; Soto and Pritzkow 2018). Protein misfolding and aggregation in other neurodegenerative diseases also follows a seeding-nucleation model involving the formation of similar intermediates and end products as in TSEs (Soto et al. 2006; Soto 2012). Indeed, acceleration of protein aggregation by the addition of seeds has been convincingly reported in vitro for several proteins implicated in diverse diseases (Krebs et al. 2004). These theoretical considerations suggest that protein misfolding processes have the inherent ability to be transmissible (Soto et al. 2006; Soto 2012). Strikingly, a series of exciting reports, using cellular and/or animal models, have provided evidence suggesting that the transmission of protein misfolding by a prion-like mechanism might be at the heart of the most common neurodegenerative diseases (Meyer-Luehmann 2006; Ren et al. 2009; Frost et al. 2009; Clavaguera et al. 2009; Münch et al. 2011; Mougenot et al. 2012; Morales et al. 2012). The similarities between TSEs and other neurodegenerative diseases in terms of their molecular mechanisms suggest that PMCA might be adapted to amplify the abnormal folding of these proteins as well. Indeed, as early as 2002, we proposed that PMCA might be adapted to amplify and detect misfolded protein aggregates implicated in other neurodegenerative disorders (Soto et al. 2002). In 2014, we adapted the PMCA principle for the detection of minute quantities of amyloid-beta misfolded oligomers in the biological fluids of patients affected by AD (Salvadores et al. 2014). Later on, various groups reported the adaptation of seed amplification to α-synculein, implicated in PD, DLB, and MSA (Fairfoul et al. 2016; Shahnawaz et al. 2017; Groveman et al. 2018). These studies have been followed up by several groups demonstrating the extraordinary ability of the adapted PMCA to detect traces of misfolded α-synuclein in several tissues, including CSF, skin, submandibular gland, skin, and olfactory mucosa of patients, sometimes at the preclinical stage of the disease (Fairfoul et al. 2016; Shahnawaz et al. 2017, 2020; Groveman et al. 2018; van Rumund et al. 2019; Garrido et al. 2019; Manne et al. 2019, 2020a, b; Bongianni et al. 2019; De Luca et al. 2019; Wang et al. 2020; Singer et al. 2020, 2021; Kang et al. 2019; Mammana et al. 2020; Rossi et al. 2020; Iranzo et al. 2021; Perra et al. 2021; Stefani et al. 2021; Donadio et al. 2021; Bargar et al. 2021; Concha-Marambio et al., 2021; Russo et al. 2021). A seed amplification assay based on the PMCA principles was also adapted to detect tau aggregates in the CSF of patients with AD and other tauopathies, including PiD, CBD, and PSP (Saijo et al. 2017, 2020, Mettrick et al. 2020; Tennant et al. 2020) and TDP-43 in the CSF of patients with ALS and frontotemporal dementia (Scialò et al. 2020).

31.6 Concluding Remarks

PMCA was first published in a Nature article in 2001 (Saborio et al. 2001) and is today widely considered as a major breakthrough in science and technology. PMCA enabled for the first time to cyclically amplify the folding and biochemical properties of a protein in a manner conceptually analogous to the amplification of DNA by PCR. PMCA has enabled the generation of infectious prions in vitro providing the strongest proof in favor of the prion hypothesis and has permitted to detect for the first time infectious prions in blood, offering a great possibility for early diagnosis. Over the past 20 years, PMCA has become a widely used and invaluable technique to study the diverse aspects of prions. The PMCA technology has been used by several groups to understand the molecular mechanism of prion replication, the cellular factors involved in prion propagation, the intriguing phenomena of prion strains and species barriers, to detect PrP^{Sc} in tissues and biological fluids, and to screen for inhibitors against prion replication. The impact of PMCA is not only restricted to the replication of prions because it represents a platform technology to amplify the process of protein misfolding of the many proteins in which this mechanism occurs. Misfolded α-synuclein, Aβ, tau, and TDP-43 are considered disease-specific biomarkers for several neurodegenerative diseases and the possibility to exploit the PMCA platform to ultrasensitively detect them in the CSF or other peripheral tissues represents a great revolution for the diagnosis of these pathologies.

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Chapter 32
Seeding Activity of Skin Misfolded Proteins as a Biomarker in Prion and Prion-Like Diseases

Wen-Quan Zou and Zerui Wang

Abstract Prion disease (PrD) and other prion-like diseases, including but not limited to Alzheimer's disease (AD) and Parkinson's disease (PD), are characterized by the accumulation and deposition of misfolded proteins in the central nervous system. The current definitive diagnosis of these diseases relies on the examination of the brain tissues obtained either by biopsy or at autopsy for the misfolded protein aggregates and their related neuropathological changes. But it is either too late or too invasive. Interestingly, some of these misfolded proteins have been observed in the skin tissues by immunohistochemistry (IHC) or immunofluorescence (IF) microscopy such as pathological α-synuclein in PD and tau in AD patients. However, the sensitivity and specificity of IHC/IF are highly variable, and they are also time-consuming and not high throughput. We and others have recently revealed that the seeding activity of prions and α-synuclein can be detected in the skin of patients with PrDs and PD, indicating that the seeding activity of skin misfolded proteins can be a novel biomarker for diagnosis of the diseases. In this chapter, we summarize these findings and highlight the implications of the skin biomarkers in the diagnosis and monitoring of the progression of the neurodegenerative diseases.

Keywords Skin · Seeding activity · Biomarker · Prions · Prion disease · Parkinson's disease · Alzheimer's disease · α-synuclein · Tau · RT-QuIC · PMCA · Immunohistochemistry

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32.1 Introduction

Neurodegenerative diseases are characterized by the accumulation of pathologically misfolded protein aggregates in the central nervous system (CNS). The misfolded neurotoxic proteins are derived from their normal cellular counterparts through a structural transition from α-helixes to β-sheet structures. Prion diseases (PrDs) are a prototype of neurodegenerative diseases. The normal soluble cellular prion protein (PrP^{C}) can be converted into an insoluble misfolded infectious conformer termed PrP^{Sc} under pathological conditions such as PrP genetic mutations, exogenous infection, or unknown reasons (Prusiner 1991). PrP^{Sc} is infectious and possesses seeding activity to propagate itself by recruiting its normal form. Notably, several lines of studies have recently demonstrated that this self-assisted propagation phenomenon is not unique to PrP^{Sc} (Costanzo and Zurzolo 2013; Aguzzi and Lakkaraju 2016; Shamsi et al. 2017). Other misfolded proteins such as amyloid-β (Aβ) and abnormally phosphorylated tau of Alzheimer's disease (AD), and pathological α-synuclein (αSyn^{P}) of Parkinson's disease (PD) have been demonstrated to spread throughout not only within the CNS but also from the peripheral tissues to the CNS in a prion-like manner with seeding activity (Costanzo and Zurzolo 2013; Goedert et al. 2014).

It is known that the key molecular event in the pathogenesis of neurodegenerative diseases is the conversion of the normal cellular proteins into the pathogenic misfolded proteins that are associated with neuronal death. The precise molecular mechanisms underlying the structural conversion and the neuronal toxicity remain unclear. The protein aggregation event that is involved in the structural conversion of proteins is often noticed mostly during middle to late age at the disease onset, even in the case of diseases associated with protein mutations. Several lines of evidence have indicated that neurodegenerative diseases have a long period of prodromal stage. Clinical symptoms and signs may not be noticeable until years, even decades after the onset of protein aggregation in the brain so that an early and effective therapeutic window may be missed. In addition, it has been proposed that neuronal death could be mediated by a loss of function or a toxic gain of function of the misfolded protein aggregates (Bucciantini et al. 2002). Conceivably, developing biomarkers that can track the events of protein misfolding, aggregation, and structural conversion of altered proteins will be critical for the development of early diagnostics and effective therapeutics of the neurodegenerative diseases.

Currently, a definitive diagnosis of neurodegenerative diseases primarily relies on the examination of brain tissues obtained at autopsy or by biopsy (Zerr et al. 2009). Analyses of 14-3-3, Aβ, tau, and α-synuclein in cerebrospinal fluid (CSF) are helpful but not specific and also invasive due to the required spinal puncture for collecting CSF samples. Neuroimaging of the misfolded proteins and neuropathological changes are useful but expensive. It mostly may not be covered by health insurance. The highly sensitive real-time quacking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA) assays have recently been demonstrated to be highly promising (Atarashi et al. 2011; Moda et al. 2014; Orrú

et al. 2015; Candelise et al. 2017; Foutz et al. 2017). They are not only ultrasensitive but also can be very specific since the two assays are mainly based on the detection of the seeding activity of the misfolded protein aggregates, a dominant pathological feature of prion and prion-like proteins. Because of invasive nature of collecting CSF via spinal tap, however, CSF may not be always available. Interestingly, the skin tissues have recently been proved as a potential convenient and less invasive source of early disease biomarkers (Miki et al. 2010; Makrantonaki et al. 2012; Donadio et al. 2014). Moreover, it is worth noting that the skin tissues are extensively innervated and possess a common developmental origin with the CNS. In this chapter, the connection between the skin and the brain and evidence regarding cutaneous markers of several neurodegenerative diseases will be discussed.

32.2 Infectivity and Seeding Activity of Misfolded Protein Aggregates in Neurodegenerative Diseases

Of all neurodegenerative diseases, PrDs are the first that were shown to be associated with a misfolded protein, PrP^{Sc} (Prusiner 1982). Moreover, it has been well demonstrated that PrP^{Sc} is the main, if not only, component of the proteinaceous infectious pathogen. It can propagate and spread from cells to cells, possibly via itself seeding activity without DNA or RNA, through which prion diseases are transmissible from individuals to individuals. In addition, prion diseases affect not only humans but also many species of animals.

Recent studies have revealed that prion-like seeding activity is shared by other misfolded proteins in many neurodegenerative proteinopathies such as Aβ, tau, and αSyn (Costanzo and Zurzolo 2013; Goedert et al. 2014; Bucciantini et al. 2002; King et al. 2012; Prusiner 2013), where the partially folded or misfolded disease-associated proteins recruit their cellular counterparts to form a beta-rich nucleus. The newly formed nucleus functions as a seed to induce conformational changes in their normal protein isoforms to further assemble into large fibrillary aggregates. Mature fibrils subsequently may be subjected to fragmentation to release additional small seeds which act as templates themselves again. Although transmission from individuals to individuals has so far only been reported in PrDs, transmission studies in animals have shown that non-prion misfolded proteins including Aβ, tau, and αSyn from the brain of patients with AD or PD can be transmitted in animal models by intracerebral or peripheral inoculations (Kane et al. 2000; Meyer-Luehmann et al. 2006; Clavaguera et al. 2009; Eisele et al. 2010; Guo et al. 2012; Watts et al. 2011; Liu et al. 2012; Lasagna-Reeves et al. 2012; de Calignon et al. 2012; Kordower et al. 2011; Mougenot et al. 2012; Luk et al. 2012; Watts et al. 2013; Morales et al. 2015). Remarkably, several lines of recent studies have suggested that actual human transmission of non-prion misfolded protein aggregates may have occurred. For instance, it has been shown that transmission of Aβ may result in new diseases such as iatrogenic cerebral amyloid angiopathy and iatrogenic AD following childhood

treatment with cadaver-derived human growth hormone (Jaunmuktane et al., 2015; Purro et al., 2018; Banerjee et al., 2022). This phenomenon could be reminiscent to a procedure that is well known to cause iatrogenic CJD reported previously (Chap. 11).

The seeding activity of prions and prion-like proteins has been mimicked and detected in vitro by PMCA and RT-QuIC assays. The two highly sensitive assays were first used for detection of prion-seeding activity and subsequently adapted for detection of other misfolded proteins. They are mainly based on the seeding model of prion formation in which misfolded proteins in the samples to be determined serve as the seeds, while their normal proteins from either normal mouse brain homogenates or *Escherichia coli* bacteria-derived recombinant forms function as the substrates. Both PMCA and RT-QuIC are able to amplify misfolded proteins from the brain, peripheral tissues, or body fluids of patients with neurodegenerative diseases (Atarashi et al. 2011; Orrú et al. 2015; Saborio et al. 2001; Castilla et al. 2005; Moda et al. this volume; Orrù et al. this volume).

32.3 Association Between the Skin and the Brain

During embryogenesis, neuronal and skin tissues emerge from a common single germ layer known as the ectoderm. Final differentiation to nervous or skin tissues occurs during neurulation 3–4 weeks after fertilization, forming the external ectoderm, neural crest, and neural tube during this stage. The former develops the epidermis while the latter two fate to become nervous tissue (Gilbert 2000). Following full differentiation, the two organs continue keeping communications. Interestingly, expression of neuronal and glial markers SOX9 and EGR2 has been found in human skin fibroblasts (Janmaat et al. 2015). The nerve growth factor RANTES is also present in keratinocytes (Raychaudhuri et al. 2000). Several of neuropeptides, such as vasoactive intestinal peptide, nerve growth factor, and catecholamines, are observed to produce in the skin, and changes in their productions have been implicated in some inflammatory skin conditions (Steinkraus et al. 1993; Steinhoff et al. 2006). It has been long recognized that mental stress is associated with cutaneous symptoms such as swelling, itching, redness, or excessive sweating (Steinkraus et al. 1993; Urpe et al. 2005). Moreover, human fibroblasts can be reprogrammed to neurons and glial cells by transcription factors (Pfisterer et al. 2011; Vierbuchen et al. 2010; Treutlein et al. 2016).

DNA damage has been proposed to play a role in neurodegenerative diseases (Rass et al. 2007). The skin is the first line of physical defense against various environmental damages and subjected daily to a variety of mutagenic insults, of which the UV light is most prominent. Given that the skin is a highly innervated organ (Urpe et al. 2005), conceivably, at least in a subset of patients, it could be the initial site of the neurodegenerative cascades. It raises the possibility of not only the cutaneous presence of diagnostic biomarkers for these diseases but also the skin as a target for prophylactics and therapeutics.

32.4 Skin PrP^{Sc}-Seeding Activity in Prion Diseases

PrP^{Sc} is the causal agent of fatal transmissible PrDs including sporadic Creutzfeldt–Jakob disease (sCJD, the most common human PrDs) in humans as well as scrapie, bovine spongiform encephalopathy (BSE) or mad cow disease, and chronic wasting disease in animals (Prusiner 1998; Das and Zou 2016). sCJD is transmissible via medical or surgical procedures due to contamination by abundant infectious prions in the brain of patients. Notably, some epidemiological studies have also associated sCJD risk with non-neurosurgeries, but experimental evidence for such a link is lacking. PrDs are currently incurable. At the onset of clinical symptoms, permanent brain damages already occurred. The absence of less invasive early diagnostic tests for the disease can result in missing the critical window for prevention and treatments, and low brain autopsy rate due to cultural constraints prevents the surveillance of sCJD that is essential for effective prevention of iatrogenic sCJD transmissions.

Currently, a definitive diagnosis of sCJD is virtually completely dependent on the examination of the diseased brain tissues obtained by biopsy or at autopsy and is impossible at the early preclinical stage, as to date there have been no tissues identified yet showing detectable PrP^{Sc} at the preclinical stage of sCJD. The recently developed CSF-based RT-QuIC analysis provides an alternative approach, but it is only for the diagnosis of clinical-stage sCJD (Foutz et al. 2017; Sano et al. 2013; Cramm et al. 2015). Moreover, lumbar puncture for CSF sampling is not feasible for all patients because of some contraindications and complications in certain conditions. Besides, not all CSF specimens from patients with prion diseases are RT-QuIC positive (Foutz et al. 2017; Bongianni et al. 2017; Groveman et al. 2016). Although skin samples may not replace CSF in routine RT-QuIC-based PrDs diagnostics, they may be helpful when prion disease is suspected but CSF is either unavailable or RT-QuIC negative. In addition, RT-QuIC or serial PMCA (sPMCA) analysis of urine and blood has not been working well for sCJD patients. For instance, PrP^{Sc} was detectable in the urine or blood from patients with variant CJD (vCJD, a distinct CJD strain that originated from mad cow disease) only but not sCJD (Moda et al. 2014; Notari et al. 2012; Bougard et al. 2016). These findings seem to be consistent with the observation that prion transmission between individuals through blood transfusion has been reported only in vCJD but not in sCJD (Llewelyn et al. 2004). In addition, nasal brushings were reported to be used for RT-QuIC analysis of PrP^{Sc} in sCJD patients (Orrú et al. 2014). However, the technique for sampling the nasal brushings is not widely used clinically as it requires highly trained personals with special license and device; moreover, the FDA approval of use of the device for collecting nasal brushings has not been reported.

PrP^{C} is detectable in the skin of normal individuals and patients affected by dermatological disease (Pammer et al. 1998). The involvement of skin damage in PrDs was first noticed in scrapie-infected sheep and goats, with repetitive scratching flanks resulting in sores and lesions, a prominent symptom of scrapie in sheep and goats (Foster et al. 2001). PrP^{Sc} has been occasionally detected in the skin tissues of

prion-infected individuals. For instance, CWD prions have been reported in the shed velvet of developing deer antler (Angers et al. 2009). Moreover, PrP^{Sc} was detected by western blotting and immunohistochemistry in the skin of animals with experimental and natural scrapie (Thomzig et al. 2007). The proteinase K (PK)-resistant PrP^{Sc} was detectable in the PrP^{Sc}-enriched skin preparation by western blotting in a single vCJD patient (Notari et al. 2010). In addition, prion infectivity was reported in the skin of BSE-affected kudu (Cunningham et al. 2004).

Although the level of PrP^{Sc} is expected to be substantially lower in the skin compared to the brain, its repeated identification across species raises the potential diagnostic utility of skin prions, especially with the advancement of the highly sensitive RT-QuIC and PMCA assays. In collaboration with the laboratories of Dr. Byron Caughey and Qingzhong Kong, our RT-QuIC assay revealed that prion-seeding activity can be detected in the autopsy skin tissues of sCJD and vCJD and transmission study with two lines of humanized transgenic (Tg) mice discovered that skin tissues from sCJD are infectious (Orrù et al. 2017). These findings raise two implications. Since skin tissues contain the infectious PrP^{Sc} in CJD patients, it is possible that even non-neurosurgeries conducted in CJD patients may pose a risk for iatrogenic transmission through contaminated reusable surgical instruments. sCJD is known to be transmissible iatrogenically via CNS-associated surgical operations (Brown et al. 2006, 2012, this volume). Notably, some epidemiological studies have also suggested that the risk of sCJD is associated with a history of having various non-CNS-related surgeries, the number of surgeries, and the age at the time of the first surgery (Collins et al. 2006; Ward et al. 2008; de Pedro Cuesta et al. 2012, 2014), implying that sCJD is also transmissible via non-CNS-associated operations. Moreover, statistically significant odds ratios were obtained for injury to, or surgery on, the head, face or neck, and trauma to other parts of the body (Davanipour et al. 1985). But a few studies reported little correlation between surgeries and sCJD incidence (Zerr et al. 2000; Hamaguchi et al. 2009). It has been proposed that there is no safe dose of prions drawn from a mathematic simulation with data of 4338 mice inoculated at doses ranging over ten orders of magnitude (Fryer and McLean 2011). Therefore, the possibility of sCJD skin PrP^{Sc} being a realistic source of iatrogenic transmission must be rigorously tested. Currently, we are evaluating the potential of transmission of CJD via skin prion-contaminated surgical instruments with animal models.

In addition to the potential iatrogenic transmission by skin prions, our findings also imply that skin prion-seeding activity can be a novel biomarker for the diagnosis of PrDs. Of 21 cases covering 9 sCJD subtypes, our study yielded the sensitivity of skin prion-seeding activity of 92% in lower back, 88% in apex, and 94% in area near ear, respectively, while the specificity was 100% in all these skin areas from 7 non-CJD controls (Orrù et al. 2017). We also revealed that the average PrP^{Sc}-seeding activity in sCJD skin was ~10^3- to 10^5-fold lower than in the corresponding brain tissue samples. Although PrP^{Sc} in both sCJD and vCJD skin samples was amplified by bank vole PrP^{C} substrate, the lag phase was much shorter in sCJD than in vCJD; moreover, with the hamster PrP^{C} substrate, the sCJD but not vCJD skin samples gave positive reactions (Orrù et al. 2017). It suggests that detection of skin

prion-seeding activity by RT-QuIC assay may be able to differentiate different prion strains.

Our finding of the detectable skin prion-seeding activity in prion-infected subjects was subsequently confirmed by other groups. Mammana et al. investigated 71 punch biopsy skin samples from 35 CJD patients, including 5 assessed in vitam (Mammana et al. 2020). Their results verified the high value of skin PrP^{Sc}-seeding activity by RT-QuIC assay for CJD diagnosis (89% sensitivity and 100% specificity), supporting its use in clinical practice. Moreover, they noticed that prion-seeding activity in the skin was higher in sCJD subtypes linked to the sCJDV2 strain (VV2 and MV2K) than in typical sCJDMM1, despite in a limited number of cases (Mammana et al. 2020). Xiao et al. studied the paired skin and CSF samples from 51 living patients including 34 probable sCJD, 14 non-CJD, and 3 genetic PrD by RT-QuIC assay using recombinant hamster PrP90–231 as the substrate (Xiao et al. 2021). They found a positive skin prion-seeding activity in 91.2% (31/34) probable sCJD and 1 genetic CJD^{T188K} (gCJD) cases and a negative activity in 85.7% (12/14) non-CJD patients. In contrast, the sensitivity of CSF RT-QuIC assay was much lower (14/34 probable sCJD patients) compared to that of skin samples. They concluded that skin RT-QuIC assay is of the higher sensitivity and specificity for diagnosis of Chinese probable sCJD compared to CSF and highlighted that skin prion-seeding activity is a reliable biomarker for premortem diagnosis of human PrDs (Xiao et al. 2021). Honda et al. compared prion-seeding activity in different samples of an autopsied sCJD case and showed a difference in SD_{50} of prion-seeding activity between the brain and skin [9.5 (frontal cortex) vs. 7.88 (scalp) vs. 5.25 (abdominal skin)], similar to our observation (Orrù et al. 2017).

To understand the feasibility of using skin for preclinical diagnosis, by using the ultrasensitive serial PMCA (sPMCA) and RT-QuIC assays, we further investigated skin samples from hamsters and humanized transgenic mice (Tg40h) at different time points after intracerebral inoculation with 263 K and sCJDMM1 prions, respectively (Wang et al. 2019). Remarkably, skin PrP^{Sc} was detectable by sPMCA as early as 2 weeks post inoculation (wpi) in hamsters and 4 wpi in Tg40h mice; the earliest skin prion-seeding activity was detected by RT-QuIC assay at 3 wpi in hamsters and 20 wpi in Tg40h mice. In contrast to the 263 K-inoculated animals, mock-inoculated animals showed detectable skin/brain PrP^{Sc} only after long cohabitation periods with scrapie-infected animals (Wang et al. 2019). We believe that our study provided the proof-of-concept evidence that skin prions could be a biomarker for preclinical diagnosis of PrDs.

To determine whether skin prion-seeding activity can be a biomarker for monitoring the therapeutic efficacy of PrDs, we investigated changes in skin prion-seeding activity in transgenic (Tg) mice expressing hamster PrP^{C} infected with the 263 K prion and then treated with TC-5RW, a compound of cellulose ethers (CEs). CEs have been widely used as food and pharmaceutical additives and recently have been shown to prolong the lifespan of prion-infected mice and hamsters (Arca et al. 2018; Alshehri et al. 2016; Teruya et al. 2016; Abdulrahman et al. 2019; Hannaoui et al. 2020; Nishizawa et al. 2019). Interestingly, the prion-seeding activity became undetectable in the skin tissues of TC-5RW-treated Tg mice by both sPMCA and

RT-QuIC assays, whereas such prion-seeding activity was readily detectable in the skin of untreated mice (Ding et al. 2021). Our results provide the first evidence that the level of prion-seeding activity in the skin may serve as a useful biomarker for assessing the therapeutic efficacy of compounds in a clinical trial of prion diseases.

In sum, skin prion-seeding activity is detectable in patients with sCJD and vCJD and it may be a biomarker for early diagnosis and monitoring the therapeutic efficacy of PrDs.

32.5 Skin α-Synuclein Seeding Activity in Parkinson's Disease and Synucleinopathies

PD is the second most common neurodegenerative disease, affecting about 1 million people in the United States and approximately 7–10 million patients worldwide. The molecular hallmark of PD is the deposition of the pathological misfolded αSyn (αSyn^P) aggregates as Lewy bodies or Lewy neurites in the brain (Spillantini and Goedert 2018; Braak et al. 1999). Currently, a definitive diagnosis of PD relies on the detection of the αSyn^P-containing Lewy bodies or Lewy neurites in autopsied brain tissues (Braak et al. 1999; Dickson et al. 2009). To date, there are no reliable and specific biomarkers that can be used for diagnosis, monitoring disease severity, or evaluating therapeutic efficacy in peripheral tissues or body fluids (Chen-Plotkin et al. 2018). Therefore, searching for biomarkers in readily accessible specimens for PD is an unmet need.

The recent CSF RT-QuIC and PMCA analyses of αSyn^P are promising for the diagnosis of PD (Fairfoul et al. 2016; Shahnawaz et al. 2017; Groveman et al. 2018). However, lumbar puncture (spinal tap) to collect CSF samples is not normally included in the routine diagnostic process outside of clinical research due to the invasive nature of the CSF collection process itself, which significantly limits its application. Moreover, it is not feasible for all patients given the contraindications and complications in certain conditions. In addition, not all CSF specimens from patients with PD are RT-QuIC or PMCA positive (Fairfoul et al. 2016; Shahnawaz et al. 2017; Groveman et al. 2018). It has been reported that the blood contamination of CSF during its collection often generates false negative results due to blood-derived unknown inhibitors that may generally reduce the seeding activity of misfolded proteins (Foutz et al. 2017; Cramm et al. 2016). Lastly, it is unclear whether and how CSF can be collected for early detection of αSyn^P during routine visits. In contrast to CSF, skin punches are more amendable to collection at regular clinics. On the other hand, the αSyn^P detection in other more easily accessible body fluids, such as blood, saliva, or urine, has not been established. As mentioned earlier, the blood has also been reported to contain inhibitors for RT-QuIC assay of specimens from CJD (Cramm et al. 2016). Therefore, it is most likely that the skin is currently the best option among the easily accessible specimens for PD diagnosis by RT-QuIC and PMCA assays. Indeed, as mentioned above, we have successfully detected

PrP^{Sc} in skin of patients with CJD (Orrù et al. 2017) and infected rodents (Wang et al. 2019; Ding et al. 2021). The ultrasensitive RT-QuIC and PMCA assays should enable premortem and even preclinical skin detection of αSyn^{P} in PD patients, thus providing an opportunity for a disease-modifying therapy of PD.

The pathologically phosphorylated αSyn^{P} deposits have been observed in the peripheral tissues including colon, salivary gland, and skin in PD patients (Donadio et al. 2014; Braak et al. 2006; Beach et al. 2014; Doppler et al. 2014). Moreover, it even has been proposed that the deposition of αSyn^{P} in the peripheral tissues may precede the brain pathology and contribute to autonomic dysfunction in PD (Braak et al. 2003; Braak and Del Tredici 2017). Previous studies showed skin αSyn^{P} deposits within autonomic and sympathetic nerve endings of PD patients by immunofluorescence (IF) microscopy and/or IHC (Donadio et al. 2014, 2017; Doppler et al. 2014; Zange et al. 2015; Gibbons et al. 2016). As autonomic dysfunction associated with skin misfolded αSyn^{P} deposition may appear long before clinical symptoms in PD patients and individuals at-risk (Gibbons et al. 2016; Antelmi et al. 2017), it is conceivable that cutaneous αSyn^{P} is a good candidate biomarker for early diagnosis and monitoring disease progression. However, IF or/and IHC examination of skin phosphorylated αSyn^{P} aggregates has been challenging because of highly variable sensitivity from 0% to 100%, which may result from methodological variability (antibody specificity, tissue fixation, antigen retrieval, proximal or distal skin areas, and tissue sectioning/embedding) (Lee et al. 2017; Visanji et al. 2017). In contrast, the RT-QuIC assay can be fully automated with higher sensitivity (positive signal in highly diluted samples), high throughput (in 96-well format), and suitable for multiple types of specimens such as tissue homogenates and body fluids monitored by a fluorescence plate reader in a real-time format. Moreover, it can be standardized and measured quantitatively for the skin αSyn^{P}-seeding activity from different stages of PD patients with varying disease severity.

In addition to PD, other non-PD synucleinopathies including dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) are also characterized by the deposition in brain of αSyn^{P} aggregates in neuronal and glial cells, respectively. Few previous studies have observed phosphorylated αSyn^{P} deposits in skin nerve fibers by IF in small numbers of DLB patients (Donadio et al. 2017). Doppler et al. reported the same positive results in the skin of MSA patients (Doppler et al. 2015), which was not confirmed by others (Zange et al. 2015). Similarly, the contradictory observations between different groups may be attributable to the inconsistent sensitivity and other variables associated with IF or/and IHC.

We extended our finding of skin prion-seeding activity detected by RT-QuIC and PMCA assays to skin misfolded αSyn in PD. We first conducted the retrospective and prospective diagnostic study to evaluate autopsy and biopsy skin samples from neuropathologically and clinically diagnosed patients with PD and non-PD controls (Wang et al. 2020). Autopsy skin samples were obtained at three medical centers and biopsy samples were collected from three institutions, respectively. Based on neuropathological or clinical diagnoses, 57 cadavers with synucleinopathies and 73 cadavers with non-synucleinopathies as well as 20 living patients with PD and 21 living controls without PD were examined. Specifically, cadavers and participants

included PD, LBD, MSA, AD, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and non-neurodegenerative controls (NNCs). A total of 160 autopsied skin specimens from 140 cadavers (85 male cadavers [60.7%]; mean [SD] age at death, 76.8 [10.1] years) and 41 antemortem skin biopsies (27 male participants [66%]; mean [SD] age at time of biopsy, 65.3 [9.2] years) were studied. RT-QuIC analysis of αSyn^P-seeding activity in autopsy abdominal skin samples from 47 PD cadavers and 43 NNCs yielded 94% sensitivity (95% CI, 85–99) and 98% specificity (95% CI, 89–100). As groups, RT-QuIC assay also exhibited 93% sensitivity (95% CI, 85–97) and 93% specificity (95% CI, 83–97) among 57 cadavers with synucleinopathies (PD, LBD, and MSA) and 73 cadavers without synucleinopathies (AD, PSP, CBD, and NNCs). PMCA revealed 82% sensitivity (95% CI, 76–88) and 96% specificity (95% CI, 85–100) with autopsy abdominal skin from PD cadavers. With the posterior cervical and leg skin biopsy samples from patients with PD and non-PD controls, RT-QuIC yielded the sensitivity and specificity of 95% (95% CI, 77–100) and 100% (95% CI, 84–100), respectively, while PMCA had 80% (95% CI, 49–96) and 90% (95% CI, 60–100), respectively (Wang et al. 2020). This study provided proof-of-concept that skin αSyn^P-seeding activity may be a novel biomarker for antemortem diagnoses of PD and other synucleinopathies.

While our manuscript submitted to the JAMA Neurology in March 2020 was in press, a similar study on RT-QuIC analysis of αSyn^P in autopsy frozen (25 cases of PD and 25 non-PD controls) and formalin-fixed paraffin-embedded (12 cases of PD and 12 non-PD controls) skin tissues from cadavers submitted to the Movement Disorders in May 2020 was published 2 weeks earlier than our study (Manne et al. 2020). The observation by Manne et al. was consistent with our findings showing a similar sensitivity (96%) and specificity (96%) in the autopsy skin samples of PD and non-PD controls (Manne et al. 2020). Kuzkina et al. examined skin punch biopsy from multiple sites (neck, lower back, thigh, and lower leg) of patients with clinically diagnosed PD ($n = 34$) and controls ($n = 30$) (Kuzkina et al. 2021). Each skin biopsy sample from a total of 198 skin tissues was divided into two parts for RT-QuIC assay in two independent laboratories. The skin αSyn RT-QuIC endorsed the clinical PD diagnosis with an 88.9% accuracy and a high degree of inter-rater agreement between the two laboratories (92.2%) (Kuzkina et al. 2021). They also noticed that higher αSyn-seeding activity was shown in patients with longer disease duration and more advanced disease stage and correlated with the presence of rapid eye movement sleep behavior disorder, cognitive impairment, and constipation (Kuzkina et al. 2021). We also examined biopsy skin samples from clinically diagnosed PD patients and non-PD controls and compared the IF and RT-QuIC assay with the same skin biopsy samples (Donadio et al. 2021). In this study, 90 patients fulfilling clinical and instrumental diagnostic criteria for synucleinopathies and non-synucleinopathies were recruited. In total, 24 patients with mainly peripheral neuropathies were used as controls. Patients underwent skin biopsy for IF and RT-QuIC analyses; CSF was blindly examined in patients subjected to spinal tap for diagnostic purposes. Both IF and RT-QuIC exhibited high sensitivity and specificity in discriminating synucleinopathies from non-synucleinopathies and controls, while

IF showed higher diagnostic accuracy. IF had a good level of agreement with RT-QuIC in both skin and CSF in synucleinopathies (Donadio et al. 2021). The blind skin RT-QuIC assay yielded 86% sensitivity and 80% specificity in determining synucleinopathies, lower than IF. This was because 9 out of 38 patients with non-synucleinopathies exhibited false positive αSyn-seeding activity. In addition, 3 out of 24 healthy control patients had a positive αSyn reaction (Donadio et al. 2021). The exact reasons for the discrepancy between skin RT-QuIC and IF in some of non-synucleinopathies and control patients remain to be determined. The two approaches seem to determine two different aspects of the αSyn^P with IF detecting phosphorylated form but RT-QuIC measuring seeding activity. A possibility needs to be excluded in the future that there are unphosphorylated αSyn^P aggregates in the skin of patients with certain conditions, and those aggregates may be detectable only by RT-QuIC for their prion-like seeding activity but not by IF with antibodies for their phosphorylation (Donadio et al. 2021).

Mammana et al. examined skin samples taken in vitam ($n = 69$) and postmortem ($n = 49$) from patients with PD, DLB, incidental Lewy body pathology, and neurological controls with RT-QuIC (Mammana et al. 2021). αSyn-seeding activity in both CSF and skin was determined in 79 patients. In general, the skin αSyn^P RT-QuIC assay distinguished DLB patients with 94.1% accuracy (sensitivity, 89.2%; specificity, 96.3%). The cervical skin samples reached 94.1% sensitivity in the 17 DLB patients. In those patients who had the two types of samples available, both CSF and skin RT-QuIC assays showed a similar diagnostic accuracy (skin, 97.5%; CSF, 98.7%) (Mammana et al. 2021).

Taken together, skin αSyn^P-seeding activity could be a biomarker for diagnosis and evaluating disease stages and progression of PD and other synucleinopathies.

32.6 Tau-Seeding Activity in Alzheimer's Disease and Tauopathies

AD is the most common neurodegenerative disease affecting more than 6.5 million Americans and at least 50 million people worldwide (Alzheimer's Association 2019; Patterson C. World Alzheimer Report 2018). It has been estimated that the prevalence of AD is expected to triple by 2050 (Brookmeyer et al. 2007), highlighting the urgent need for its improved diagnostics and therapeutics. The disease manifests progressive degeneration of the limbic and cortical structures associated with deposition of the extracellular amyloid β plaques and intracellular phosphorylated tau-containing neurofibrillary tangles in the brain (Karran and De Strooper 2022). Clinically, it is characterized by deterioration in memory, impaired executive functions, and alterations in mood and behavior. As other neurodegenerative diseases, currently AD is not curable.

The deposition of disease-associated tau aggregates in the brain is the pathological hallmark of AD and other tauopathies including Pick's disease (PiD), PSP, and CBD. It is known that six tau isoforms are expressed in the human brain. The tau

isoforms contain three or four microtubule-binding repeats (3R or 4R tau) and 0–2 N-terminal inserts (0 N, 1 N, or 2 N tau) (Goedert et al. 1989). Notably, the composition and morphology of the varied tau filaments can differ between tauopathies, which may be associated with the existence of distinct tauopathy strains. In AD, the neuronal tau inclusions contain both 3R and 4R isoforms, while PiD mainly contains the 3R isoforms in the neuronal deposits. PSP and CBD are characterized by the accumulation of the 4R tau assembly in the brain. The recent advances in brain imaging tests and immunoassays of phosphorylated and total tau in the plasma and CSF have made a definitive AD diagnosis in living patients possible. However, CSF sampling requires the highly invasive lumbar puncture while the brain imaging is expensive and/or involves radioactivity. Newly developed ultrasensitive technologies including RT-QuIC and PMCA have now made it possible to identify new biomarkers in readily accessible specimens for early diagnosing and assessing disease progression. As mentioned above, misfolded prion and αSyn^{P} have been detected in the skin of PrD and PD patients by RT-QuIC and/or PMCA (Orrù et al. 2017; Mammana et al. 2020, 2021; Xiao et al. 2021; Wang et al. 2019, 2020; Ding et al. 2021; Manne et al. 2020; Kuzkina et al. 2021; Donadio et al. 2021; Bargar et al. 2021).

Interestingly, pathologically phosphorylated tau has been reported in the skin of AD patients. Dugger et al. examined tau by western blotting and ELISA not only in the brain but also in peripheral tissues from 18 cases with the pathological tau deposits in the spinal cord (Dugger et al. 2016). Examined samples included frontal cortex gray matter, sigmoid colon, scalp, abdominal skin, liver, and submandibular gland. ELISA revealed the highest total tau levels in the brain, followed by submandibular gland, sigmoid colon, liver, scalp, and abdominal skin. Western blotting with antibodies directed against tau phosphorylated at threonine 231(p231), serine 396 and 404 (PHF-1), and an unmodified total human tau between residues 159 and 163 (HT7) showed multiple bands, some of which predominated in peripheral tissues. Two bands migrating at approximately 60 kDa and 30 kDa p231 were detected in peripheral tissues including skin by antibody p231. This study provided evidence that certain tau species are present in skin tissue of AD patients (Dugger et al. 2016).

A recent study further characterized tau expression in biopsy skin samples of patients clinically diagnosed with synucleinopathies (PD and MSA), tauopathies (PSP and CBD), and in healthy control subjects (Vacchi et al. 2022). In all groups, tau was detectable along both somatosensory and autonomic nerve fibers in the epidermis and dermis layers by IF. Western blotting revealed the presence of mainly two different bands migrating at 55 kDa and 70 kDa, co-migrating with 0N4R/1N3R and 2N4R isoforms, respectively (Vacchi et al. 2022). The main transcript tau variants were found to be 2 N and 4R, whose expression level was increased in PSP/CBD. Also, ELISA revealed significantly higher levels of total tau in skin lysates of PSP/CBD than that of the other groups. Multivariate regression analysis and ROC curves analysis of tau amount exhibited a clinical association with tauopathies diagnosis and high diagnostic value for PSP/CBD vs. PD (sensitivity 90% and specificity 69%) and PSP/CBD vs. MSA (sensitivity 90%, specificity 86%).

Intriguingly, increase in tau correlated with cognitive impairment in PSP/CBD (Vacchi et al. 2022).

Like misfolded prions and αSyn^P, pathological tau from the brain or/and CSF of patients with PiD, AD, chronic traumatic encephalopathy (CTE), and frontotemporal lobar degeneration also has been found to have seeding activity detected by RT-QuIC with different truncated tau isoforms, either 3R, 3R/4R, or 4R as substrates (Saijo et al. 2017, 2019; Kraus et al. 2019; Metrick 2nd et al. 2020). Saijo et al. first developed an assay called tau RT-QuIC that detected misfolded tau seeds in PiD brain (Saijo et al. 2017). This test was significantly less responsive when seeded with brain homogenates containing predominant 4R tau aggregate seeds from cases of CBD, argyrophilic grain disease, and PSP. The AD brain containing 3R/4R tau deposits also exhibited much weaker responses than the PiD brain. CSF tau RT-QuIC discriminated PiD from non-PiD cases (Saijo et al. 2017). Thus, abnormal tau aggregates can be detected for their seeding activity with high sensitivity and specificity in crude tissue and fluid samples. Using a mixture of K19CF with a point mutation from cysteine to serine at residue 322 and τ306 (residues 306–378) as the substrate, the RT-QuIC assay detected higher tau-seeding activity in the brain of AD and CTE than in that of other tauopathies including PiD, CBD, and PSP with comparable loads of predominant 3R or 4R tau aggregates (Kraus et al. 2019). Moreover, they observed that tau RT-QuIC assay was highly sensitive and able to detect as little as 16 fg of synthetic tau fibrils. Using a recombinant 3R tau substrate termed K12CFh, Metrick et al. recently reported a modified tau RT-QuIC assay called K12 RT-QuIC that enabled sensitive detection of tau-seeding activity in the brain homogenates of PiD, AD, and CTE (Metrick 2nd et al. 2020). It was revealed that PiD could be differentiated from AD and CTE cases by the quantitative differences in their thioflavin T responses. This difference was further confirmed by the distinct structural properties of the associated reaction products. The single K12 RT-QuIC assay is believed to be able to detect and discriminate tau aggregates comprised mainly of 3R or both 3R/4R tau isoforms.

The above all RT-QuIC assays of the brain or CSF tau involved differently truncated tau substrates. To evaluate the feasibility of the six recombinant full-length wild-type tau isoforms as substrates to amplify misfolded tau, we determined how individual tau isoforms worked as substrates for the RT-QuIC assay of brain tau-seeded aggregation (Wu et al. 2022). The tau seeding activities of brain samples from AD and non-AD patients as seeds were examined in the presence of individual six recombinant tau isoform substrates, respectively. We observed that the activities started at approximately 10–30 h and reached a plateau at about 60 h (Wu et al. 2022). In contrast, non-AD brain samples showed no or minimal seeding activities for the entire 60 h of reaction period, and the tau seeding activities were significantly lower than that in AD samples at ~60 h ($p < 0.001$ for all isoforms). Blank controls containing tau isoform substrate only without brain homogenates showed no seeding activities. All six recombinant wild-type human tau isoforms exhibited nearly 100% specificity while sensitivity varied and ranged from 60% to 100% among different isoform substrates (Wu et al. 2022).

Consistent with our above finding, Tennant et al. recently confirmed that RT-QuIC assay is able to detect tau-seeding activity in the brain of patients with tauopathies using some of full-length human tau substrates (Tennant et al. 2020). The seeding activity was detectable in AD ($n = 11$) and other tauopathy brain samples, including PiD ($n = 4$), PSP ($n = 3$), and frontotemporal lobe dementias ($n = 2$), but significantly less in age-matched normal human ($n = 2$) brain samples using 2N3R, 2N4R, or 2N3R/2N4R r-tau substrate mixture. It was observed that the equimolar 2N3R/2N4R r-tau mixture produced the best combination of sensitive and specific detection. In the same study, they also detected tau-seeding activity ahead of the onset of clinical symptoms in longitudinally sacrificed mice expressing human tau with mutation at residue P301S (Tennant et al. 2020). In the meanwhile, they pointed out that the small cohort of normal controls available in this study was a limitation. Currently, we are using RT-QuIC and PMCA to detect skin tau-seeding activity in samples from AD and non-AD tauopathies with various full-length and truncated tau isoforms as substrates.

In summary, since tau-seeding activity has been detected in the brain or CSF of AD and other tauopathies and also phosphorylated tau has been found in the skin tissues of AD patients, it is most likely that detection of skin tau-seeding activity will be a useful biomarker for diagnosis of AD and tauopathies.

32.7 Conclusion

The development of affordable and accessible biomarkers for early diagnosis, assessing disease progression, and monitoring therapeutic efficacy in clinical trials of neurodegenerative diseases is critical for battling against these devastating diseases. Due to the common developmental origins, highly neurotropic feature of the misfolded proteins, and rich in nerve endings, it is most likely that the skin tissues are the highly promising specimen for developing biomarkers for the diseases. Moreover, studies from peripheral misfolded αSyn^{P} in PD have raised a possibility that misfolded protein aggregates in the skin play a role in the pathogenesis of neurodegenerative diseases. Therefore, continuous research on skin misfolded proteins is highly potential not only for diagnostics and therapeutics but also for understanding the pathogenesis of various neurodegenerative diseases.

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Chapter 33
Diagnosis of Prion Disease: Conventional Approaches

Inga Zerr and Peter Hermann

Abstract Prion diseases are characterized by the deposition of PrP^{Sc}, an abnormal form of the normal cellular protein, PrP^{c} in the brain. The unique nature of human prion diseases includes their pathogenesis, mode of transmission, and neuropathology. In humans, a long incubation time, rapid and dramatic evolution of the disease course, and always a lethal outcome are key features of the clinical syndrome. The clinical diagnosis in sCJD is supported by detection of periodic sharp and slow wave complexes (PSWCs) in the electroencephalogram, 14-3-3 proteins, the detection of the abnormal PrP of in the cerebrospinal fluid (CSF) via RT-QuIC, and hyperintense signal changes in the basal ganglia, thalamus and cortical areas on magnetic resonance imaging (MRI). These tests became part of the clinical diagnostic criteria for CJD. Elevated levels of brain-derived proteins in plasma such as neurofilaments or tau might contribute to the clinical diagnosis in the future. The sensitivity of diagnostic tests varies across molecular CJD subtypes. Alzheimer's disease and Lewy body dementia are the most frequent differential diagnoses in elderly patients, while chronic inflammatory CNS disorders and autoimmune mediated encephalitis have to be considered in younger patients.

Keywords 14-3-3 proteins · PrPSc · Cerebrospinal fluid · Neurofilaments · Tau · Plasma · Diagnosis · Diagnostic criteria · EEG · Molecular disease subtype · MRI · PSWCs

33.1 Introduction

Human prion diseases share many common features—transmissibility in animal experiments, fatal progressive disease course, neuronal loss, astrogliosis, and PrP^{Sc} deposition in the brain. Despite this, several forms are distinguished depending on assumed pathophysiology: genetic, acquired, and sporadic disease forms. In

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addition, sporadic disease forms display clinicopathological diversity, which origins in codon 129 *PRNP* genotype and PrP^{Sc} type (see Molecular disease subtypes). In clinical terms, signs and symptoms of the disease are heterogeneous and comprise a wide spectrum of neurological and psychiatric abnormalities. Because of this and because of the fact that a definite early clinical test or biomarker is still lacking, several diagnostic investigations have to be taken into account and considered in the context of comprehensive clinical examination, thoughtful evaluation of the clinical history, and consideration of other differential, potentially curable diagnoses.

A definite and final diagnosis requires invasive procedures such as brain biopsy or analysis of brain material at autopsy. Early detection will become increasingly important once forthcoming effective therapies are available (Krammer et al. 2009). Clinical diagnostic criteria for sCJD were first suggested 40 years ago, using a combination of distinctive clinical features and best available investigations, which at that time was EEG (Masters et al. 1979). In recent years, substantial progress in developing other specialized investigations, including useful surrogate biomarkers in the cerebrospinal fluid, plasma and brain imaging, and clinical diagnostic criteria, have been amended (Collins et al. 2006; Zerr et al. 2000, 2009, 2022; Hermann et al. 2021) (Fig. 33.1). Blood-based biomarker are currently under investigations and might become important tools for monitoring disease progression and potentially even response to therapy once disease-modifying drugs become available (Vallabh et al. 2020).

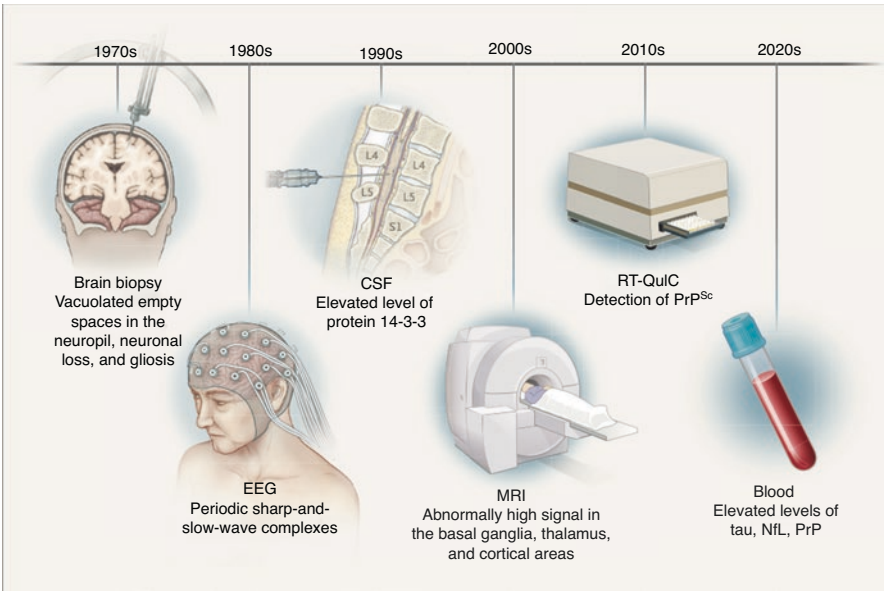


Fig. 33.1 Progress in the development of diagnostic techniques for Creutzfeldt–Jakob disease (CJD). (Zerr 2022)

33.2 Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is the main component of the brain extracellular space and participates in the exchange of many biochemical products in the central nervous system (CNS). Consequently, CSF contains a dynamic and complex mixture of proteins, which reflects physiological or pathological state of the CNS. CSF analysis is an important part in clinical neurology and is used to diagnose various inflammatory and malignant disorders and recently also neurodegenerative disorders. The alterations in CSF composition are also discussed to reflect pathological changes in the brain and thus contribute to a better understanding of the pathophysiology of the underlying disorders affecting CNS.

For many years, CSF analysis in CJD has been used to exclude brain inflammation in patients with rapid progressive dementia. Since modern proteomic technologies allow us to identify proteins and protein patterns in human fluids, the CSF analysis in dementia disorders has become even more important. Historically, first CSF abnormalities in human prion diseases were reported by Harrington et al. (1986), who identified two proteins spots, named p130/131 in the CSF of CJD patients. Decades later, these proteins became known as 14-3-3 proteins and were the first CSF biomarker ever used in clinical criteria in patients with a neurodegenerative dementia.

33.2.1 Routine Tests

The routine examination of CSF from patients with CJD or GSS usually reveals normal results. An unspecific increase in total protein, the presence of oligoclonal IgG bands or raised cell count is an extremely rare finding (see Table 33.1). In the most comprehensive study on this subject, data from 450 patients with sporadic CJD and 47 patients with other TSEs were analyzed as part of an EC-supported multinational study. Raised white cell counts of >5 cells/ml were found in three out of 298 patients with sporadic CJD, in two with cell counts of 7 cells/ml and in one of 20 cells/ml. Total protein concentrations of >0.9 g/l were found in 5 of 438

Table 33.1 Frequency of abnormal CSF white cell counts, raised total proteins, and the presence of oligoclonal IgG

Diagnosis	CSF white cells count > 5 µl (%)	CSF total protein > 0.6 g/l (%)	CSF total protein > 0.9 g/l (%)	Presence of oligloconal IgG (%)
Sporadic CJD	3 (1.0)	44 (10.0)	5 (1.1)	8 (4.4)
Genetic CJD	3 (13.0)[a]	1 (3.1)	0 (0)	0 (0)
FFI	2 (66.7)[a]	1 (12.5)	0 (0)	1 (20.0)

[a]Significant different from sporadic CJD, p = 0.01. Fisher exact test two sided.

patients with sporadic CJD, but none had a concentration of >1 g/l. CSF oligoclonal IgG was detected in 8 out of 182 sporadic CJD patients. Among patients with other TSEs, six had elevated cell counts ranging from 6 to 14 cells/ml, but none had total protein concentrations of >0.9 g/l and one patient had detectable oligoclonal IgG. None of the patients with sporadic CJD or other TSEs had abnormalities in all three tests (Green et al. 2007).

As a rule, inflammatory CSF findings exclude the diagnosis of a human prion disease.

33.2.2 14-3-3 Proteins

14-3-3 proteins were initially described as abundant, acidic brain proteins, and their names are derived from the combination of its fraction number on DEAE-cellulose chromatography and migration position in the subsequent starch gel electrophoresis (Hsich et al. 1996). Despite the fact that the pathology behind the elevated level of 14-3-3 in CJD is still a question of debate, the detection of 14-3-3 protein in CSF is part of clinical diagnostic criteria for probable sCJD because of its high sensitivity and, even more important, high predictive values in clinical setting. A large number of studies proved that in appropriate clinical circumstances, a positive 14-3-3 is highly sensitive and specific for sCJD diagnosis. A meta-analysis showed a sensitivity of 92% and a specificity of 80% of Western Blot 14-3-3 analyses (Muayqil et al. 2012). The recently developed γ14-3-3 ELISA was reported with 88% sensitivity and 96% specificity (Schmitz et al. 2016a, b) and showed superior diagnostic accuracy in a comparative evaluation (Leitão et al. 2016). However, the specificity may be substantially lower in the differentiation from acute non-neurodegenerative encephalopathies (Stoeck et al. 2012).

The sensitivity of 14-3-3 varies among TSE types. Whereas very high sensitivity was shown in gCJD and iatrogenic CJD, similar to sCJD, 14-3-3 positivity is substantially less frequent in GSS and rarely present in FFI (Llorens et al. 2020a, b, c; Schmitz et al. 2022). In variant CJD (vCJD), only about 49% of patients are positive (Green et al. 2001; Gmitterová et al. 2009; Green et al. 2002; Van Everbroeck et al. 2003; Castellani et al. 2004).

Biological parameters significantly influence the sensitivity of 14-3-3 test in patients with sCJD, that is, disease duration, codon 129 genotype, age at onset, and time of the lumbar puncture (Sanchez-Juan et al. 2007). In general, the 14-3-3 test displays best sensitivity in patients older than 40 years with short disease duration, homozygous at codon 129 genotype, and when lumbar puncture is performed at later disease stages (Sanchez-Juan et al. 2006). Differences in the sensitivity of 14-3-3 test are also observed between classical and nonclassical CJD types (see Molecular disease subtypes). In classical CJD (which basically fulfills the criteria of having the tendency to be older, homozygous for methionine at codon 129, short disease duration, and rapid progression), 14-3-3 test sensitivity is superior to nonclassical (or atypical) cases.

Although this test was often found to be positive at onset of the first neurological symptoms, higher sensitivity was reported in the middle or late stage of the disease. Moreover, in the terminal stage of disease, 14-3-3 level might decrease in CSF, but this observation is based on case reports and might reflect extremely long disease duration.

33.2.3 Tau/p-tau

Tau concentration in CSF of CJD patients is highly increased, and its quantitative analysis is a good diagnostic tool for CJD (Kovacs et al. 2017c). Determination of tau has shown to yield specificity and sensitivity comparable to those for 14-3-3 testing, and several studies revealed that the optimum cut-off point for CJD is at 1.300 pg/ml (Hermann et al. 2021). This cut-off is three times higher than levels reported for Alzheimer's dementia. In the latter, extreme tau levels have been reported occasionally, especially in atypical or rapidly-progressive Alzheimer's dementia. Therefore, a ratio of phosphorylated and total t-tau has been proposed to improve the diagnostic accuracy (Skillbäck et al. 2014, Hermann et al. 2022) Concerning the phosphorylated tau isoforms in CSF of CJD, tau phosphorylated at threonine 181 (p-tau) was significantly raised in sCJD as well as in vCJD. Interestingly, tau concentration was lower in vCJD when compared to sCJD, whereas p-tau concentration was much higher in vCJD than in sCJD.

33.2.4 RT-QuIC

The Real-Time Quaking-induced conversion (RT-QuIC) is a new method to detect PrPSc in various tissues and body fluids. The method is described in detail in chapter 30. Since 2011, it has been evaluated in the context of the clinical diagnosis of sCJD in several retrospective and prospective studies. Because all studies reported good to excellent Sensitivity with outstanding specificity of 99%–100% (see a summary in Table 33.2), it was integrated as a new diagnostic criterion in recent consensus criteria. If available, the test should be performed in all patients with suspected prion disease (Hermann et al. 2021).

33.2.5 Other CSF Markers

Besides common TSE markers, several other proteins have been proposed as possibly useful in the diagnosis of the human TSE. So far, they were tested in small numbers of patients and need further rigorous testing and thoughtful validation of their potentials to be classified as biomarkers in human prion disorders (Table 33.3). Hermann et al. Lancet Neurol 2021.

Table 33.2 Diagnostic accuracy of CSF RT-QuIC in retrospective and prospective studies (Hermann et al. 2021)

	Cases		Controls				
	n	type	*n*	type	Sensitivity	Specificity	Protocol
Atarashi et al. (2011)	34	Definite sCJD	49	OND+	85%	100%	1st Gen
McGuire et al. (2012)	123	Definite sCJD	103	RPD	89%	99%	1st Gen
Orrú et al. (2014)	30	Probable + definite sCJD	46	non-CJD	77%	100%	1st Gen
Orrú et al. (2015)	48	Probable + definite sCJD	39	OND+	96%	100%	2nd Gen
Cramm et al. (2016)	110	Definite sCJD + gCJD	400	OND+	85%	99%	1st Gen°
Groveman et al. (2017)	113	Probable + definite sCJD	64	OND+	73%	100%	1st Gen
Groveman et al. (2017)	113	Probable + definite sCJD	64	OND+	94%	100%	2nd Gen
Park et al. (2016)	81	Probable + definite sCJD	100	non-CJD	77%	100%	1st Gen
Franceschini et al. (2017)	145	Probable + definite sCJD + gCJD	42	RPD	97%	100%	2nd Gen
Bongianni et al. (2017)	49	Probable + definite sCJD	71	OND+	73%	100%	1st Gen
Bongianni et al. (2017)	22	Probable + definite sCJD	71	OND+	86%	100%	2nd Gen
Lattanzio et al. (2017)	225	Definite sCJD	348	RPD	84%	99%	1st Gen
Foutz et al. (2017)	126	Definite sCJD + gCJD	67	RPD	92%	99%	2nd Gen
Rudge et al. (2018)	171	Definite sCJD	47	RPD	89%	100%	1st Gen
Foutz et al. (2017)	65	Definite sCJD + gCJD	14	RPD	95%	100%	2nd Gen
Hermann et al. (2018)	65	Definite sCJD	118	RPD	89%	100%	1st Gen°
Abu-Rumeileh et al. (2019)	65	Definite sCJD + gCJD	62	RPD	82%	100%	1st Gen
Abu-Rumeileh et al. (2019)	65	Definite sCJD + gCJD	62	RPD	96%	100%	2nd Gen
Fiorini et al. (2020)	102	Probable + definite sCJD	80	RPD	96%	100%	2nd Gen
Mammana et al. (2020)	24	Probable + definite sCJD	12	RPD	88%	100%	1nd Gen
Rhoads et al. (2020)	439	Definite sCJD	69	RPD	93%	99%	2nd Gen

1st paragraph (Atarashio et al. to Rudge et al.): retrospective studies; 2nd paragraph (Foutz et al. to Rhoads et al.): prospective studies. definite sCJD, neuropathological confirmed diagnosis of sporadic Creutzfeldt–Jakob disease; probable sCJD, clinical diagnose of sporadic Creutzfeldt–Jakob disease based on syndrome and biomarkers;[4] gCJD, genetic Creutzfeldt–Jakob disease; OND+, other neurological diseases including dementia syndromes; RPD, rapidly progressive dementia, clinically suspicious for CJD; non-CJD, including non-neurologic disorders, neurologic disorders, and dementia syndromes; 1st Gen, first-generation tests;[8] 2nd Gen, second generation test[31].

Table 33.3 Other reported cerebrospinal fluid candidates for markers of human prion diseases

Study	Proposed CJD-marker	Level in CSF
Manaka et al. (1992) [44]	Ubiquitin	Elevated
Choe et al. (2002) [9]	ApoE	Elevated
Minghetti et al. (2002) [51]	Prostaglandin E(2)	Elevated
Guillaume et al. (2003) [26]	H-FABP	Elevated
Kettlun et al. (2003) [35]	Matrix metalloproteinase	Elevated
Schmidt et al. (2004) [63]	LDH-1	Elevated
Cartier et al. (2004) [6]	Fibronectin, Thrombospondin, Heparan sufhate proteoglycan	Elevated
Zerr et al. (2004) [82]	Plasminogen	
Sanchez et al. (2004) [61]; Piubelli et al. (2006) [57]	Cystatin C	Elevated
Stoeck et al. (2005) [68]	IL-4, IL-8, and IL-10	Elevated
Silveyra et al. (2006) [67]	Acetylcholinresterase	Altered glycolysation pattern
Holsinger et al. (2006) [30]	BACE1	Increased activity
Stoeck et al. (2006) [69]	TGF-β	Reduced
Albrecht et al. (2006) [3]	Beta-nerve growth factor	Elevated
Jesse et al. (2009) [32]	GFAP	Elevated
Alberti et al. (2009) [2]	Neurofilament heavy chain	Elevated
Gawinecka et al. (2012)	Desmoplaskin	Elevated
Singh et al. (2011)	Transferrin	Lowered
Oeckl et al. (2012)	cAMP and cGMP	Lowered
Kasai et al. (2014)	α-Synuclein	Elevated
Dorey et al. (2015)	Total Prion protein	Lowered
Schmitz et al. (2016b)	Malate dehydrogenase 1	Elevated
Oeckl et al. (2016)	β-Synuclein	Elevated
Llorens et al. (2017)	YKL-40	Elevated
Kovacs et al. (2017)	Neurofilament light chain	Elevated
Ermann et al. (2018)	Nonphosphorylated	Elevated
Blennow et al. (2019)	Neurogranin	Elevated
Li et al. (2019)	mtDNA	Elevated
López-Pérez et al. (2020)	BAMBI	Elevated
Diaz-Lucena et al. (2021)	sTREM2	Elevated

33.2.6 Blood-Based Biomarkers

Blood-based biomarkers to detect disease or to monitor disease progression are highly desirable because they would replace CSF puncture or at least support the diagnostic process. In addition, blood samples can be taken regularly and repeatedly. Attempts to develop blood-based biomarkers have been carried out for many years (Otto et al. 1998; Völkel et al. 2001) and have experienced a renewed upswing in recent years. With the improvement of detection technology, some brain proteins can be detected in plasma with good reproducibility. These

Table 33.4 Diagnostic performance of serum or plasma markers for sCJD (Hermann et al. 2021)

Proposed CJD marker	Studies
s-100b	Otto et al. (1998); Steinacker et al. (2016)
t-Tau	Steinacker et al. (2016); Kovacs et al. (2017); Thompson et al. (2018); Abu-Rumeileh et al. (2020a); Zerr et al. (2021)
NfL	Steinacker et al. (2016); Kovacs et al. (2017); Thompson et al. (2018); Abu-Rumeileh et al. (2020a); Zerr et al. (2021)
t-PrP	Llorens et al. (2019)
YKL-40	Villar-Piqué et al. (2019)
Beta-synuclein	Oeckl et al. (2020); Halbgebauer et al. (2022)
Small RNA-seq read	Norsworthy et al. (2020)
sTREM2	Diaz-Lucena et al. (2021)

primarily include the proteins tau, NFL (Steinacker et al. 2016), and PrP (Villar-Piqué et al. 2019). Elevated levels may correlate with disease stage and progression but, unfortunately, the specificity of tau and NFL in the differential diagnosis from other rapidly progressive encephalopathic syndromes is not very high (Kovacs et al. 2017; Abu-Rumeileh et al. 2020a; Zerr et al. 2021). See Table 33.4 for a summary of recent studies. However, the sensible use may be to monitor the disease progression or as a surrogate marker to check the therapy effects in clinical trials (Thompson et al. 2021).

33.3 Magnetic Resonance Imaging

33.3.1 General Introduction

MRI has played an important role in the diagnosis of CJD (Tschampa et al. 2005; Urbach et al. 1998; Nozaki et al 2018; Young et al. 2005; Seror et al. 2010). In 1988, a hyperintense signal of the basal ganglia on T2-weighted images was first described as a characteristic finding in sCJD patients, followed by further case reports. Subsequently, systematic studies on the sensitivity and specificity of hyperintense signal changes in the striatum in sCJD were performed. Along with the availability of methods, the early MR studies mainly focused on T2-weighted, proton density weighted (Finkenstaedt et al. 1996; Schröter et al. 2000), and to a lesser extent on FLAIR (Choi et al. 2009) imaging, while current studies and criteria mainly rely on FLAIR and especially DWI MRI (Zerr et al. 2009; Vitali et al. 2011; Bizzi et al. 2020; Lee et al. 2010).

With the emergence of more sensitive MRI techniques, such as fluid-attenuated inversion recovery (FLAIR) and diffusion-weighted image (DWI), cortical signal increase was additionally observed in sCJD patients and hyperintense basal ganglia were detected more frequently (Fig. 33.2). Using FLAIR- and DWI, signal increase

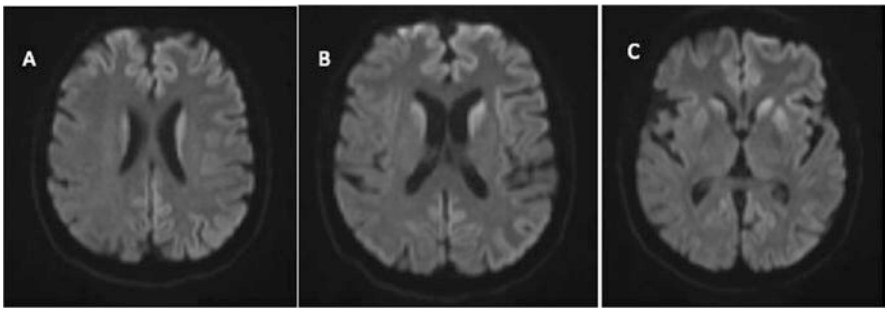


Fig. 33.2 DWI from patient with CJD representing. (**a**) Hyperintensities cortical. (**b**) Symmetric both caudate nucleus plus cortical. (**c**) striatum right> left plus cortical

in the cortex has been reported even more frequently than basal ganglia signal increase. Apart from the cortex and basal ganglia hyperintensity, signal increase has also been reported for the hippocampus, thalamus, and cerebellum and for the mesencephalon. In general, the most sensitive technique to date seems to be DWI, followed by FLAIR and T2 imaging.

33.3.2 Test Performance

With the introduction of diffusion-weighted imaging, MR changes are detected earlier in the disease (Collins et al. 2006; Heinemann et al. 2007b; Josephs et al. 2009) and interobserver reliability was improved (Demaerel et al. 1999, Zerr et al. 2009, Vitali et al. 2011). The diagnostic accuracy was reported by several studies with a sensitivity ranging from 80% to 98% and a specificity ranging from 74% to 98% (Hermann et al. 2021). However, discrepant reports of the test performance may be explained with the use of different criteria, different control groups, and, in particular, may be highly dependent on the image reader's experience (Carswell et al. 2012).

33.3.3 Changes During the Disease

Data on serial MR examinations in CJD are limited in the literature. In early disease stages, characteristic basal ganglia lesions are not found in up to one-third of the patients (Meissner et al. 2008). According to Ukisu and colleagues (Ukisu et al. 2005), cortical DWI changes (9/9 cases) preceded the hyperintensities in the basal ganglia (5/9 cases at early stage). During the course of the disease, there is generally an expansion of the signal changes and progressive cerebral atrophy (Tribl et al. 2002; Eisenmenger et al. 2016), displaying lesion propagation from cortex to basal

ganglia in MM1 and vice versa in VV2 (Pascuzzo et al. 2020). In the late stage of the disease, the diffusion changes may disappear (Arruda et al. 2004; Tribl et al. 2002). Some very interesting case studies reported very early and even preclinical MRI changes in genetic and sporadic CJD up to two years before disease onset (Alvarez et al. 2005; Zanusso et al. 2016; Novi et al. 2018; Koizumi et al. 2021).

33.4 EEG

For decades, periodic sharp wave complexes (PSWCs) in the EEG were reported to represent the most typical finding in the course of sCJD. The apparent advantages of the EEG are: This investigation is widely available, noninvasive, and can easily be repeated several times. At onset, the EEG might show only nonspecific changes such as background slowing of alpha activity and dysrhythmia. As the disease progresses, slow periodic complexes might appear occasionally; later, the typical periodic pattern is seen. In end stage of CJD, the EEG might show an isoelectric line. PSWCs might be provoked by acoustic or tactile stimulation. Typical periodic patterns (Fig. 33.3) are observed in 60%–70% of all cases after about 12 weeks (median) from disease onset but might occur as early as three weeks after onset.

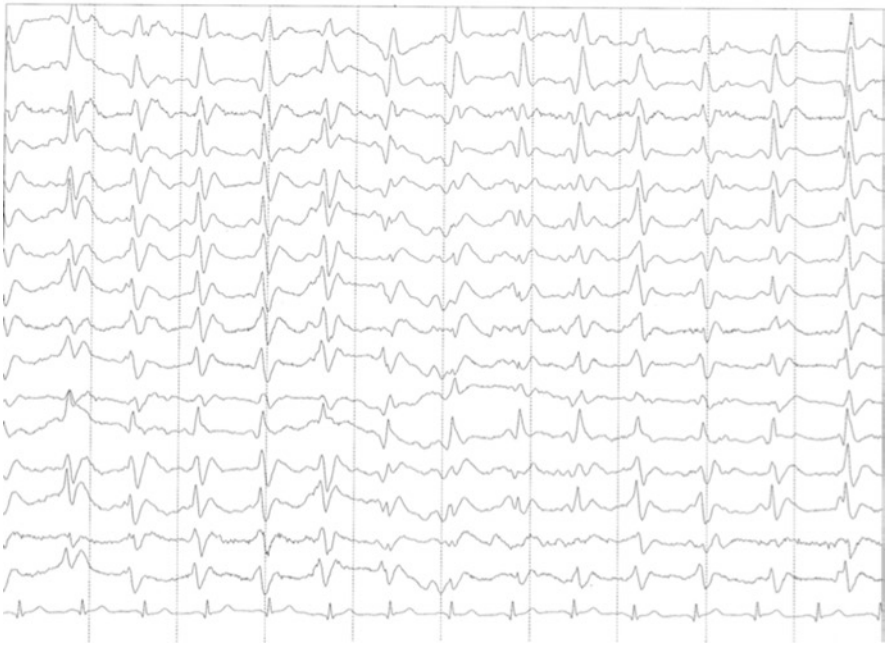


Fig. 33.3 Typical periodic sharp wave complexes

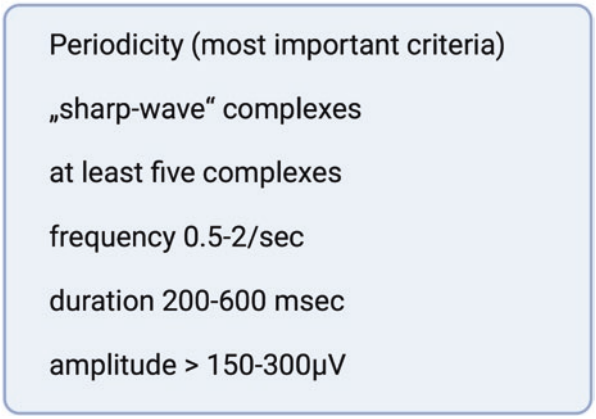


Fig. 33.4 EEG criteria (according to Steinhoff et al. 2007)

They may disappear at later disease stages. Since the term PSWCs has not been operationalized before, sensitivity of the detection of this abnormality varied among studies. EEG criteria have been suggested (Fig. 33.4). According to these criteria, PSWCs are detectable in two-thirds of CJD patients at mid and late disease stages (sensitivity 64%) with a specificity of 91% (Steinhoff et al. 2004). However, they are not able to differentiate CJD from nonconvulsive status epilepticus (Marquetand et al. 2017) and in addition, recent observations reported a lower sensitivity (around 40%) (Hermann et al. 2018), most likely due to the fact that modern diagnostics allow the identification of CJD in early disease stages when PSWCs are not present.

33.5 Molecular Disease Subtype-Specific Diagnosis

Some years ago, a molecular basis has been defined, which might explain the clinical and pathological disease heterogeneity (Parchi et al. 1996). The polymorphism for methionine (M) or valine (V) at codon 129 of *PRNP* gene has been shown to influence the clinical features of sCJD. In 1996, two PrP^{Sc} subtypes in brain homogenates of sCJD patients were identified. The polymorphism at codon 129 and the prion protein types 1 and 2 were the basis for a new molecular classification of sCJD, which replaced the previous attempts (Parchi et al. 1999). Currently, patients with the MM1/MV1 subtype, who display a short disease duration, dementia, myoclonus, and typical EEG pattern, are frequently referred to as having “classical” or “common” CJD subtype. Other (“nonclassical” and “atypical”) subtypes are rare (Fig. 33.5).

The discovery of several distinct molecular CJD subtypes explains many features observed in sporadic CJD patients. The clinical presentation at early disease stage is

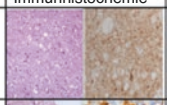
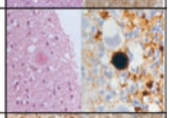
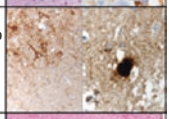
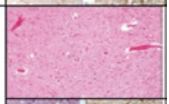
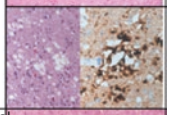
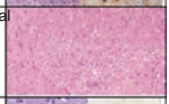
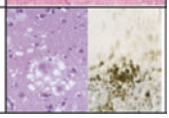
	molecular subtype	clinical signs	neuropathological findings	PrP-Immunhistochemie
frequent	MM1/MV1	dementia, cortical anopia, myoclonus, short disease duration (average 4 month)	severe damage of the occipital cortex (spongiosis, neuronal loss, astrogliosis), synaptic PrP–deposition	
	MV2	ataxia, dementia, extrapyramidal symptoms, long disease duration (average 18 month)	focal cortical damage, amyloid-(„Kuru"-) plaques, focal plaque-like Prp-deposition	
	VV2	ataxia at onset, late dementia (average disease duration 7 month)	severe damage of subcortical structures and brainstem, spongiosis often restricted to deep cortical layers, plaque-like as well as perineuronal Prp-deposition	
rare	MM2-thalamic (sFI)	insomnia, autonomic dysfunction, late ataxia and cognitive decline	atrophy of thalamus and nucleus olivaris, spongiosis may be missing	
	MM2-cortical	dementia over a longer period (month)	focal and confluent vacuoles with coarse perivacuolar PrP deposition	
	VV1	early dementia, late ataxia and extrapyramidal symptoms	spongiosis, gliosis and neuronal loss of cortical structures except brainstem and cerebellum	
VCJD	MM2b	early psychiatric symptoms, dysesthesia, late ataxia and dementia	spongiosis, gliosis and neuronal loss, PrP deposition (florid plaques)	

Fig. 33.5 Molecular CJD subtypes

Table 33.5 Different values of the technical investigations EEG, CSF and MRI stratified by CJD subtype

			MM1/MV1	VV1	MM2	MV2	VV2
EEG	PSWCs		+				
CSF	14-3-3		+	+	(+)	(+)	+
	RT-QuIC		+	(+)	(+)	+	+
MRI	Cortex		+	+	+	+	
	Basal ganglia		+		(+)	+	+
	Thalamus	hyperintensity				+	+
		pulvinar sign				(+)	

peculiar in most disease subtypes, and the detailed investigation of the clinical syndrome often allows the assignment to the distinct CJD subtype. This observation is supported by EEG, CSF, and MRI results, which appear in subtype-distinctive pattern as described below. Table 33.5 gives an overview of the diagnostic investigations in distinct molecular CJD subtypes (Heinemann et al. 2007a).

EEG is abnormal in all disease subtypes, but the typical periodic sharp and slow wave pattern (PSWC) is observed in MM1/MV1 subtype only and is rare in MM2/

MV2/VV1-2 patients. Because CJD diagnosis was based on the triad: dementia, myoclonus, and PSWC in EEG for a long time, we might speculate that the frequency of so-called classical myoclonic CJD type was overestimated in earlier studies due to the selection bias. As mentioned above, results of various CSF tests vary considerably by disease subtype. 14-3-3 test sensitivity is best in MM1 and VV patients (>90%) and has the lowest sensitivity in MV2 (65%) and MM2 (78%) patients. Similar results were obtained for CSF tau protein (Sanchez-Juan et al. 2006) and for the detection of PrPSc via RT-QuIC (Franceschini et al. 2017; Rhoads et al. 2020).

The most characteristic MRI lesion patterns are found in MV2 and VV2, showing predominant involvement of thalamus and basal ganglia. Limited cortical signal increase was significantly related to PrP^{Sc} type 2. A further possible characteristic lesion pattern was found in VV1 showing widespread cortical hyperintensities and absence of basal ganglia signal alterations. In the other subtypes, there was a higher overlap between cortical and subcortical involvement. MV2 subtype was characterized by basal ganglia and thalamic involvement (Krasnianski et al. 2006). The pulvinar sign, according to current criteria, was identified in the MV2 subtype only (Collie et al. 2003). Due to the generally high frequency of thalamic hyperintensities in MV2, this subtype is the most likely to be mistaken for variant CJD (vCJD) on MRI. A multicenter international study evaluated MRI scans in 211 CJD patients with various sCJD disease subtypes (Meissner et al. 2009). Although basal ganglia hyperintensities on the MRI represented a consistent finding in all subtypes (except VV1), the frequency and location of cortex hyperintensities as well as the presence or absence of thalamus involvement varied between the subtypes. Across all molecular subtypes, VV2 patients showed the most frequent involvement of basal ganglia and thalamus. Cerebral cortical signal increase was usually restricted to less than three regions and most frequently found in the cingulate gyrus (Table 33.6 and Fig. 33.6). Recent studies validated these findings and reported that in the most common MM1 subtype, DWI abnormalities are predominantly present in cortical regions and caudate nucleus. VV2 and MV2 subtypes show primary involvement of the striatum and the thalamus, whereas in the rare MM2 and VV1 subtypes, often

Table 33.6 MRI findings and subtypes

Subtype	More than 3 cortical regions	Basal ganglia	Insula	Thalamus
MM1	30%	66%	18%	**7% p = 0.004**
MM2-cortical	78%	**22% p = 0.04**	22%	11%
MV1	**67% p = 0.01**	67%	16%	20%
MV2	32%	65%	16%	**35% p = 0.001**
VV1	**86% p = 0.03**	**14% p = 0.02**	71%	0%
VV2	**17% p = 0.04**	72%	14%	**31% p = 0.057**

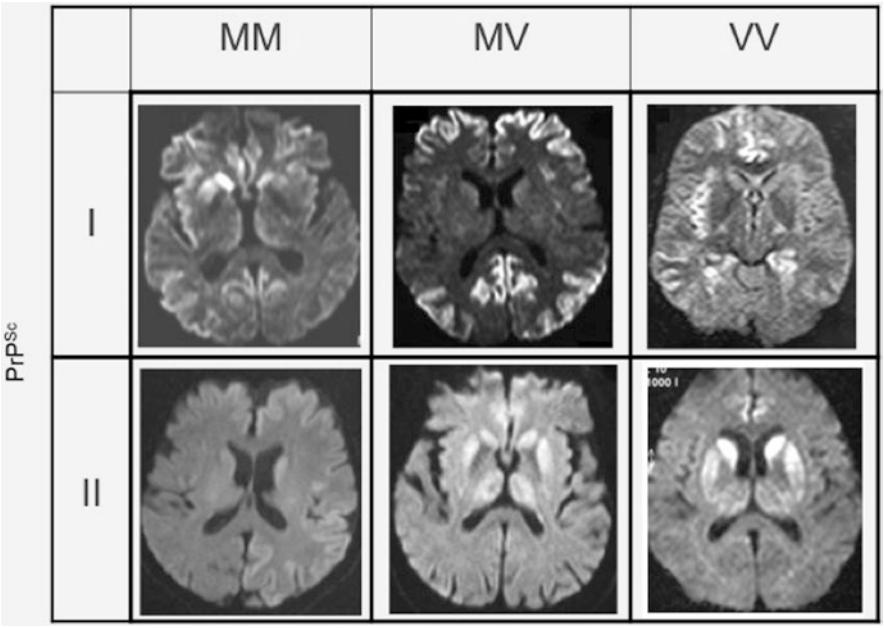


Fig. 33.6 Characteristic MRI findings in sporadic CJD subtypes

only cortical abnormalities can be detected (Pascuzzo et al. 2020). Based on these results, Bizzi et al. (2021) proposed an MRI-based prion subtype classification algorithm.

33.6 Genetic TSE

Patients with inherited forms of human prion diseases are diagnosed by genetic analysis of the *PRNP* gene. However, the family history of a prion disease might be absent in a considerable number of patients, thus it is important to know the outcomes of conventional tests such as EEG, CSF, and MRI. The most comprehensive studies have been carried out in genetic CJD with E200K and V210I mutations and in fatal familial insomnia (FFI), whereas only case reports or very small cohorts have been investigated in rare PRNP mutations.

Genetic transmissible spongiform encephalopathies (gTSE) represent 10%–15% of human TSEs. However, there is a special interest in studying biochemical and imaging markers in CSF to improve diagnosis and to monitor disease progression in genetic forms, especially when disease phenotype differs from that of typical sporadic CJD (Ladogana et al. 2009).

Table 33.7 Sensitivity of CSF markers in genetic TSE with most common PRNP mutations

	14-3-3 (ELISA)	Total-tau	α-Synuclein	RT-QuIC
E200K (gCJD) n=112	82%	81%	87%	93%
V210I (gCJD) n=47	94%	96%	96%	87%
D178N (FFI) n= 68	13%	18%	21%	28%
P102L (GSS) n=14	43%	43%	43%	43%
5-OPRI Insert n=10	90%	80%	80%	60%

PSWCs are not recorded in Gerstmann–Sträussler–Scheinker (GSS) syndrome, fatal familial insomnia, and in transmitted forms of the disease such as Kuru, iCJD, and vCJD. In patients with genetic prion diseases, PSWCs are only occasionally seen, with exception of patients with the mutation at codon 200 and 210 (Ladogana et al. 2005). In these patients, the sensitivity of the EEG is almost the same as in sCJD (Zerr et al. 1998a).

Concerning CSF 14-3-3 testing, sensitivity varies across the spectrum of genetic mutations (Rosenmann et al. 1997). Apparently, the types of mutation significantly influence the biomarker concentration in the CSF and, thus, test sensitivity. According to current information from a large multicenter study, changes in the CSF of patients with familial genetic forms of CJD (gCJD) are comparable to those found in sCJD samples. Table 33.7 gives an overview. For example, 14-3-3 proteins are detectable in patients with an E200K and V210I mutation but only in rare cases in FFI and GSS (Schmitz et al. 2022). In an another study on biomarkers in CJD (Ladogana et al. 2009), the crude analyses of disease-modifying factors on 14-3-3 test in gCJD revealed that age at onset and *PRNP* codon 129 genotype influenced sensitivity. Age at onset correlated significantly with 14-3-3 test sensitivity in gCJD, being lower in those patients with disease onset before 40 years. These data parallel the results of the same analysis performed on sporadic CJD (Ladogana et al. 2009; Sanchez-Juan et al. 2006). Interestingly, the *PRNP* codon 129 genotype seemed to influence 14-3-3 sensitivity in gCJD in a different way as in sporadic CJD. Valine homozygous gCJD patients had a statistically significant lower sensitivity in 14-3-3 test than heterozygous patients, but sensitivity was not significantly lower when adjusted for the mutation. This might be due to the fact that the *PRNP* mutations coupled with valine alleles (P105T, R208H, D178N, and E196K) yielded lower sensitivity to 14-3-3 (Table 33.8).

In recent years, more and more data have become available concerning MRI changes in gCJD. Of special importance, thalamostriatal and cortical diffusion reductions have been shown to precede disease onset in E200K and other prion mutation carriers and might therefore serve as an early diagnostic marker (Lee et al. 2009; Cohen et al. 2015; Koizumi et al. 2021; Fulbright et al 2006; Fulbright et al. 2008; Tsuboi et al. 2005). Restricted diffusion seems to be observed less frequently in gTSE, especially in FFI GSS, than in sCJD (Krasnianski et al. 2016). A Japanese study that included 216 gTSE patients and reported an overall

Table 33.8 Differential diagnoses of RPD reported by tertiary referral centers (Zerr and Hermann 2018)

	Athens, Greece [1]	Zhejiang, China [2]	Sao Paulo, Brazil [3]	Chandigarh, India [4]
	n = 68*	*n* = 310**	*n* = 61	*n* = 187
Infectious encephalitis	5.9%*	21.9%	19.7%	20.6%
Immune-mediated disease	8.8%	9.0%	45.9 %	18.2%
Creutzfeldt–Jakob disease	13.2%	7.1%	11.5%	7.5%
Neurodegenerative diseases:	47.0%	24.8%	8.2%	14.4%
Alzheimer's disease	17.6%	14.5%	n.a.	n.a.
Others	29.4%	10.3%	n.a.	n.a.
Vascular dementia	13.2%	**	n.a.	9.6%
Toxic + metabolic	*	10.3%	n.a.	16.0%
Others	11,8%	26.9%	14.7%	13.4%

*Acute infectious diseases and toxic-metabolic disorders had been excluded. **Cerebrovascular diseases had been excluded

sensitivity of 79% (Nozaki et al. 2010). However, CJD-typical signal patterns are quite common in gTSE associated with typical gCJD mutations such as E200K (Gao et al. 2019).

An important point of interest for biomarkers in gTSEs is to analyze their potential use as surrogate parameter for disease progression in clinical trials. These data might be used for selection of homogenous patient groups when testing new drugs to obtain a more reliable assessment of their effects on the disease progression and to reduce the sample size needed in clinical trials. In addition, such biomarkers might be used to monitor the disease progression (Vallabh et al. 2020, Hermann et al. 2020, Thompson et al. 2021).

33.7 Differential Diagnosis

The differential diagnosis of sCJD includes a large number of neurological and psychiatric diseases (Van Everbroeck et al. 2004; Schmidt et al. 2010; Maat et al. 2015; Chitravas et al. 2011; Papageorgiu et al. 2009). In most cases, the diagnosis of CJD as the primary diagnosis is not taken into account when patients are admitted to hospital. Alzheimer's disease is the most important differential diagnosis in older patients. Rapid disease courses, in particular, can rarely be discriminated from CJD, especially when myoclonus is present. Dementia with Lewy bodies is another neurodegenerative dementia that must be considered (Gaig et al. 2011). Because CJD typically presents as a rapidly evolving

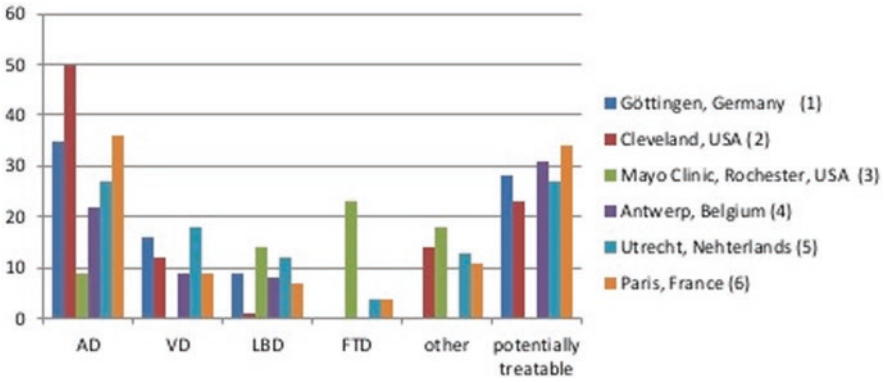


Fig. 33.7 Diagnosis of rapid progressive dementia in % in various retrospective analyses of surveillance/neurology units

neurological disorder, the spectrum of differential diagnosis also comprises some treatable or reversible diseases or acute conditions. Figure 33.7 gives an overview on differential diagnoses of CJD in specialized centers. It indicates that potentially reversible conditions may be present in about 30% of suspected prion disease cases (Kelley et al. 2008). Moreover, studies from nonspecialized centers reported that immune-mediated and infectious encephalitides were the most frequent diagnosis among patients with rapidly progressive dementia (Zhang et al. 2017; Studart Neto et al. 2017; Anuja et al. 2018). Thus, a clinical suspicion of prion disease has to be validated through biomarker-based diagnostics and a thorough exclusion of potential mimics before a final diagnosis is made.

33.8 Criteria

The symptoms and signs of disease in patients with prion diseases are heterogeneous. This heterogeneity is the result of the involvement of various brain structures and still undefined biological determinants influencing disease course. The classification criteria are based on the etiology of the disease, which can be divided into four categories: sporadic, iatrogenic, familial/genetic, and variant CJD (WHO 2003; Will et al. 2000; Zerr et al. 2009; Hermann et al. 2021). Criteria for sporadic CJD have been amended by 14-3-3 CSF test, MRI, and more recently, detection of the PrPSc. They are displayed in Fig. 33.8.

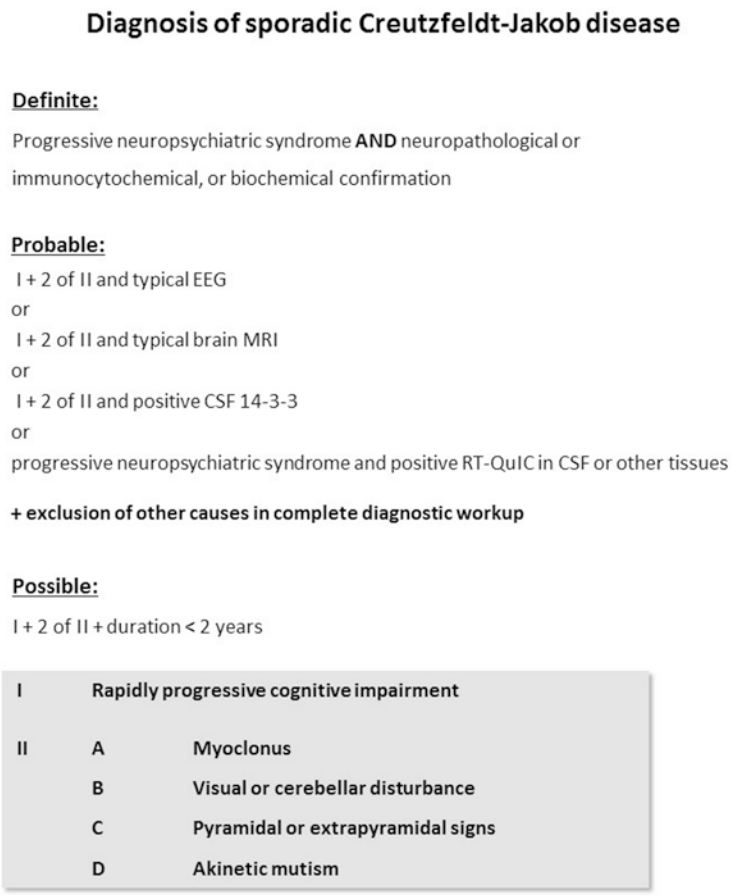


Fig. 33.8 Criteria for sporadic CJD. http://cjd-goettingen.de/en/for-doctors/criteria-of-sporadic-cjd

33.9 Conclusions

Creutzfeldt–Jakob disease is a frequent cause of rapid progressive dementia. Achieving a correct early diagnosis has important implications for (1) distinguishing prion disease from other, potentially treatable diseases, (2) preventing infectious material from being distributed via blood transfusions, surgery, or organ donations, and (3) selecting homogeneous population for upcoming drug trials. The clinical diagnosis of sCJD is supported by detection of biomarkers in blood or CSF, including the biomarkers such as 14-3-3 and tau/phosphorylated tau and recently detection of the abnormal PrP in the CSF. Clinical diagnostic criteria were amended and validated (Hermann et al. 2021, Watson et al. 2022). Advanced brain imaging techniques significantly contribute to the clinical diagnosis, on the one hand, but might

also help in the early differentiation of molecular disease subtypes in sporadic CJD, on the other hand.

Progress in development of blood-based biomarker is substantial and will further add to the development of monitoring strategies once powerful anti-prion drugs will appear on the horizon.

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Chapter 34
Human Prion Disease Surveillance

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Abstract Human prion diseases are characterized by rapid fatal neurodegeneration caused by pathologic prion proteins that are transmissible under specific circumstances. Although the minority of human prion diseases are acquired through transmission, all prion diseases have the potential for transmission. The main goals of human prion disease surveillance are to reduce the public health burden by helping to recognize and prevent acquired prion diseases and to increase prion disease-related knowledge (e.g., identifying possible new prion diseases, new diagnostics, and treatments). Most international human prion disease surveillance programs were initially created in response to concerns about the zoonotic potential of an outbreak of a new cattle disease, bovine spongiform encephalopathy (BSE), which was identified in the 1980s and the discovery of its human form, variant Creutzfeldt–Jakob disease (CJD), first reported in 1996. Continued surveillance is warranted because of the continued public health threats of variant and iatrogenic CJD and concerns about the zoonotic potential of other animal prion diseases (e.g., chronic wasting disease and camel prion disease). These two animal prion diseases present many challenges to animal health and could potentially become a threat to human public health. The incidence of human prion disease has risen over time in most countries, prompting investigations of unrecognized methods of transmission. In this chapter, the authors will review known and potential transmissible causes of human prion diseases. Methods of human prion disease surveillance and the rationale for ongoing surveillance activities will be reviewed. Finally, the authors will offer recommendations for continued surveillance.

Keywords Prion disease · Creutzfeldt–Jakob disease · Surveillance · Public health · Chronic wasting disease

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34.1 Introduction

Human prion diseases, also known as transmissible spongiform encephalopathies, are invariably fatal, rapidly progressive (death usually within 1 year of onset), neurodegenerative conditions caused by disease-causing prion proteins. Unlike conventional transmissible diseases that require nucleic acid for replication, prions employ template-directed protein misfolding to convert normal cellular prion protein (PrP^{c}) into the disease-causing isoform (PrP^{D}) (Prusiner 1982). Prion diseases have been detected in several animals and include scrapie (goat and sheep), bovine spongiform encephalopathy (BSE), chronic wasting disease (cervids), transmissible mink encephalopathy (mink), and camel prion disease (camels).

Neuropathologically, prion diseases exhibit astrogliosis, spongiform changes, and neuronal loss as well as protease-resistant prion protein deposition (Appleby et al. 2018). Neuropathologic changes and biochemical properties of PrP^{D} vary between prion diseases.

There are three main epidemiological categories of human prion diseases: sporadic, genetic, and acquired. Most human prion diseases (~85%) occur sporadically with no identifiable environmental etiology and include sporadic Creutzfeldt–Jakob disease (sCJD), sporadic fatal insomnia (sFI), and variably protease-sensitive prionopathy (VPSPr). Presumptive theories for the cause of sporadic prion diseases include the stochastic production and reduced clearance of the replicating PrP^{D} as well as possible somatic mutations. Approximately 10–15% of human prion diseases are due to a genetic mutation in the prion protein gene (*PRNP*) that reduces the stability of PrP^{C}. These latter diseases include genetic Creutzfeldt–Jakob disease (gCJD), Gerstmann–Straussler–Scheinker disease (GSS), and fatal familial insomnia (FFI). Over 50 different pathogenic mutations have been described with various probabilities of causing disease and are usually the result of point mutations or deletions/insertions within an octapeptide region of *PRNP* (Kim et al. 2018). The remainder of cases (<5%) are acquired prion diseases, in which an individual develops prion disease due to exposure to exogeneous prions. Acquired prion diseases include kuru (now extinct), iatrogenic CJD (iCJD), and variant CJD (vCJD).

Because of the transmissible nature of prions and the recognition of acquired forms of the disease, surveillance has important public health implications. In this chapter, we will review the known causes of prion disease transmission and potential threats for prion disease transmission. Various methods of surveillance will be described and compared. Lastly, the rationale and need for continued long-term surveillance of human prion disease will be reviewed (Budka and Will 2015; Ward et al. 2018; Watson et al. 2021).

34.2 Acquired Human Prion Diseases

34.2.1 Kuru

Understanding the transmissible elements of prion diseases commenced with the discovery and study of kuru, a rapidly progressive neurodegenerative condition that affected the Fore linguistic group of Papua New Guinea (Gajdusek and Zigas 1957). Characterized by a rapidly progressive ataxia that culminated in dementia, myoclonus, and akinetic mutism, kuru primarily affected women and young children who participated in mortuary ritualistic endocannibalism (males over 8 years old did not participate in this ritual). Endocannibalism is the most likely means of disease transmission within the tribe. The neuropathology of kuru resembled scrapie, prompting further investigation of its potential transmissible properties (Hadlow 1959; Chandler 1961). Subsequently, Carleton Gajdusek and colleagues were able to successfully transmit a kuru-like illness in chimpanzees by intracranially inoculating them with brain tissue from individuals affected by kuru (Gajdusek et al. 1966). This discovery led to similar experimental studies using brain tissue of patients affected by various neurodegenerative conditions. CJD, sharing similar neuropathologic characteristics as scrapie and kuru, was successfully transmitted to chimpanzees through intracranial inoculation and was therefore associated with what were previously called "slow viral illnesses" (Gibbs et al. 1968). The moniker of "slow viral illnesses" denoted the transmissible nature of the diseases that included prolonged incubation periods.

In addition to the transmissible nature of these diseases, kuru was also the first human prion disease in which incubation periods could be estimated. Because Australia colonized Papua New Guinea and banned endocannibalism during a specific time frame, investigators could determine incubation periods with a fair degree of accuracy. Although the mean incubation period for kuru is estimated to be approximately 12 years, incubation periods ranged from as short as 4–5 years to 50 or more years in some cases (Collinge et al. 2006). Additionally, the *PRNP* codon 129 polymorphism affected susceptibility to kuru as well as the length of its incubation period (Mead et al. 2008). Heterozygosity (methionine–valine) at codon 129 provided relative resistance to kuru and prolonged incubation periods for those who did succumb to the disease. The prolonged incubation period and the influence of codon 129 polymorphism are pertinent to all acquired human prion diseases. The long incubation periods also increase the public health importance of early identification of new or highly suspected environmental sources of human prion infections and instituting preventive measures early.

34.2.2 Iatrogenic Creutzfeldt–Jakob Disease

Iatrogenic CJD (iCJD) is the inadvertent transmission of CJD through medical treatments and procedures. Several routes of iatrogenic transmission are recognized, including the use of cadaveric human growth hormone (hGH) and pituitary gonadotropins, dura mater grafts, and corneal transplants, as well as via contaminated neurosurgical instrumentation. Transmissibility via blood transfusions appears to be unique to variant CJD (vCJD) and will be discussed later. Although precautions are currently taken to prevent iatrogenic transmission, such as using recombinant hGH, synthetic dura mater grafts, and screening corneal transplant donors and neurosurgical patients prior to surgery, iCJD cases still occur primarily because of very long incubation periods, extending greater than 40 years in some cases (Brown et al. 2012).

Certain characteristics of iCJD are worth noting to inform future surveillance efforts. Peripheral (e.g., non-central nervous system) exposures, such as treatment via intramuscular injections of cadaveric hGH and pituitary gonadotropin, are associated with the longest incubation periods (mean ~17 years), a mean that is increasing as cases with longer incubation periods continue to occur. The duration of the incubation periods also varies inversely to the level of prion contamination. Such peripheral exposures also commonly present at illness onset with cerebellar symptoms. Cadaveric dura mater graft-associated iCJD has a relatively longer mean incubation period (~12 years) compared to other central nervous system exposure cases (e.g., neurosurgical instrumentation) (~1.6 years), although this relative short period may be influenced by the likely greater difficulty in confirming the exposures of rare cases with long incubation periods (Bonda et al. 2016). *PRNP* codon 129 polymorphism appears to affect disease susceptibility with methionine homozygotes being overrepresented in iCJD cases. In general, methionine–valine heterozygotes have longer incubation periods. The prompt recognition of iCJD and at-risk procedures through surveillance activities was pivotal to the prevention of further exposures and the subsequent development of additional iCJD cases.

34.2.3 Variant Creutzfeldt–Jakob Disease

Variant Creutzfeldt–Jakob disease (vCJD) is a human prion disease which is believed to be due to eating beef products contaminated with bovine spongiform encephalopathy (BSE). The BSE epidemic mainly affected the United Kingdom, but also affected other areas of the world, primarily other European countries. Despite millions of individuals likely exposed to BSE, as of November 2021, the number of vCJD cases worldwide is 232, with most cases occurring in the UK (n = 178) (eurocjd.ed.ac.uk/data_tables). Although the origin of BSE is unknown, its source is likely due to the contamination of cattle feed with another animal prion disease (e.g., scrapie) during the feed rendering process (Kimberlin and Wilesmith

1994). The BSE epidemic was perpetuated by refeeding cattle BSE-contaminated meat-and-bone meal. Once this feeding practice was banned and increased prohibitions established to prevent other feed from becoming contaminated with such meat-and-bone meal, BSE was virtually eliminated from cattle herds.

BSE was first suspected of infecting humans in the mid-1990s when a series of atypical human prion diseases were discovered in the United Kingdom. Initially termed new variant CJD (vCJD), these cases were marked by several clinical characteristics (Will and Ironside 1996). The reported cases were much younger compared to typical cases of sporadic CJD, ranging in ages from 16 to 39 years. Survival time was longer than typical CJD cases, and early symptoms were typically psychiatric or sensory in nature. Additionally, the neuropathology of these cases demonstrated the presence of florid plaques. The biochemical characteristics of prions isolated from these cases strongly resembled those found in BSE (Collinge 1999). The peak incidence of vCJD occurred in 2000, but additional cases still occur from time to time. All but one definite case of vCJD has been homozygous for methionine at codon 129 of *PRNP*, strongly indicating that this genotype is a risk factor for disease. This one definite case was heterozygous at codon 129 and reflects that disease susceptibility and incubation period may vary by codon 129 genotype, as observed in other acquired prion diseases.

There have been four cases of secondary transmission of vCJD through non-leukodepleted blood transfusions (Llewelyn et al. 2004; Peden et al. 2004; Wroe et al. 2006). Unlike other prion diseases, vCJD appears to be transmitted via blood transfusions, which is likely due to its presence in lymphoreticular tissue. The incubation periods in three of the patients ranged from 6.5 to 8.5 years. The fourth patient had laboratory evidence of vCJD after dying from a non-neurologic condition >5 years after receiving blood transfusion from a donor who developed vCJD. A fifth case developed asymptomatic infection due to factor VIII treatment for hemophilia, died of unrelated causes, and was found to have prion protein deposition in a single spleen sample (Peden et al. 2010). These discoveries, enabled through surveillance activities, led to many countries creating blood donor deferral policies to secure the safety of the blood supply from vCJD contamination.

34.3 Methods of Surveillance

Public health surveillance is the ongoing, systematic collection, analysis, interpretation, and dissemination of data regarding a health-related event for use in public health action to reduce morbidity and mortality and to improve health (Centers for Disease Control and Prevention 2001). The purpose of prion disease surveillance is to ascertain cases to determine if there are trends in the frequency, distribution, and/or types of prion diseases within a given population. A robust surveillance system should be able to detect as many cases of prion diseases as possible reliably and accurately, with the realization that 100% capture rate is unlikely. Common features of prion surveillance programs are described in the World Health Organization

document, "WHO manual for surveillance of human transmissible spongiform encephalopathies including variant Creutzfeldt–Jakob disease" (World Health Organization 2003).

Surveillance entails education, outreach, and collaboration with entities that are likely to encounter prion diseases. Most cases of prion diseases are diagnosed clinically while the patient is still living. Surveillance programs that educate, communicate, and aid clinicians in diagnosing prion diseases can provide a reasonable accounting of case frequency as well as the identification of cases with known acquired prion disease risk factors and atypical prion diseases. Hence, many surveillance programs offer clinical diagnostic testing or consultations for suspected prion disease cases. This is often in the form of diagnostic cerebrospinal fluid (CSF) testing, genetic testing, brain MRI consultation, and clinical evaluations. Such an active engagement with clinicians improves clinical management of patients and alerts the surveillance system about suspected cases of prion disease. Making available laboratory diagnostic resources and consultation services as part of a surveillance program allows for direct outreach to clinicians caring for suspected cases to inform them of crucial surveillance activities, such as post-mortem evaluation. Use of these services can be paired with data collection tools that inquire about known acquired prion disease risk factors (e.g., cadaveric human growth hormone) or possible risk factors (e.g., venison consumption). These data can be actively collected on potential prion disease cases by including survey questions as part of the test requisition or autopsy consent forms. Such an approach standardizes data collection, which may not be systematically collected or documented the same way in medical records.

Some countries' surveillance systems have a clinical component in which an expert team is dispatched to evaluate suspected cases of prion disease for a comprehensive clinical evaluation and interview. Benefits of this approach include standardized collection of medical history, known and potential risk factor data, and clinical features. Surveillance using this approach was established in the United Kingdom in 1990 because of concerns about the zoonotic potential of an outbreak of BSE first recognized there in 1986. This surveillance enabled the quick identification and dispersal of information regarding the clinical phenotype associated with vCJD (Will and Ironside 1996). However, employing and distributing expert clinical teams can be time-consuming and costly. A surveillance system's ability to partake in these activities is partially dependent on healthcare and public health systems as well as population size and geography of the area undergoing surveillance. For example, clinical means of surveillance are likely to be easier and more complete in countries that employ national health care, where healthcare data can be assessed from a central repository. Additionally, sending clinical teams out to evaluate cases of suspected prion disease may not be feasible for large countries with a geographically dispersed population. Telemedicine can address some of these challenges (Appleby et al. 2019b; Watson et al. 2020). Examinations may be limited with this approach, but medical histories pertaining to known and potential acquired prion disease risk factors are more complete when conducted via telemedicine compared to medical record review alone (Appleby et al. 2019b).

A neuropathologic component of prion disease surveillance is crucial as it is the only way to achieve a definitive diagnosis as well as the only way to confirm clinically suspected or diagnosed specific types of prion diseases (e.g., vCJD). Because of the clinical heterogeneity observed in sCJD, diagnosis can be difficult and may overlap with several other disease entities. Autopsy confirmation of prion disease is important to verify the diagnosis and monitor the emergence of novel variants. This is especially important when investigating clusters of suspected prion disease, as recently demonstrated in New Brunswick, Canada, where a variety of non-prion disease pathologies were present on post-mortem evaluation. Neuropathologic evaluation can also uncover prion diseases with an incongruent clinical phenotype, as seen in the recent codon 129 heterozygous vCJD case that clinically resembled sCJD (Mok et al. 2017). Disease-causing prions can be isolated from brain tissue and strains can be characterized using biochemical analyses and bioassays, something that is not possible with purely clinical surveillance activities. The collection of tissues and clinical and epidemiological data on patients with clinically suspected prion disease that are subsequently confirmed as having or not having a prion disease can be used to facilitate research and evaluations of clinical diagnostic tests (e.g., MRIs, CSF RT-QuIC assays). Longitudinal and widespread neuropathologic surveillance is important to establish historical controls separated by time and geography for analyses of potential transmissible risk factors (e.g., exposure to CWD through contaminated venison). Screening of non-prion disease brain banks, such as those used in Alzheimer's disease and related dementia registries, could also be useful to detect unrecognized and/or atypical clinical presentations of prion disease (Peden et al. 2019). Similarly, autopsies should be considered in children and adolescents with progressive neurodegenerative decline as several acquired prion diseases occur in younger populations (Verity et al. 2019). Neuropathology is the gold standard for diagnosis and surveillance and should be heavily featured in any prion disease surveillance program.

There are a variety of epidemiology-based surveillance methods that are reliant on the accuracy of the database systems from which the data are collected. In many countries, analyses of routinely collected death certificate data can be a cost-effective mechanism for estimating prion disease incidence rates and assessing the temporal, geographic, and demographic features of identified cases. Unfortunately, several studies have demonstrated that death certificate data alone require cautious interpretations because they include both a majority of the valid prion disease cases and many non-prion disease cases as well. For more accurate data, it is best to combine such mortality data with multiple other mechanisms of surveillance (Brandel et al. 2011; Barash et al. 2014). For example, prion disease surveillance based on death certificates can be very useful when combined with surveillance mechanisms based on neuropathologic findings or CSF RT-QuIC results (Maddox et al. 2020). Hospital discharge records may also be used, but similar to diagnoses on death certificates, may be affected by pending laboratory results at the time of discharge that reduce their reliability as a sole source of prion disease surveillance. (Barash et al. 2014; Kotkowski et al. 2020) Local public health departments in key geographic

areas can be used to further investigate specific cases and educate local clinicians (Sánchez-González et al. 2020).

Because earlier criteria for probable sCJD required detailed clinical history that included clinical symptoms and various diagnostic test results, surveillance of prion disease cases lacking autopsy was difficult and time-consuming. However, with the recent change in criteria for probable CJD that requires a positive RT-QuIC result in the setting of a neuropsychiatric syndrome, ascertainment of probable CJD cases is more easily achieved (Centers for Disease Control and Prevention 2019). Laboratory-based ascertainment of probable CJD cases as detected by positive CSF RT-QuIC increased overall detection of prion disease based on autopsies alone by over 90% in one study (Rhoads et al. 2020). Similar increases in CJD incidence using amended RT-QuIC criteria have been demonstrated in other studies (Hermann et al. 2018).

34.4 Rationale for Surveillance

34.4.1 Zoonotic Threats

34.4.1.1 Variant Creutzfeldt–Jakob Disease

Although the peak incidence of vCJD appears to have passed, it remains an important focus of prion disease surveillance. As demonstrated with kuru and iatrogenic CJD, acquired prion diseases are characterized by prolonged incubation periods measured by decades. Similarly, new cases of vCJD might occur from BSE exposure in the 1980s and 1990s. Acquired prion diseases are also characterized by shorter incubation periods in codon 129 homozygotes and longer incubation periods in heterozygotes. All but one case of definite vCJD have been codon 129 methionine homozygotes, which implies that we may see further cases from the same exposure in individuals with different codon 129 polymorphisms (i.e., valine homozygotes and heterozygotes). The clinical phenotype may also differ by codon 129 polymorphism. For example, the one autopsy confirmed vCJD case that was heterozygous at codon 129 presented with a clinical phenotype and brain MRI that were characteristic of sCJD and did not meet clinical criteria for possible or probable vCJD (Mok et al. 2017). These findings make autopsy confirmation of cases even more important, as prion disease subtyping by clinical criteria alone is not entirely accurate. Prior assumptions such as young age and psychiatric symptoms at initial presentation being suggestive of vCJD may not be reliable. As we get further away from the initial exposure period, patients with vCJD are expected to be older compared to prior cases. Additionally, psychiatric presentation at illness onset may be more related to age at disease onset as opposed to clinical characteristics specific for vCJD (Appleby et al. 2007).

Continued surveillance for vCJD is also necessary because of secondary transmission through blood products. Millions of people were likely exposed to BSE, yet only 232 cases of vCJD have been detected to date, which suggests that a certain

proportion of the exposed population is asymptomatically infected. Blood infectivity from such possibly infected people would be likely at least in part due to its deposition in lymphoreticular tissue. Several studies have estimated the prevalence of asymptomatic infectivity in UK individuals by screening lymphoreticular tissues. The first study screened tonsillectomy (n = 1739) and appendectomy (n = 14,964) samples for abnormal prion proteins. Three appendectomy samples demonstrated prion protein deposition, resulting in an estimated prevalence of 1 in 4000. A second study detected 16 positive appendectomy samples out of 32,441 screened, with an estimated asymptomatic infectivity prevalence of 1 in 2000. The most recent study addressed the prevalence of asymptomatic infectivity in individuals born after the presumed end of the BSE epidemic and in those who had appendectomies before the presumed start of the BSE epidemic (Gill et al. 2020). Positive samples were detected in both groups and the prevalence did not vary significantly from the two prior studies, suggesting that either some of the appendices were falsely positive for vCJD or that the duration of the BSE epidemic was longer than what was initially presumed. These studies also demonstrated asymptomatic infectivity in all codon 129 polymorphisms. Two studies only screened tonsil specimens and detected one possible prion positive sample; however, this finding could be explained by the possibility that tonsil infectivity may be a relatively late stage finding (Clewley et al. 2009; Marco et al. 2010; Watson et al. 2021). In general, these studies justify a cautionary approach of continuing blood donation deferral programs and prion disease surveillance because of the risk of secondary transmission of vCJD.

Although there appears to be little increased risk for developing classic forms of CJD among various occupations, there are two cases of vCJD that may be attributed to occupational exposure through prion disease laboratory work. One individual handled frozen brain sections from transgenic mice that overexpressed human prion protein that were infected with sheep-adapted BSE (Brandel et al. 2020). She had a documented puncture injury during her laboratory work and developed symptoms consistent with vCJD 7.5 years later. The diagnosis of vCJD was confirmed at autopsy, and she was methionine homozygous at codon 129. Given the incubation period between the occupational injury and disease onset, which is consistent with transfusion-related vCJD, it is likely that the occupational injury involving BSE-contaminated tissue caused her illness. An additional Italian laboratory worker that worked with BSE infected brains developed vCJD, but this individual had no documented occupational injury and little is published about this case (Brandel et al. 2020).

34.4.1.2 Atypical Bovine Spongiform Encephalopathy

Classical BSE has been linked to vCJD, but other forms of BSE have also been detected. Cattle surveillance programs uncovered additional forms of BSE termed atypical BSE that could be separated into L-BSE and H-BSE, named for low and high molecular weights of protease resistant PrP on western blot analyses, respectively. Unlike classical BSE, atypical BSE cases are found in low frequency and

among older cattle that usually do not have symptoms (Houston and Andréoletti 2019). Because of these features, atypical BSE would be more likely to make it into the human food supply compared to classic BSE, although preventive measures to protect consumers from classic BSE would presumably reduce the risk of exposures to the prions of atypical BSE as well. Transmission studies demonstrate differences between the two atypical BSE types. L-BSE, but not H-BSE, was successfully transmitted to macaques and transgenic mice expressing the human prion protein (Comoy et al. 2008; Béringue et al. 2008; Ono et al. 2011). As opposed to sCJD strains, 25% of transgenic mice expressing the human prion protein that were inoculated with L-BSE had prion deposition in the spleen, suggesting that L-BSE is lymphotropic (Kong et al. 2008). Strain typing comparisons between L-BSE and sCJD subtypes have failed to demonstrate an association between the two (Jaumain et al. 2016). In summary, L-BSE appears to have a higher zoonotic potential than H-BSE and may be more virulent than classical BSE. Although the study by Jaumain and colleagues is encouraging that atypical BSE does not appear to be a cause of sCJD, continued vigilance is warranted given the results of animal studies involving L-BSE.

34.4.1.3 Chronic Wasting Disease

Chronic wasting disease (CWD) is a prion disease that affects cervids (e.g., deer and elk). First identified in 1967 in a Colorado research facility, it has since been detected in at least 27 states, 2 Canadian provinces, South Korea, Norway, Finland, and Sweden. Infectious prions are known to be secreted in urine, saliva, and feces, resulting in significant horizontal transmission in domesticated and free-ranging cervids (Haley et al. 2009). Cervids affected by CWD are potentially infectious before illness onset because of extensive prion protein deposition in lymphoreticular tissue prior to spread to the central nervous system (Hoover et al. 2017). Long-term contamination of the environment with CWD prions is a concern for continued propagation of infectivity among cervids, and there is currently no robust management plan in place to control the CWD epidemic in free-ranging animals (Osterholm et al. 2019).

A significant portion of the US population consumes venison, including venison derived from free-ranging deer and elk, raising concern of possible transmission of CWD to humans. A survey conducted by the Foodborne Diseases Active Surveillance Network from 2006 to 2007 indicated that more than two-thirds of respondents had ever consumed venison and 18.5% had hunted cervids, with 1.2% having a history of hunting cervids in a CWD-endemic area (Abrams et al. 2011). Given the continuing spread of CWD (a total of 27 states have had documented CWD in free-ranging cervids as of January 2022), the risk of exposures of people to CWD-infected animals undoubtedly is increasing. CWD prions have been detected in skeletal muscle of infected cervids, even in those without clinical symptoms, and would be the most likely tissue source for transmission to humans (Daus et al. 2011). Primate CWD transmission studies have demonstrated mixed results. Several studies have demonstrated transmission of CWD to squirrel monkeys via intracranial (IC) and oral (PO)

routes (Marsh et al. 2005; Race et al. 2009). Cynomolgus macaques, which are more related to humans, were not successfully infected by CWD in one study (Race et al. 2018). An unpublished study, presented at several international conferences, has reported successful transmission of CWD to macaques using IC CWD-contaminated steel wire implants and oral consumption of skeletal muscle from asymptomatically infected deer (Osterholm et al. 2019). In vitro and transgenic animal models have also produced mixed results. Using protein misfolding cyclic amplification (PMCA), CWD prions were able to transform human PrP^{c} into a novel form of human PrP^{Sc} following serial passages, suggesting that CWD can theoretically be transmitted to humans, especially in the setting of strain adaptation that is likely occurring with the extensive spread of CWD (Barria et al. 2011). A robust species barrier to CWD was demonstrated in a transgenic mouse model expressing the human prion protein, in which no infected animals developed disease after prolonged post-inoculation periods (Kong et al. 2005). There has not been any evidence that CWD has transmitted to humans and caused symptomatic prion disease. Five epidemiologic studies using various methodologies conducted between 2000 and 2016 failed to demonstrate CWD-related human prion disease (Waddell et al. 2018). There have been no novel prion diseases associated with venison consumption or hunting in areas affected by CWD. However, one study comparing prion protein properties of CWD with CJD subtypes found that there were biochemical similarities between CWD and sCJD of the MM1 subtype (Xie et al. 2006). Thus, it is important to thoroughly evaluate cases of sCJD that resemble known subtypes in case of the possibility that CWD transmitted to humans resembles recognized sCJD phenotypes.

In addition to the primary transmission of CWD to humans, secondary transmission through other animal sources is also a concern. The extensive environmental contamination of CWD prions by infected cervids, oftentimes in unknown geographical distributions, could potentially infect other domestic animals that may enter the human food supply. Multiple transmission studies have been performed in a variety of wild type and transgenic animals. Although CWD has been successfully transmitted to many animals via IC inoculation, few animals outside of cervids have been infected through a PO transmission route (Kurt and Sigurdson 2016). Abnormal prion protein seeding activity was detected in the central nervous system (CNS) and occasionally in peripheral tissues when cattle were IC inoculated with CWD (Haley et al. 2016). Swine infected with CWD via IC and PO routes demonstrated infectivity of brain and lymphoid tissues, but only one pig developed symptoms suggestive of disease. These findings suggest that some infected non-cervid animals may harbor infectivity without clinical symptoms and could easily enter the human food supply (Moore et al. 2017).

CWD presents unique challenges and has the potential for human transmission, which is best addressed by continued human prion disease surveillance. Threats to human public health include primary transmission of CWD from infected deer or elk as well as secondary transmission from other animal sources. Current human transmissibility data suggest that the likelihood of CWD transmission to humans is low but not impossible. As most deer are free-ranging animals, controlling disease

spread is more difficult compared to prion disease outbreaks in domestic animals. With the widespread dissemination of CWD and newly developing strains emerging, the CWD landscape is evolving (Tranulis et al. 2021). Transmissibility across animal species is strain dependent, and as more strains of CWD are discovered, the possibility of human transmission may also change (Pritzkow et al. 2021). The continued spread of CWD, with no end in sight, evolving CWD strains, and prolonged incubation periods of prion disease call for continued long-term human prion disease surveillance. Additionally, historic tissue collected from time periods and geographical regions unaffected by CWD are crucial for comparison studies should suspected CWD transmission to humans occur.

34.4.1.4 Camel Prion Disease

The newly discovered camel prion disease raises significant concerns regarding the completeness of our knowledge concerning animal prion diseases. In 2018, Babelhadj and colleagues reported a prion disease of dromedary camels in Algeria (Babelhadj et al. 2018). PrP^{Sc} deposition was demonstrated in the brains of three symptomatic camels, and it was estimated that approximately 3% of camels presenting to a specific abattoir were affected. Notably, prions were also detected in lymphoreticular tissue, mirroring that of other highly infectious animal prion diseases (e.g., scrapie and chronic wasting disease). Little is known about camel prion disease, such as its origin and transmissibility risk to other animals, including humans. Camel meat and milk are commonly consumed in the Middle East and Africa, but neither geographic region has a surveillance program for human prion disease. Lack of human prion disease surveillance in these areas substantially affects our ability to investigate the transmissibility risks to humans. Future studies should examine primate and transgenic animal models for the potential transmission to humans. International human prion disease surveillance programs should be aware of this new disease and its possible pertinence to individuals with prion disease who have lived in this area of the world.

34.4.2 Increasing Incidence of Prion Disease Over Time

A core feature of human prion disease surveillance is examining its incidence to detect any increase in cases. The annual incidence of human prion disease is estimated to be between 1 and 2 new cases per million individuals, but is heavily influenced by surveillance intensity (Klug et al. 2013). Most countries have observed an increased incidence of prion disease over time, which is likely multifactorial in origin. Like other neurodegenerative diseases, age is a significant risk factor for sCJD. In the United States, the average annual incidence in those 65 years of age or older is 5.9 per million, compared to the average annual incidence of all age groups of 1.2 per million (Maddox et al. 2020). As many countries have an aging

population, there would be an expected increase in prion disease cases, specifically in older individuals. Clinical ascertainment has also greatly improved over the last couple of decades with the identification of novel prion diseases with atypical clinical presentations such as fatal insomnia (FI) and variably protease sensitive prionopathy (VPSPr) (Cracco et al. 2018; Notari et al. 2018). The ability to diagnose cases has greatly improved over this time period with the introduction of highly sensitive and specific diagnostic tests, including brain MRI and RT-QuIC (Bizzi et al. 2020; Rhoads et al. 2020). Clinician and public awareness have also improved because of increased case ascertainment due to better diagnostic tests and because of the outbreaks of BSE, vCJD, and CWD. Although there are multiple valid explanations for the rising incidence of human prion diseases, surveillance remains critical to ensure that other explanations are not missed.

34.4.3 Investigation of Unrecognized Transmission Routes

Most prion diseases (>85%) are sporadic in etiology, meaning that there is no recognized environmental source of infection. Other age-related neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease) are also mostly comprised of sporadic cases. Common explanations of sporadic prion disease include post-translational modification of PrP^c that may be due to age-related protein dysmetabolism as well as possible somatic mutations of *PRNP*. However, it is also possible that some cases of sCJD may be due to unrecognized transmission routes or sources of infection. Surveillance programs are crucial to examining these questions through the collection of standardized information on confirmed cases of prion disease.

One of the most frequently examined possible source of sCJD is the use of contaminated surgical instruments. Highly sensitive seeding assays have detected abnormal seeding activity in many tissues of sCJD patients, including skin and ocular tissues (Orrù et al. 2017, 2018). However, most tissues in the body are considered to have low or no detectable levels of infectivity as generally determined by bioassays (World Health Organization 2005). Multiple epidemiologic studies have reported on the possibility of surgical risks for the transmission of prion diseases, the majority of which are confounded by a variety of study biases. A recent meta-analysis on the subject found that the quality of evidence was low across studies (López et al. 2017). A positive association was found between sCJD and heart, vascular, and eye surgery. No association was detected between sCJD and tonsillectomies, appendectomies, and neurosurgeries, despite the latter being known to have transmitted CJD. One study reported an increased risk of sCJD that depended on the age at first surgery, which is in agreement with known prolonged incubation periods of prion diseases (de Pedro-Cuesta et al. 2014). In total, data are of low quality but suggestive that if a prion disease is transmitted through unrecognized surgical exposure, then it is likely occurring at a very low rate.

Another theoretical route of transmission is via blood transfusion. As previously discussed, secondary transmission of vCJD via blood transfusion has been

demonstrated. Multiple studies have examined the potential risk of transmission of non-vCJD prion diseases through blood transfusion. Multiple case control and look-back studies, in which blood recipients from donors who succumbed to CJD were followed to determine if they developed prion disease, have been conducted. None of these studies examining non-variant forms of prion diseases found any epidemiologic evidence of transfusion transmitted prion diseases (Wilson et al. 2000; Crowder et al. 2017). One case–control study found a significant risk of developing sCJD in individuals who had blood transfusions more than 10 years prior to their onset of sCJD; however, these results could be the result of study biases (Puopolo et al. 2011). Like the risk from surgical exposures, if a non-variant form of prion disease is being transmitted via blood transfusion, it is likely at a very low frequency.

Several studies have examined occupational risk factors for developing sCJD. Most studies have not demonstrated an increased risk of sCJD among healthcare workers (Wientjens et al. 1996; van Duijn et al. 1998; Cocco et al. 2003; Ruegger et al. 2009; Alcalde-Cabero et al. 2012). Although healthcare professionals were not at increased risk in one study, people working at physicians' offices were at increased risk of developing sCJD (Alcalde-Cabero et al. 2012). A study in Germany indicated an increase rate of sCJD in physicians in recent years and most of these physicians were surgeons (64%) (Hermann et al. 2020). One study found an increased risk of sCJD among workers in animal laboratories compared to age-, sex-, and education-matched controls, but other studies have not documented any increased risk in such occupations (Ruegger et al. 2009). Like the surgery and blood transfusion studies, findings from studies examining occupation as a risk factor are somewhat contradictory. Although mostly negative, the studies are likely confounded by multiple biases.

34.4.4 Investigation of Disease Clusters

Infectious diseases tend to form case clusters due to common sources of transmission. Several clusters of prion disease cases have been described in the literature and are an important part of surveillance. When investigating disease clusters, it is important to verify the final diagnosis, preferably by autopsy. Genetic prion diseases should also be ruled out as families often live in close proximity and individuals may not be aware that they are related to one another. Diagnostic bias should also be considered. Individuals may live in an area where there is prion disease or behavioral neurology expertise that would make diagnoses of prion diseases more likely. Finally, common exposure sources should be investigated. Common travel destinations, surgeries, and healthcare settings should be explored (Moreno et al. 2013). Several studies have found geographic clusters of prion diseases, but none have determined a common source of infectivity (Barash and Dziura 2007; Chamosa et al. 2014; Nakatani et al. 2015; Puopolo et al. 2020). These findings may be due to study biases and prolonged incubation periods that make such investigations

difficult. A robust surveillance program can be helpful in the investigation of case clusters and can provide the necessary tools for the investigation.

34.4.5 Provide a Research Platform

Prion disease surveillance programs based on autopsies and collection of clinical and epidemiological data have additional utility outside of conventional surveillance activities, such as providing a platform for collaborative research. Characterization of prion disease cases is essential to have adequate historical controls for case–control comparison studies. Surveillance has also resulted in special studies that led to the discovery of several prion diseases, including the identification of E200K gCJD, which was initially thought to be a disease cluster, FI, and VPSPr. International surveillance efforts have also allowed for a standardized subtyping of sCJD cases (Cali et al. 2006). International tissue banks, largely from surveillance centers, allow for large-scale collaborative research that is impossible to do using data from a single site. Genetic analyses typically require large sample sizes to generate reliable results, and this can be difficult for a rare disease. International collaboration between surveillance centers allowed researchers to investigate important genetic findings such as penetrance among different genetic mutations and to look for polymorphisms outside of *PRNP* that may affect sCJD risk (Minikel et al. 2016; Jones et al. 2020; Brennecke et al. 2021). Surveillance centers will undoubtedly become important for the efficient roll-out of large-scale international clinical trials, for which timely diagnosis and communication of results and study options will be crucial (Appleby et al. 2019a; Watson et al. 2021).

34.5 Conclusions and Recommendations

Human prion disease surveillance has been successful, and it has imparted many lessons. Findings and benefits from prior surveillance activities have demonstrated the need for continued and long-term human prion disease surveillance. There was initial disagreement on whether BSE could transmit to humans, but human prion disease surveillance readily detected this occurrence and likely saved thousands of lives and helped prevent a healthcare catastrophe. Although the incidence of vCJD has declined significantly, there are vCJD-related reasons to continue surveillance. Emerging animal prion diseases such as CWD and camel prion disease raise many questions and pose uncertainty regarding their threat to human health. Incomplete transmission, prolonged incubation periods, novel and rapidly disseminating animal prion diseases, and detection of atypical clinicopathologic phenotypes argue for continuing human prion disease surveillance programs. Failure to continue surveillance may result in extremely late recognition of acquired prion diseases and unnecessary spread, potentially causing preventable morbidity and mortality. The authors

Table 34.1 Aspects of a comprehensive case investigation

Item reviewed	Characteristics examined
Clinical history	Age, duration, clinical presentation, and progression, recognized and potential acquired prion disease risk factors
Diagnostic tests results	Surrogate markers of neurodegeneration (e.g., tau and 14-3-3), RT-QuIC, brain MRI lesion profiles
Western blot analyses of brain tissue	Prion protein type & characteristics (e.g., Types 1, 2, 1 and 2, vCJD, genetic cases, VPSPr)
Histology/ immunohistochemistry	Histotype (e.g., MM(MV)1, VV2)
Genetics	*PRNP* mutations and codon 129 polymorphism
Aspects that may cause variations	Specimen quality, laboratory error
Atypical cases	Consensus conference, literature review, further investigations as needed

RT-QuIC real time quaking induced conversion, *PRNP* prion protein gene

recommend a multi-tier approach to prion disease surveillance that, in addition to analyses of multiple-cause-of-death mortality data, includes collection and analyses of neuropathologic and epidemiological studies, physician outreach and education, centralized diagnostic testing if possible, and collection of risk factor data in a standardized fashion. Each case should be examined for congruency across all investigative elements, and incongruencies should be further investigated (Table 34.1). Continued human prion disease surveillance is necessary to help protect public health from the threat of known and unknown causes of prion disease transmission.

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Part X
Treatment

Chapter 35
Overview on Treatment of Prion Diseases and Decontamination of Prions

Richard Knight

Abstract Currently, there are no prophylactic or disease-modifying therapies for prion diseases with proven, significant efficacy. The discovery of treatments by design is hampered by incomplete understanding of prion disease pathogenesis. However, therapeutic considerations have broadly centered on a loss of function of the normal prion protein or possible toxicity of abnormal prion proteins. Potential disease-modifying treatments have been assessed by in vitro cell-free studies, cell-culture studies, in vivo animal experiments, and in human clinical trials. The last of these poses several problems, including the rarity of prion diseases, variations in the rates of clinical progression, difficulties in measuring this clinical progress, and the difficulty of early diagnosis at a time before significant neurological damage has already occurred (unless preventative treatment is considered in those at risk but not currently ill). Given the transmissibility of prion diseases, one aspect of their prevention involves decontamination of potentially contaminated medical instruments. Unfortunately, prion infectivity is particularly difficult to remove or inactivate, with variations between different prion agent strains and methodological problems in the assessment of the effectiveness of any proposed method. The general principles underpinning prion disease treatment and decontamination are reviewed with reference to past research and current knowledge.

Keywords Efficacy · Diagnosis · Prion decontamination · Prion protein · Treatment of prion diseases

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35.1 Introduction

The prevention of prion disease depends on the type of disease concerned. In acquired forms, protecting human diet from infection, avoiding the use of potentially contaminated materials (including blood), and the satisfactory decontamination of materials or medical instruments are important. Specific therapies could either prevent disease in those at particular risk of it (by exposure to infection or by virtue of inheritance) or treat clinically ill individuals. Prevention is particularly important in the absence of any effective disease treatment. This is an overview of the key concerns in the areas of therapy and decontamination.

35.2 Treatment

35.2.1 Treatment: General Principles

Medical treatment may be preventative, symptomatic, and disease modifying. Given the rarity of prion diseases, preventative measures would be considered for only those at particular risk of illness: known carriers of pathogenic *PRNP* mutations and those known to have been exposed to a relevant risk (such as cadaveric-derived human growth hormone or recipients of blood from a vCJD donor). Various manifestations of human prion disease may be considered for symptomatic treatment (such as agitation or myoclonus), but such symptomatic treatment is not specific to prion diseases and follows general principles. This overview will address mainly prophylactic and disease-modifying treatments. The rational treatment of disease requires diagnosis, and, in general, the earlier a disease is diagnosed, the more efficacious treatment is to likely be; unfortunately, early diagnosis is often problematic in prion diseases. Potential treatments need to be discovered and then assessed (for efficacy and potential toxicity).

35.2.2 Diagnosis

Diagnosis is an important and (in prion diseases) difficult precursor to treatment. There are situations where individuals are known to be at risk of such disease and therefore can, at least in principle, be monitored in order to recognize disease at an early clinical stage. However, in most cases, the diagnosis is generally made relatively late in the illness. This is particularly so in the commonest form, sCJD, where the diagnosis is made typically when there is severe neurological impairment, often only shortly before death. As a general principle, even very effective treatments may not be of much benefit if given late in a disease process. Moreover, even if a treatment halted the progression of prion disease, it would not necessarily undo existing

neurological damage; this might not be advantageous (and might even be regarded as disadvantageous) if it simply left the patient in a severely disabled state.

In the case of sCJD, the presentation is typically neurological and indicates a serious, progressive encephalopathy. However, there are commoner causes of, say, dementia with ataxia, than sCJD. The process of exclusion of other diagnoses necessarily takes time and sCJD is rapidly progressive, with a median duration from first symptom to death of only around 4 months (in most countries). The EEG and CSF 14-3-3 assay show abnormalities not specific to prion disease but have well-established supportive roles in diagnosis (Chaps. 33 and 34). Cerebral MR imaging is important in excluding other possible diagnoses but also often shows abnormalities characteristic of prion disease, varying with disease type (Chaps. 18 and 33). However, the relevant abnormalities are not always present and are not entirely specific for prion diseases. The most important human prion disease diagnostic developments have centered on amplification techniques (such as RT-QuIC and PMCA) that may allow detection of low levels of abnormal prion protein in various tissues (CSF, blood, urine, skin, and nasal brushings) (Chaps. 33 and 34). As these are based on fundamental mechanisms in prion disease, they are specific tests, although only one has been validated sufficiently for routine clinical use: CSF RT-QuIC in sCJD (Atarashi et al. 2011; McGuire et al. 2012) (Chaps. 33 and 34).

In vCJD, the illness progression is typically slower, with a median illness duration of around 14 months. The presentation of vCJD is very nonspecific, typically consisting of psychiatric features without specific neurological symptoms or signs for several months (Spencer et al. 2002). Early diagnosis is potentially very difficult, but it is often made at a stage of lesser neurological disability than in the case of sCJD. Cerebral MR imaging is very useful, but the characteristic abnormalities are not entirely specific to vCJD (Chaps. 33 and 34). There is a potentially useful, disease-specific (albeit somewhat invasive), test in the form of tonsil biopsy (Chaps. 33 and 34). Less invasive tests have been developed, based on protein amplification techniques, and these include three blood tests, although they have not been validated over large numbers in routine clinical practice (Bougard et al. 2016; Edgeworth et al. 2011a, b). One blood test proved positive in the asymptomatic, pre-clinical, phase of vCJD (although in only two individuals), which could be important in considering early treatment; it is difficult to see how a large-scale assessment could be undertaken (Bougard et al. 2016).

The diagnosis of genetic prion disease can be more straightforward: a family history is usually present, and genetic testing for a relevant *PRNP* mutation can be undertaken. The current status of diagnostic test development is detailed in Chaps. 33 and 34.

35.2.3 Disease-Modifying Treatment in Humans

35.2.3.1 The Period Up to 2008

A systematic review summarized the published data concerning prion disease therapy in humans over the period 1971–2007 (Stewart et al. 2008). It found reports of a total of 149 patients treated with 14 drugs. However, most publications concerned single case reports of a few patients, only four were comparative studies with only one of these being a randomized controlled trial (RCT). The reported drugs included Interferon, Acyclovir, Vidarabine, Amphoteracin, Clomipramine, Venlafaxine, Antioxidants, Amantadine, Topiramate, Phenytoin, Levetiracetam, Flupirtine, Quinacrine, and Pentosan Polysulphate; the therapeutic choices reflecting various ideas including possible viral causation, effects on protein aggregation, and possibilities of neuroprotection. In most, there was no convincing evidence of efficacy, but, given the small numbers treated and the poor methodology (including lack of controls), it was often not possible to form an absolutely definitive opinion. The single RCT showed some improvement in the group treated with Flupirtine, compared with placebo. However, this was only a small study (13 patients with active treatment; 15 controls) with the same overall survival in both groups; whether this reflected a symptomatic or a partial disease-modifying effect is uncertain (Otto et al. 2004).

35.2.3.2 Quinacrine

Quinacrine was suggested as a treatment on the basis of in vitro work (Korth et al. 2001). Subsequently, an animal experiment showed no evidence of efficacy (Collins et al. 2002).

Haik and colleagues reported on the open, compassionate use of Quinacrine in France between 2001 and 2002, in 32 patients (30 sCJD; 2 vCJD). There was no evidence of treatment efficacy clinically or pathologically (Haik et al. 2004). A later human trial in the United Kingdom followed a patient-preference protocol; patients were offered the choice of 300 mg of Quinacrine a day, no treatment, or randomization to immediate or deferred Quinacrine treatment (Collinge et al. 2009). Overall, 107 patients with various forms of CJD (including 45 with sporadic and 18 with variant CJD) entered the trial, but only two patients chose randomization. There was no evidence of disease-modifying treatment effect. A further American study of Quinacrine in sCJD managed to enroll 69 patients into a double-blinded, placebo-controlled, stratified randomisation trial, with a primary endpoint of survival from the time of randomization to month 2; there was no evidence of treatment efficacy (Geschwind et al. 2013).

35.2.3.3 Pentosan Polysulphate (PPS)

Animal experiments involving intra-cerebro-ventricular administration of Pentosan Polysulphate (PPS) showed promising results (Doh-ura et al. 2004). In the United Kingdom, several patients were treated on an open, compassionate basis, including sporadic, iatrogeic, variant, and genetic forms of disease. There was a suggeston of slowing-but not halting- of disease progression in some cases (most convincingly in vCJD) (Bone et al. 2008). A report of 11 CJD cases treated in Japan (genetic, iatrogenic, and sporadic) suggested the possibility of longer survival in some cases (Tsuboi et al. 2009). In neuropathological studies of the Japanese treated cases, lower than expected levels of brain PrP^{res} were seen, but whether or not this reflected PPS treatment is uncertain (Honda et al. 2012; Terada et al. 2010). One UK case of PPS-treated vCJD was studied at autopsy with no evidence of reduction of the overall neuropathological changes in the brain (Newman et al. 2014). Overall, there is no evidence of a significant clinical benefit from PPS in humans, and the requirement for intra-cerebro-ventricular administration makes it a difficult treatment to deliver.

35.2.3.4 Doxycyline

Doxycyline (100 mg/day) was studied in a joint French/Italian randomized, double-blinded, placebo-controlled trial between 2007 and 2010. A total of 121 patients (sporadic and genetic prion disease) were recruited; no clinical efficacy was seen (Haik et al. 2014).

35.2.3.5 PRN 100

PrP^{C}reduction being a potential therapeutic option in prion disease (as discussed in 35.2.5 below) led to the development of a human prion protein antibody (designated PRN 100) by the MRC Prion Unit in London. A single batch of clinical-grade PRN 100 was manufactured and given to patients on an open, compassionate basis between 2018 and 2019. The results from these treatments (which were not given in the context of a clinical trial) have been reported; it was a very limited treatment program mostly centred on establishing safety and dose determination. Some minor beneficial effect was suggested but there was with no definite treatment efficacy (Mead et al. 2022).

35.2.4 Preventative Treatment in Humans

Given the often fulminating disease course (e.g., in sCJD) and the established neurological damage by the time of diagnosis, the greatest likelihood for effective treatment might well be in preventing disease in those at significant risk of developing it. Unfortunately, the commonest form of disease (sCJD) is not a reasonable candidate for prophylactic therapy. The two main areas for this consideration are carriers of known pathogenic *PRNP* mutations and those at risk of disease through known exposure to infection. In both instances, treatment would be given to healthy individuals and, therefore, lack of toxicity is a more important consideration than in the treatment of clinically ill individuals. The assessment of efficacy is potentially problematic: those at risk via exposure may never develop disease or only after possibly very long incubation periods; with genetic mutations, disease penetrance and age at disease onset may be variable. A study of potentially preventative therapy (using doxycycline 100 mg/day in a randomized, placebo-controlled protocol) in presymptomatic *PRNP*-D178N mutation carriers is underway in Italy (EudraCT 2010-022233-38).

35.2.5 Treatment: Potential Treatment Targets

The discovery of disease therapies can be fortuitous or by design. In the latter case, one needs a reasonable understanding of disease mechanism. Unfortunately, while much is known about the molecular underpinning of prion disease, its precise pathogenesis (what actually leads to neuronal dysfunction and death) is not completely understood. Theories of pathogenesis have very broadly involved the possible effects of loss of function of the normal cellular protein PrP^{C} (due to its conversion to PrP^{Sc}), possible toxicity of aggregated deposits of the abnormal, disease-related PrP^{Sc}, and possible toxicity of intermediate forms between PrP^{C} and PrP^{Sc}, with a current tendency to favour the last of these (Weissmann and Aguzzi 2005; Zanusso and Monaco 2005; Aguzzi and Falsig 2012).

Potential treatment targets include:

(i) *Reduction of PrP^{C}*. Experimental work has shown that successful transmission of prion disease requires PrP^{C} in the exposed animal, and, while the normal roles of PrP^{C} are uncertain, its acquired absence may not be significantly deleterious to animal health (Mallucci et al. 2002). In one study, depleting PrP^{C} in an animal infection model prevented progression to clinical disease and even reversal of early neuropathological changes (Mallucci et al. 2003). In a later study, using the same animal model, the early neuropathological changes were associated with cognitive/behavioural deficits that also reversed with PrP^{C} depletion (Mallucci et al. 2007). As a result, one therapeutic approach is based on endogenous PrP^{C} depletion; methods have included antibodies against PrP^{C} and RNA interference (White et al. 2003, 2008; White and Mallucci 2009).

More recently, a study showed that Pharmacological Protein Inactivation by Folding Intermediate Targetting (PPI-FIT) could modulate target protein levels by acting on their folding pathways using PrP^{C} (Spagnolli et al. 2021).

(ii) *PrP^{C} to PrP^{Sc} conversion*. Another set of approaches is to identify molecules that could interfere with PrP^{C}–PrP^{Sc} interaction, reducing or preventing conversion to PrP^{Sc}.

(iii) *Inhibition of PrP^{Sc} oligomer formation.* Since oligomers, intermediate between PrP^{C} and the amyloid aggregations of PrP^{Sc}, may be of particular importance not only to PrPSc formation but also to toxicity and neurodegeneration, this is another potential treatment target.

(iv) *Facilitation of PrP^{Sc} degradation.* Autophagy and lysosomal degradation have been implicated in PrP^{Sc} clearance (Heisece et al. 2010; Marzo et al. 2013).

(v) *Breaking down tissue accumulation of aggregates of PrP^{Sc}*. Breaking down aggregations of PrP^{Sc} is reasonable if aggregated deposits are harmful and/or it aids the breakdown of PrP^{Sc}, but could be useless or potentially harmful if the aggregates are not intrinsically toxic and if more toxic prion protein forms are released (Aguzzi et al. 2018).

(vi) *Potential consequent neurodegeneration mechanisms.* One example is trying to reverse the persistent translational repression of protein synthesis induced by the unfolded protein response to abnormal prion protein, as described by Moreno and colleagues (Mallucci et al. 2003; Mallucci GR Prion 2009; Moreno et al. 2012). Synaptic dysfunction appears to be a key process in the evolution of many neurodegenerative diseases, including prion disease, potentially reversible in early disease and, therefore, with the underlying synaptic pathogenic mechanisms being a therapeutic target (Mallucci 2009).

Any consideration of therapeutic targets needs to take into account three factors: multiple mechanisms of action, treatment strain specificity, and treatment resistance. First, it is the case that some potential treatments may have more than one action, so they do not necessarily fit neatly into the above target scheme. This is not just a point about classification: action at more than one step in the disease process may be of additional therapeutic benefit. Of course, in any case, the precise modes of action of potential drug therapies are not always fully understood. Second, experimental evidence indicates that certain treatments are disease-strain specific; evaluation with one prion disease may not translate to another and monotherapy might select for a resistant strain, as may happen in anti-bacterial chemotherapy (Berry et al. 2013). Clearly, therapies that target either PrP^{C} or downstream neurodegenerative processes common to all prion strains may be more applicable to all types of prion disease and may avoid the potential problem of strain resistance. Strain resistance provides an additional argument for considering combination therapy for prion diseases. However, there is one report of a scrapie-mouse model, where a new resistant strain developed to a combination of two drugs, despite being susceptible to monotherapy (Burkew et al. 2020).

Several useful reviews of therapeutic approaches to prion diseases have been published (Weissmann and Aguzzi 2005; Teryua and Doh-ura 2017; Aguzzi et al. 2018).

35.2.6 Treatment: Identifying Possible Treatments

There have been many approaches taken to identify possible treatments: animal bioassays (Watts and Prusiner 2014), various cell lines (Priola 2018), and cell-free conversion assays (Ferreira and Caughey 2019). One important development for studying human prion infection is the successful use of human induced pluripotent stem cell (iPSC) derived astrocytes (Krejciova et al. 2017). However, the use of cell lines to identify treatments has been disappointing and it has been argued that the use of human cerebral otrganoids (which have been successfully infected with sCJD) is more promising (Groveman et al. 2019, 2021; Aguzzi et al. 2018).

These entirely reasonable, desirable, steps have potential limitations: success in a chemical setting, a cell line, or even an organoid, is not success in a whole organism, and treatment results in animals (even transgenically modified ones) may not be directly transferable to humans. A particular difficulty with animal experiments is that typically treatment is given relatively close in time to the inoculation of infection with efficacy often expressed in terms of the number of animals, which either fail to become ill or do so with prolonged incubation periods. This is not the same situation as treating clinically ill individuals. Quite aside from these irreducible facts, laboratory experiments have to use selected strains of prion disease and treatments may have prion strain specificity. There is a useful systematic review (up to 2006) of experimental models in prion disease therapeutics (Trevitt and Collinge 2006). Cell-based or organoid assays at least allow for relatively rapid, high-throughput searches for anti-prion disease compounds (Kocisko and Caughey 2006).

35.2.7 Treatment: Assessing the Efficacy of Potential Treatments in Humans

Since prion diseases are uniformly fatal with a relatively predictable course, it might be thought that assessing treatment efficacy would be much more straightforward than in diseases with a highly variable course and prognosis, such as multiple sclerosis. However, there are significant, interacting methodological problems:

(a) Dramatic or curative efficacy would not be difficult to demonstrate. However, initial therapies may be only partially beneficial; a relatively minor effect may be more difficult to confirm, especially in the light of other factors detailed below. While minor efficacy may not be immediately valuable, it may be an important lead in the development of more effective drugs.

(b) How is efficacy to be measured? At present, any measures probably need to be clinical ones as there are no validated para-clinical tests or disease markers of progression. Clinical improvement may not be expected even if disease progression is halted, due to the typically established neurological damage at diagnosis. Slowing of disease progression or even clinical stability may be difficult to confirm if there is already severe neurological impairment. Total illness duration is a simple measure but one that may be affected by a number of factors, as discussed in (c) below. If significant impairment "milestones" (such as inability to walk, mutism, requirement for tube feeding) have not already been reached, then the time taken to reach them could be used (Bone et al. 2008; Mead et al. 2011).
(c) Concerning clinical measures, there is variation within the prion diseases. For example, vCJD has a slower progression and longer duration than sCJD. Even within one form of prion disease, there can be significant variation in simple clinical measures such as total illness duration. Within sCJD, a variety of factors are known to influence survival: age at onset, sex of the patient, *PRNP*-129 genotype, and disease-associated prion protein type (Pocchiari et al. 2004). There are, therefore, good arguments for dividing patients into appropriate subgroups before treatment. Naturally, aside from these essentially biological factors, different disease management approaches (such as the use of feeding tubes and the treatment of intercurrent chest infections) may also affect disease duration.
(d) These are rare diseases, with annual mortality rates of around 1–2 per million population. While international collaboration in treatment trials could at least partially overcome this problem, the need for subgrouping (including within sCJD) exacerbates the numerical problem.

35.2.8 Treatment: Assessing the Toxicity of Potential Treatments in Humans

Given the severe, progressive, and ultimately fatal nature of these diseases, one might be prepared to consider relatively toxic treatments if there is a chance of benefit. While this is an arguable position for the treatment of clinical illness, it is certainly not so for prophylactic therapy. For example, if one were considering treating currently healthy *PRNP* mutation carriers, especially with uncertainties about disease penetrance or age of illness onset, then treatment toxicity would be an important consideration. There is the additional problem of assessing neurotoxicity in ill patients when the illness itself is so neurologically devastating. There is always the theoretical possibility that treatments aimed at disease mechanisms may exacerbate the disease process, and the detection of this is subject to the same considerations as those listed above for assessing efficacy.

35.2.9 Ethical Considerations

The possibility of slowing or halting progression of a disease that has already caused serious and potentially irreversible brain damage is something that doctors, patients, and families need at least to reflect upon. In addition, with an inevitably progressive and fatal disease, is it right and/or possible to run a control group for comparison? There are sound arguments for having a control group: treatment requires time-consuming interventions (medical supervision with assessments); treatment may be toxic; clinical measures (including simple disease duration) are subject to individual variations as outlined above. The acceptability of a control arm to prion disease patients or families trials is uncertain. The UK Prion-1 Trial did not manage to recruit significantly into a control arm (Collinge et al. 2009). However, other trials have succeeded in this (Otto et al. 2004; Geschwind et al. 2013; Haik et al. 2014).

35.3 Decontamination

35.3.1 The Background to Decontamination Concerns

The existence of iatrogenic CJD justifies the development of decontamination procedures for prion disease (Chap. 11).

A number of factors are relevant: the type of prion disease, the tissue spatial distribution of infectivity (which varies with disease type), the temporal tissue distribution of infectivity (which may be different at different disease stages), the amount of infectivity likely to be found on any relevant material or instrument, and the difficulties of removal or inactivation of prion infectivity. The prion, as an infectious agent, is considered to consist mostly or entirely of some form of prion protein, without constituent nucleic acid, and this underpins the observation that prion infectivity is notoriously resistant to routinely employed sterilizing methods: germicidal light, glutaraldehyde, formaldehyde, alcohol, and certain autoclaving settings are all of negligible effect (McDonnell and Burke 2003). Resistance to very high temperatures has also been demonstrated (Brown et al. 2000). Certain methods such as exposure to 2 M sodium hydroxide are effective but not practical in routine practice (ACDP REF appropriate format http link in refs.). Various autoclaving protocols involving 134–137 C reduce infectivity but cannot be relied upon for its complete removal (ACDP ref. appropriate format http link in refs.). Prion infectivity has been considered resistant to gamma-irradiation, which was listed as ineffective in the WHO 2000 Guidelines (ref. given in http form in ref. list). In addition to these biological considerations, there are epidemiological and practical factors to take into account. In terms of the former, it is a question of the risk of infection being present in the population, and this varies with disease and country. For example, studies have suggested the existence of a significant number of individuals with potential vCJD infection in the United Kingdom (Hilton et al. 2004; de Marco et al.

2010; Gill et al. 2020). In terms of the latter, quite aside from any theoretical considerations and laboratory demonstrations of decontamination efficacy, there are important practical and logistic considerations. Success on the laboratory small scale does not automatically lead to the adoption of a method into real-life clinical practice. Any decontamination method of practical merit needs to be one that can be used on a large scale, in routine clinical settings, on instruments or materials as they are currently employed, without possible corrosive or destructive effects on the items being treated. In addition, the actual costs and opportunity costs of any general decontamination protocols need to be taken into account.

Decontamination may be considered in two intertwined but separable parts: cleaning and inactivation of infection. Cleaning is an important aspect as obvious remnants of tissue or bodily secretions may contain infectious material and make inactivation of infection more difficult. However, even with rigorous macroscopic cleaning, protein residues that may remain are particularly important in prion disease (Murdoch et al. 2006). The precise nature of the prion (the infectious agent) is still uncertain, but the current view is that it is entirely, or largely, composed of PrP^{Sc}, the disease-related, abnormally folded prion protein. There is evidence that prion protein is firmly adsorbed to steel surfaces, with associated infectivity (Zobeley et al. 1999). There is another factor of importance, namely the effect of drying of items prior to decontamination processes, with drying making decontamination more difficult (Secker et al. 2011; Lipscomb et al. 2007).However, the combinatorial effects of pre-cleaning, cleaning, and inactivation steps may be inconsistent, and any assessment of a decontamination process needs to include all of the individual steps (McDonnell et al. 2013).

There are two broad decontamination situations: decontamination of items with known exposure and general decontamination methods of universal application. In either case, an alternative to decontamination is disposal of the item. In considering a single item (e.g., a specific surgical instrument used in someone with a prion disease, or at known increased risk of prion disease), the risk of reuse needs to be balanced against the cost of disposal and replacement of the item. In considering universal measures, the particular circumstances of a country may be relevant. For example, in the United Kingdom, because of estimates of vCJD subclinical infection prevalence in the population, with the potential involvement of reticuloendothelial tissues, disposable instruments for various procedures have been considered; however, the general use of disposable instruments is not without possible problems. For example, in England, when disposable instruments were introduced for tonsillectomy (because of the possibility of vCJD transmission), there was a consequent rise in surgical morbidity (Maheshwar et al. 2003; Nix 2003). In the case of brain biopsy for a non-focal cerebral illness, especially a dementing one, it is possible to quarantine the instruments until the biopsy pathological report confirms or excludes prion disease. A concern about possible prion contamination via waste water has been raised, perhaps particularly relevant in areas of endemic CWD (Chronic Wasting Disease), which might not be inactivated by standard waste-water treatments (Ding et al. 2013).

35.3.2 Methods of Decontamination

There are various decontamination methods. A review in 2006 detailed the methods recommended by the WHO and the UK ACDP (Advisory Committee on Dangerous Pathogens); the USA CDC recommends following the WHO guidelines (appropriate ref. to ACDP & WHO http links in ref. list). Updated UK ACDP guidelines can be found on the relevant website (Http link in ref. list and Sutton et al. 2006).

In recent years, a variety of new approaches have been developed, including radio-frequency gas-plasma treatment, hydrogen peroxide gas plasma treatment, nitrogen gas plasma treatment, and an enzyme-detergent method (Baxter et al. 2005; Rogez-Kreuz et al. 2009; Jackson et al. 2005; Shintani 2012). Sodium dodecyl sulfate (SDS), combined with autoclaving, was found to be effective in a steel-wire model (Peretz et al. 2006). A combitorial approach (using sodium percarbonate, SDS, and proteinase K) was found to reduce but not eliminate infectivity in an RML scrapie model (Smith et al. 2013). Hypochlorous acid proved useful in one study (Hughson et al. 2016). Hypochlorous acid being the acid conjugate of hypochlorite (sodium hypochlorite being effective for decontamination at levels that are generally corrosive, but hypochlorous acid is non-corrosive). One study showed that ozone may be useful in treating potentially contaminated waste water (Ding et al. 2013). While gamma-irradiation has been deemed ineffective, as mentioned above, there are two reports of partial efficacy. It has been shown to reduce prion infectivity in albumin (without important degradation of the albumin product) (Miekka et al. 2003). This was in a hamster-scrape model and showed reduction rather than elimination. Another study, using a mouse-scrapie model, reported a non-neglible reduction of prion infectivity using gamma-irradiation, and the authors suggested that gamma-irradiation might be considered as part of a combination decontamination process (Gominet et al. 2007).

35.3.3 Assessment of Decontamination Methods

As the ultimate nature of prion infectivity remains uncertain, determination of infectivity and the effectiveness of decontamination processes has been done by protein detection methods, cell-based assays, or by bioassay of infectivity. Protein detection methods have included western blotting, fluorescent microscopy, scanning electron microscopy, energy-dispersive spectroscopic analysis, and quantitative total amino acid analysis (following acid stripping and hydrolysis) (Howlin et al. 2010; Baxter et al. 2005, 2006). A cell-based assay has been described and employed in a comparative assessment of commercially available prion decontamination reagents (Edgeworth et al. 2009, 2011a, b). Bioassay methods involve attempted transmission to experimental animals and are, therefore, a more direct assessment of infectivity and have been used (Smith et al. 2013). However, they are expensive and time-consuming. It has been argued that cell-free assays based on

protein misfolding cyclic amplification (PMCA) could complement, or even replace, animal bioassay as assessments of decontamination techniques, based on the correlation of PMCA and animal bioassay in measuring prion concentrations (Moudjou et al. 2020). Another cell-free assay, real-time quaking-induced conversion (RT-QuIC) has been used in decontamination assessment (Hughson et al. 2016).

Steel wires have often been used in the experimental assessment of decontamination processes, but concerns have been expressed as to whether this is an entirely valid method (Lipscomb et al. 2006; Giles et al. 2017; Moudjou et al. 2020).

One potential problem with the assessment of decontamination methods is the evidence that inactivation of infection varies between different strains of prions (Taylor et al. 2002; Somerville et al. 2002; Moudjou et al. 2020). Therefore, general extrapolation of any specific experimental determination of decontamination is not necessarily valid. There are recent useful reviews of methods of assessment of potential decontamination methods (Sakudo et al. 2011; Giles et al. 2017; Moudjou et al. 2020).

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Chapter 36
Gene Therapy Strategies for Prophylactic and Therapeutic Treatments of Human Prion Diseases

Manuel Camacho and Qingzhong Kong

Abstract Prion disease is a diverse family of fatal and usually transmissible and progressive neurodegenerative diseases that strike humans and many other mammal species, such as cattle, sheep, and cervids. The cellular PrP (PrP^{C}) is the substrate for the replication of misfolded prion protein aggregates (PrP^{Sc}) that serve as the transmissible prion agents. PrP^{C} is also essential for prion pathogenesis. No treatments are available for prion diseases. Numerous efforts with various anti-prion compounds or antibodies have not produced meaningful benefits for prion patients in clinical trials so far. The gene therapy technology has matured in the last several years and offers great hopes for effective treatment and prevention of prion diseases. Here, we review the current literature on prion gene therapy development and propose a few promising gene therapy strategies targeting various aspects of prion replication and pathogenesis.

Keywords Prion disease therapeutics · Combination of gene therapy · RNA interference · Prion protein fragments and cleavages · Prion replication inhibition

36.1 Overview of Human Prion Diseases

Prion disease is a family of progressive neurodegenerative diseases affecting humans and many other mammal species, such as cattle, sheep, goat, cervids, mink, cats, and rodents. It is always fatal and usually transmissible. It is a rare disease that occurs at a prevalence of 1–2 cases per million.

Human prion diseases are highly diverse and heterogeneous. They can be familial (due to inherited mutations in the PrP gene), acquired (due to infection by prion

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agents), or sporadic (with unknown etiology) (Kong et al. 2004; Gambetti et al. 2011). The sporadic form is primarily Creutzfeldt–Jakob disease (CJD) and accounts for 85%–90% of all human prion disease (Parchi et al. 1999; Cali et al. 2006; Gambetti et al. 2011), of which at least four sporadic CJD strains (Bishop et al. 2010), the variably protease-sensitive prionopathy (VPSPr) strain (Gambetti et al. 2008, 2011; Zou et al. 2010) and the sporadic fatal insomnia strain (sFI) have been identified. The sCJDMM1/MV1 strain is the most common. The genetic forms include familial CJD, Gerstmann–Sträussler–Scheinker disease (GSS), and fatal familial insomnia (FFI) (Kong et al. 2004). The acquired forms include Kuru, iatrogenic CJD, and variant CJD (vCJD) (Ironside 2012). The clinical and histopathological features as well as the characteristics and tissue regional distribution of the prion agent often overlap between different subtypes and show heterogeneity within the same subtype, which is one of the challenges in the diagnosis and therapy development of prion diseases (Baiardi et al. 2019). The rarity of this disease is another big hurdle in clinical trials.

There is no treatment or prevention for CJD or any other prion diseases. There have been numerous attempts to develop or test treatments or prevention against prions in preclinical studies or clinical trials, most targeting prion replication. Some have shown great potential in extending the lifespan in animal models (White et al. 2003, 2008; Bradley et al. 2017; Mead et al. 2022), such as treatments with antisense oligonucleotides (Nazor Friberg et al. 2012; Raymond et al. 2019; Minikel et al. 2020) or cellulose ethers (Teruya et al. 2016; Hannaoui et al. 2020; Ding et al. 2021), but their safety and efficacy in human prion patients remain to be tested and all clinical trials so far have failed to provide meaningful benefits to the patients (Forloni et al. 2015, 2019).

Gene therapy, a technique long held as a possible true solution to many difficult diseases, has finally overcome many hurdles and achieved clinical applications using recombinant adeno-associated viral (rAAV) vectors for a few single-gene genetic diseases in the last few years (Wang et al. 2019; Sayed et al. 2022). Gene therapies address the root causes of diseases through correcting a mutated gene, expressing a functional protein of the mutated gene in the host or a beneficial protein or RNA that enhances a desired biological activity, suppressing an unwanted activity, or eliciting killing or clearance of dysfunctional proteins, cells (such as cancer cells), or invading pathogens (such as virus or bacteria). The sustained nature of the gene therapy approach will also ensure a long-lasting effect after a single treatment, which is a huge advantage over traditional therapies that generally require regular and continuous treatments. We believe that gene therapy represents a highly promising answer to the treatment and prevention of various human prion diseases.

We will review the current status of the development of gene therapies against prion diseases and discuss a few promising gene therapy strategies, including combination therapies simultaneously targeting multiple aspects of prion replication and pathogenesis that may have a better chance to succeed.

36.2 General Strategies for Development of Therapies Against Prion Diseases

The development of effective treatments is dependent on our understanding of the target disease. Our current knowledge on the structure, replication, clearance, and pathogenesis of prions is still limited overall, but some aspects are well established. First, the prion agents are composed of PrP^{Sc}, which is primarily (if not exclusively) misfolded aggregates of the normal cellular prion protein (PrP^{C}) that replicates using PrP^{C} as the substrate. PrP^{C} is a ubiquitously expressed normal cellular glycoprotein that is attached to the outer layer of the cell membrane via the glycosylphosphatidylinositol (GPI) anchor. Second, prion pathogenesis requires GPI-anchored cell surface PrP^{C} ($csPrP^{C}$) (Brandner et al. 1996; Chesebro et al. 2005; Puig et al. 2019). Third, there are multiple prion strains encoded by the conformations of the PrP^{Sc} aggregates. Fourth, just like in Alzheimer's disease and several other protein misfolding diseases, the soluble oligomers of PrP^{Sc}, not the large PrP^{Sc} aggregates, appear to be the main cytotoxic molecules that mediate prion neurodegeneration. Fifth, the conversion of PrP^{C} to PrP^{Sc} requires the conformational transition from α-helices to β-sheets for a significant portion of the C-terminal globular domain of PrP^{C}. Sixth, PrP^{Sc} aggregates are heterogeneous and their precise structures remain under debate, although several models have been proposed (Cobb et al. 2007; Glynn et al. 2020; Spagnolli et al. 2020; Wang et al. 2020, 2021; Kraus et al. 2021; Serpa et al. 2021).

From the generation of prions to neurodegeneration takes many steps. First, the *PRNP* gene that encodes PrP^{C} is transcribed to PrP mRNA, which is then translated to the PrP protein in the endoplasmic reticulum. The nascent PrP protein undergoes post-translational modifications (removal of signal peptide, glycosylation at the asparagine side chains, and addition of GPI anchor at the C-terminal end) before it is translocated to the cell surface as the mature glycosylated and GPI-anchored PrP^{C}. For sporadic prion patients, the first prion seeds occur in an age-dependent process with unknown mechanisms. For genetic prion disease patients with a mutated *PRNP* allele, the first infectious prion seeds are generated spontaneously from the mutated PrP protein in a poorly defined process. For acquired prion disease patients, the first prion seeds are acquired from an external source, through oral, surgical, medical, or accidental exposure to prions from contaminated food, drug, transplant, transfused blood, or surgical tools. Once the first PrP^{Sc} seeds are formed or acquired, PrP^{C} is recruited by the existing PrP^{Sc} seeds (PrP^{Sc} oligomers and small PrP^{Sc} aggregates) and converted to the PrP^{Sc} conformation in a repetitive PrP^{Sc}-seeded process to create large aggregates, which are subsequently broken down to smaller PrP^{Sc} aggregates, thereby amplifying the number of seeds for further amplifications. The PrP^{Sc} aggregates are subject to cellular clearance mechanisms, such as proteasomal degradation and autophagy. The toxic PrP^{Sc} molecules, generally believed to be the PrP^{Sc} oligomers, will interact with the cell surface PrP^{C} ($csPrP^{C}$) to activate a neurotoxic signaling pathway or work with astrocytes to damage and eventually kill the neuronal cells. This multistage process provides numerous

opportunities for interventions with different approaches. Potential intervention strategies include reduction of PrP^{C} levels, inhibition of PrP^{Sc} replication, enhancement of PrP^{Sc} clearance, prevention of cytotoxic signaling, inhibition of neuroinflammation, and restoration and regeneration of neuronal functions. Some of these strategies have been tested in gene therapy experiments for prion prophylaxis or treatment in cultured cells or animal models.

36.3 Reported Gene Therapy Tests Against Prion Diseases in Cell or Animals

36.3.1 Reduction of PrP^{C} Levels by RNAi

PrP^{C} is central to both prion replication and prion pathogenesis. PrP^{C} is the only required substrate for prion replication, although some host factors may facilitate the process. Reducing the PrP^{C} levels will inhibit prion replication and diminish the cytotoxic PrP^{Sc} species (believed to be PrP^{Sc} oligomers). Since the PrP^{C} is also essential for prion pathogenesis as the receptor for toxic PrP^{Sc} oligomers, lowering PrP^{C} levels will also attenuate the cytotoxic effects of existing PrP^{Sc}. Fortunately, PrP^{C} is not essential for cell survival and there are no significant abnormalities in PrP knockout mice or cattle (Sailer et al. 1994; Weissmann and Flechsig 2003; Richt et al. 2007), although some subtle defects are discovered in these animals. These observations make reducing PrP^{C} levels in the CNS a highly enticing strategy to combat prion diseases. The safety of this approach is strongly supported by the excellent therapeutic effect of controlled inhibition or knockout of the PrP gene (*PRNP*) in transgenic mice infected by scrapie prions (Tremblay et al. 1998; Mallucci et al. 2003). More recent reports of very significant extension of survival (up to 98% extension) with antisense oligonucleotides against PrP gene in prion mouse models further attest to the effectiveness and safety of the PrP^{C} reduction strategy (Nazor Friberg et al. 2012; Raymond et al. 2019; Minikel et al. 2020). It is worth noting that none of these in vivo experiments were done on animals infected with human prions.

RNA interference (RNAi) is an effective technique to knock down the target gene expression through enhancing the degradation of the target gene mRNA using a small RNA complementary to the target mRNA (Kong 2006; Pereira and Lopes-Cendes 2012). Anti-PrP RNAi was found to greatly reduce PrP expression and prion accumulation in cultured cells infected with scrapie prions (Daude et al. 2003). Pfeifer et al. (2006) generated chimeric mice with ES cells treated with lentivector-mediated RNAi against PrP and demonstrated that knocking down PrP expression in the brain prolonged the survival by up to 39% in a prion mouse model. Subsequent RNAi experiments by other groups through liposome or viral delivery confirmed the effectiveness of the anti-PrP RNAi approach, with one experiment showing extension of survival by up to 24% (White et al. 2008; Ahn et al. 2014; Bender et al.

2019). The less than stellar effectiveness of RNAi in animals appears to be a result of limited suppression of PrP expression due to poor RNAi delivery into the brain. In addition, Stobart et al. (2009) demonstrated that hybrid hammerhead ribozymes were also able to effectively knock down human PrP mRNA in cultured cells.

36.3.2 Inhibition of PrP^{Sc} Replication

Several anti-PrP antibodies effectively inhibit prion replication in cultured cells. A couple of PrP variants also dominant negatively inhibit prion replication. A few research groups have tested such anti-PrP antibodies and dominant negative PrP variants for gene therapy against prions in mouse models.

Wuertzer et al. (2008) injected the thalami and striata of mice with recombinant adeno-associated (rAAV) type 2 vector carrying a secretable anti-PrP scFv antibody and then inoculated the mice intraperitoneally with prions. They found that the rAAV2-anti-PrP scFv treatment led to about 30% increase in survival time along with improvements in clinical signs and rotarod performance as well as reduced PrP^{Sc} accumulation in the brain. Fujita et al. (2011) transduced an immortalized brain-engraftable murine microglial cell line (Ra2) with a lentiviral vector carrying an anti-prion single chain Fv fragment and showed that intracerebral injection of such ex vivo modified anti-PrP scFv expressing microglia cells led to marginal extension of life, if done before or at early time points after experimental prion infection, which could be partially due to the limited survival time of the injected cells in vivo. Similarly, Moda et al. (2012) showed a 7% extension in survival in mice treated with rAAV9 carrying an anti-PrP scFv antibody. Mead et al. (2022) recently reported the result of the first clinical trial using a humanized monoclonal anti-PrP antibody in six CJD patients, and they found that the treatment was well tolerated and some signs of treatment effect were observed, raising hope that the anti-PrP antibody therapy may work if delivered via a gene therapy vector. The 37/67 kDa laminin receptor (LRP/LR) is also a target for prion gene therapy since it is believed to act as a receptor for prions. Zuber et al. (2008) found that rAAV-mediated intrahippocampal delivery of anti-LRP/LR single-chain antibodies failed to prolong survival despite some reduction of PrP^{Sc} level in the spleen.

The Perrier research group tested a dominant negative PrP variant for gene therapy against prions in cell or mouse models and found strong inhibition of PrP^{Sc} replication in cells and extended survival (up to 20%) in mice infected with prions (Crozet et al. 2004; Toupet et al. 2008). The Aguzzi group reported strong prophylactic and therapeutic effect of lentiviral vector mediated delivery and expression of a soluble prion antagonist PrP-Fc(2) to the brains of prion infected mice, extending survival by 41% when given before prion inoculation or 14% when given at 30 days after prion inoculation (Genoud et al. 2008).

36.4 Promising Gene Therapy Strategies Against Prion Diseases

There are numerous potential interventions among the key players and multiple steps of prion replication and pathogenesis. For example, PrP^{Sc} replication requires the PrP^{Sc} seeds, the PrP^{C} substrate, direct interactions between PrP^{C} and PrP^{Sc}, conversion of PrP^{C} to the PrP^{Sc} conformation, and some host cofactors. For prion pathogenesis, it requires the toxic PrP^{Sc} species, the cell surface PrP^{C} and the laminin receptor (LRP/LR) as the receptors for the toxic PrP^{Sc} species, and the downstream signaling pathways. Each of the steps can be targeted for interventions.

Prion replication and pathogenesis both require PrP^{C}. PrP^{Sc} is also necessary for prion replication, and prion pathogenesis depends on some form of PrP aggregates related to PrP^{Sc} replication, likely PrP^{Sc} oligomers. Therefore, reduction of PrP^{C} levels, inhibition of PrP^{Sc} replication, and enhancement of PrP^{Sc} clearance have been the main focus in anti-prion therapy development efforts (Giles et al. 2017; Abdelaziz et al. 2019; Forloni et al. 2019; Teruya and Doh-Ura 2022; Zattoni and Legname 2021). Reduction of PrP^{C} can be achieved at multiple levels. The *PRNP* gene can be knocked out by CRISPR or its transcription suppressed by specific transcriptional inhibitors. The PrP mRNA can be marked by anti-sense oligonucleotides or shRNA/siRNA for enhanced degradation. The turnover of PrP^{C} protein can be elevated through augmented degradation or processing, the latter includes α-cleavage, β-cleavage, and shedding, of which the α-cleavage and shedding are beneficial and of potential therapeutic values (Liang and Kong 2012; Dexter and Kong 2021a, b). The strategies to control PrP^{Sc} replication could include, aside from reduction of PrP^{C} levels, enhancing the clearance of PrP^{Sc}, blocking the interactions between PrP^{C} and PrP^{Sc} (such as using anti-PrP antibodies or dominant-negative PrP), stabilizing large PrP^{Sc} aggregates to inhibit the generation of smaller PrP^{Sc} seeds, and stabilizing the PrP^{C} structure to prevent its conversion to PrP^{Sc} conformation.

Many of the steps and players in prion replication and pathogenesis are amenable for the gene therapy approach. The anti-PrP antibodies or dominant negative PrP approach seem promising. Here, we discuss a few other gene therapy strategies that we believe hold great promises for effective treatment and prevention of the various prion diseases in humans regardless of the specific prion strains involved.

Sustained reduction of PrP^{C} levels through delivery of a gene therapy vector to broad areas of the brain is no doubt the most attractive strategy, because reduced PrP^{C} levels in the CNS is very well tolerated and its threefold effects are all very beneficial: diminishing PrP^{Sc} replication within the cells due to reduced PrP substrate levels, retarding prion spread between brain regions due to blunted PrP^{Sc} replication in each cell, and attenuated cellular toxic signaling due to the reduced amount of cell surface PrP^{C} that serves as the receptor for toxic PrP^{Sc} species.

As described earlier, RNAi-mediated reduction of PrP^{C} levels is safe and highly effective in controlling prions in cells, and it is one of the most promising prophylactic and therapeutic strategies against prion diseases when effectively delivered

through an efficient gene therapy vector. In an effort to utilize the PrP^C reduction strategy to treat and prevent prion diseases in human patients via gene therapy, my laboratory has developed a panel of shRNA constructs harbored on rAAV plasmids that show excellent efficiency against human PrP mRNAs in cultured cells and in mouse brains.

PrP^C levels can also be reduced by other approaches. ADAM10 is the primary enzyme responsible for the shedding of cell surface PrP to release the nearly full-length soluble PrP (Altmeppen et al. 2011; McDonald et al. 2014). Overexpression of ADAM10 led to reduced PrP^C levels and prolonged survival (Endres et al. 2009), whereas knocking out ADAM10 resulted in elevated PrP^C levels and accelerated the disease course in prion mouse models (Altmeppen et al. 2015). It is reasonable to expect that a gene therapy vector that can overexpress ADAM10 in the brain may be a viable strategy for prion treatments. However, overexpressing ADAM10 carries significant risks, including inducing cancer, autoimmunity and inflammation in the brain (Dexter and Kong 2021b). Nevertheless, modest overexpression of ADAM10 might be safe and still effective since transgenic mice overexpressing ADAM10 do not show significant abnormality (Postina et al. 2004; Endres et al. 2009; Prinzen et al. 2009). An alternative approach has emerged recently. A large panel of PrP antibodies were assessed in cells and organotypic brain slide cultures, and many were found to reduce cell surface PrP levels by enhancing ADAM10-mediated PrP shedding or causing surface clustering, endocytosis, and degradation of PrP^C (Linsenmeier et al. 2021), suggesting that the PrP antibodies may achieve prion inhibition through mechanisms other than blocking the interactions between PrP^C and PrP^{Sc}. It may be safer and more effective to overexpress one of these PrP antibodies in the brain from an efficient gene therapy vector such as rAAV.

We have demonstrated that ADAM8 is the primary enzyme responsible for the highly beneficial and neuroprotective PrP α-cleavage in muscles (Liang et al. 2012, reviewed by Dexter and Kong 2021a), and we have recently shown that ADAM8 is also active in PrP α-cleavage in the brain. A gene therapy approach overexpressing ADAM8 in the brain should be effective in prevention and treatment of prion diseases, but significant caveats similar to those of ADAM10 also exist (Dexter and Kong 2021b).

The side effects of the ADAMs are largely due to the fact that they all have multiple cellular substrates in addition to PrP^C (Dexter and Kong 2021b). Direct expression of a secreted form of the highly neuroprotective PrP N1 fragment or similar PrP N terminal peptides will bypass this problem (Dexter and Kong 2021b). We and others (Mohammadi et al. 2020) have been working on this strategy and made significant progress.

Each of the above strategies usually target only one aspect of prion replication or pathogenesis. We (Dexter and Kong 2021b) and others (Zattoni and Legname 2021) believe that combination therapy strategies targeting multiple aspects of prion replication and pathogenesis will be more effective and more likely to succeed in the prevention and treatment of prion diseases. We are actively working on a combination gene therapy approach by simultaneously expressing an anti-human PrP shRNA and a secreted neuroprotective PrP N-terminal peptide from rAAV vectors delivered

to the brain locally or systematically (Dexter and Kong 2021b). We are highly hopeful that this strategy will be safe and effective, because it will concurrently knock down the PrP^{C} levels to reduce prion replication, blunt toxic signaling, and provide active neutralization of the toxic PrP^{Sc} species and direct neuroprotection from the sustained high levels of an extracellular PrP N-terminal peptide in the brain.

One common challenge for the prion gene therapy strategy is that, as is the case for all other gene therapy efforts in the brain, the CNS distribution of the delivered therapeutic gene constructs, such as shRNA or siRNA, is often quite limited, thereby diminishing the overall effectiveness. However, recent advances in rAAV vector research offer hope that this hurdle will be overcome soon (Deverman et al. 2016; Chan et al. 2017).

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Chapter 37
Immunomodulation

Thomas Wisniewski and Fernando Goñi

Abstract The underlying pathogenesis of prion diseases (prionoses) is related to an autocatalytic conformational conversion of a normal membrane, GPI-anchored self-protein, the PrP^{C} (C for cellular) to a pathological and infectious conformer known as PrP^{Sc} (Sc for scrapie) or PrP^{Res} (Res for Proteinase K resistant) (Prusiner, Science 216(4542):136–144. https://doi.org/10.1126/science.6801762, 1982; Carlson and Prusiner, Int J Mol Sci 22(9). https://doi.org/10.3390/ijms22094861, 2021). However, a small number of cases have been linked to changes in conformation along with a specific glycosylation pattern, producing a protease sensitive prion protein in what is termed as Variably Protease-Sensitive Prionopathy (VPSPr) (Zou et al., Ann Neurol 68(2):162–172, 2010; Gambetti et al., Ann Neurol 63(6):697–708. https://doi.org/10.1002/ana.21420, 2008). Currently, all prion diseases are without effective treatment and are universally fatal (Trevitt and Collinge, Brain 129(Pt 9):2241–2265, 2006; Wisniewski and Goni, Vaccination strategies. In: Manson J, Pocchiari M (eds) Human prion diseases, Handbook of clinical neurology, vol. 153. Elsevier, New York, pp 419–430. https://doi.org/10.1016/B978-0-444-63945-

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5.00023-4, 2018; Forloni et al., Curr Opin Pharmacol 44:53–60. https://doi.org/10.1016/j.coph.2019.04.019, 2019; Ma and Ma, Pathogens 9(3). https://doi.org/10.3390/pathogens9030216, 2020; Zattoni and Legname, Expert Opin Ther Pat 31(12):1097–1115. https://doi.org/10.1080/13543776.2021.1945033, 2021; Mathiason, Cell Tissue Res. https://doi.org/10.1007/s00441-022-03590-4, 2022). The conformational change of PrP in all prion diseases is associated with a negative gain of function and self-propagation. "Prion like" protein conformational changes are increasingly being recognized also to be part of the pathogenesis of most neurodegenerative diseases, such as Alzheimer's disease (AD) and α-synuclopathies (Carlson and Prusiner, Int J Mol Sci 22(9). https://doi.org/10.3390/ijms22094861, 2021; Jucker and Walker, Nat Neurosci 21(10):1341–1349. https://doi.org/10.1038/s41593-018-0238-6, 2018). The growing understanding of these protein conformational changes and post-translational modifications such as glycosylation in biological processes creating or modifying PrP epitopes opens the possibility of immune-therapeutic targeting when this phenomenon occurs in association with disease. The past experience with bovine spongiform encephalopathy and variant Creutzfeldt–Jakob disease (vCJD), as well as the current epidemic of chronic wasting disease (CWD), has highlighted the need to develop prophylactic and/or therapeutic approaches. Any effective therapeutic intervention for prion disease could have significant implications for similar neurodegenerative diseases. Conversely, therapeutic approaches that are effective in overcoming the conformational changes that occur to amyloid β (Aβ) and the tau protein as part of AD, might also be beneficial for prion disease. AD, like prion disease, has no effective therapy in spite of recent data from human trials suggesting that immunotherapeutic approaches using some anti-Aβ monoclonals can partially ameliorate amyloid plaque and tau pathology (Cummings et al., Alzheimers Res Ther 13:98. https://doi.org/10.1186/s13195-021-00838-z, 2021; Mintun et al., N Engl J Med 384(18):1691–1704. https://doi.org/10.1056/NEJMoa2100708, 2021; Kim et al., J Alzheimers Dis. https://doi.org/10.3233/JAD-215699, 2022). Human prion diseases are most commonly sporadic; hence, the therapeutic need would be primarily to stop progression; however, in animals the majority of prionoses are infectious and the emphasis is on prevention of transmission (Mathiason, Cell Tissue Res. https://doi.org/10.1007/s00441-022-03590-4, 2022; Gallardo and Delgado, Open Vet J 11(4):707–723. https://doi.org/10.5455/OVJ.2021.v11.i4.23, 2021; Orge et al., Biomolecules 11(3), https://doi.org/10.3390/biom11030466, 2021). These infectious prionoses are typically acquired via the alimentary tract as a major portal of infectious agent entry. This makes mucosal immunization a potentially attractive method to produce a local immune response that partially or completely prevents prion entry across the gut barrier, while at the same time producing a modulated systemic immunity that is unlikely to be associated with toxicity (Goni et al., Vaccine 33(5):726–733. https://doi.org/10.1016/j.vaccine.2014.11.035, 2015). In addition, this same approach has the potential to be used to retard or ameliorate human familial prionoses when given years ahead of the expected onset of disease. A critical factor in any immunomodulatory approach aimed at a self-antigen is the need to finely balance an effective humoral immune response with potential auto-immune toxicity. Our

results using an attenuated Salmonella vaccine strain expressing the prion protein showed that mucosal vaccination could protect against prion infection from a peripheral source even in large ungulate cervids, suggesting the feasibility of this approach (Wisniewski and Goni, Vaccination strategies. In: Manson J, Pocchiari M (eds) Human prion diseases, Handbook of clinical neurology, vol. 153. Elsevier, New York, pp 419–430. https://doi.org/10.1016/B978-0-444-63945-5.00023-4, 2018; Mathiason, Cell Tissue Res. https://doi.org/10.1007/s00441-022-03590-4, 2022; Goni et al., Vaccine 33(5):726–733. https://doi.org/10.1016/j.vaccine.2014.11.035, 2015; Wisniewski and Goni, Transmissible spongiform encephalopathies. In: Reiss CA (ed) Neurotophic viral infections, 2nd ed. Springer, New York, pp 221–248, 2016). The current epidemic of CWD and emergent camel prion disease (CPD), with their potential to spread to human populations, emphasizes the importance of developing such immunomodulatory approaches more fully.

Keywords Prion · Chronic wasting disease · Creutzfeldt–Jakob disease · Amyloid β · Oligomers · Vaccine · Conformational disorders

Abbreviations

BSE	Bovine Spongiform Encephalopathy
CPD	Camel Prion Disease
CWD	Chronic Wasting Disease
vCJD	Variant Creutzfeldt–Jakob Disease

Interest in developing potential therapeutics for prionoses was greatly increased since the emergence of bovine spongiform encephalopathy (BSE) and the resulting appearance of variant CJD (vCJD) in human populations, as well as the more recent epidemic of chronic wasting disease (CWD) and the relatively new camel prion disease (CPD) (Orge et al. 2021; Babelhadj et al. 2018). BSE was first identified among cattle in the UK in 1985, with its emergence being related to the practice of feeding meat-and-bone meal from animal carcasses to cattle; subsequently, BSE was found in 25 additional countries in Europe, North America, the Middle East, and Asia (Collee and Bradley 1997; Harman and Silva 2009; Kumagai et al. 2019; Watson et al. 2021). The rendering of BSE contaminated bovine carcasses into meat-and-bone meal amplified transmission, which peaked in 1992, during which time more than 3,000 cases per month were being recorded (Orge et al. 2021; Harman and Silva 2009; Kumagai et al. 2019). This led to the emergence of variant CJD (vCJD), with the first case being recognized in 1996 (Will et al. 1996; Mackay et al. 2011; Ritchie et al. 2021). Since the original report in 1996, a total of 232 probable or confirmed cases of vCJD have been diagnosed, from which 178 alone

occurred in Great Britain (Gallardo and Delgado 2021; Ritchie et al. 2021). An important proof of the zoonotic transmission of BSE is the maintenance of its distinctive molecular signature on Western blots following proteinase K (PK) digestion (Collinge et al. 1996). Two concurrent studies identified a distinct PrP^{Res} pattern that had not been previously observed in human prion disease (apart from some rare familial cases) (Collinge et al. 1996; Parchi et al. 1997). This new PrP^{Res} was termed Type 4 (Collinge classification) or Type 2B (Parchi classification); it is characterized by an ~19 kDa unglycosylated fragment and a clear predominance of the diglycosylated PrP^{Res} fragment; a pattern that is identical to PrP^{Res} in BSE and transmitted BSE to mice, non-human primates and domestic cats (Collinge et al. 1996). From 1988, a number of rigorous control measures were implemented in the UK (which were subsequently copied in many other countries) to limit BSE. As a consequence, BSE is now considered a rare disease, with only two cases recorded in the UK in the last 5 years (https://www.gov.uk/government/publications/active-tse-surveillance-statistics#full-publication-update-history). Although the hype of BSE transmission to humans has receded, and there is a strenuous screening of cattle all over most of the world, the possibility of the emergence of new strains that are more virulent still looms.

All the vCJD cases identified so far who were genetically characterized were homozygous for methionine (MM) at codon 129 on PRNP, this being a well-known risk factor for human prion disease. However, an exception was the last reported case of vCJD in the UK in 2017 occurred in a methionine/valine (MV) heterozygous patient (Mok et al. 2017). In addition, a possible case of vCJD occurred in a MV subject in 2009, but this case was never confirmed as vCJD as there was no autopsy (Kaski et al. 2009). The fact that such transmission could occur was not surprising, as in various experimental settings, it had been shown that the BSE agent could be passaged to other human PRNP genotypes besides MM, albeit with a greater species barrier (Bishop et al. 2006; Jones et al. 2007). The molecular signature of the PrP^{Res} of vCJD in the MV patient was the same as in the other MM patients; however, the MV individual had a clinical presentation more suggestive of sporadic CJD (Mok et al. 2017). This case highlights the need for continued surveillance of CJD cases, in particular, in the UK but also globally. There is also the risk of secondary human-to-human transmission of vCJD via blood transfusion. That BSE could be transmitted by blood collected during the asymptomatic phase of the disease has been demonstrated in experimentally infected sheep (Houston et al. 2000, 2008; Hunter et al. 2002). Thus far, there have been three cases of vCJD following blood transfusion, all in patients with MM at PRNP codon 129 (Llewelyn et al. 2004; Hewitt et al. 2006; Wroe et al. 2006). However, in addition, there has been one preclinical case in an individual who was MV at codon 129, who died 5 years after receiving a vCJD contaminated blood transfusion, from a condition unrelated to prion disease (Peden et al. 2004). The patient had PrP^{Res} in the spleen and cervical lymph nodes but not in the brain. It is not known if they would have developed vCJD if they had lived longer. Furthermore, vCJD blood transmission studies using both mice and macaques have shown passage of prion related disease that lacked classical features such as PrP^{Res} deposition (Comoy et al. 2017). Hence,

surveillance for vCJD should be maintained with the caution that the scope should include neurological conditions that do not fit typical prion disease criteria.

However, the biggest concern currently is the uncontrolled spread of chronic wasting disease (CWD), the most infectious prionoses to date, affecting free-ranging and farmed ungulates (white-tailed deer, mule deer, elk, moose, and reindeer) (Mathiason 2022; Gallardo and Delgado 2021; Williams 2005; Sigurdson 2008; Gilch et al. 2011). CWD was first described in 1967 and was recognized to be a prion disease in 1978 on the basis of brain histopathology in a captive population of mule deer which were not thriving (Williams 2005; Williams and Young 1980, 1982). CWD has been detected in the United States (26 states), Canada (four provinces), South Korea, Norway, Finland, and Sweden (Mathiason 2022; Gallardo and Delgado 2021; Gilch et al. 2011; Tranulis et al. 2021). In some captive cervid populations up to 90% of animals have been reported to be prion positive, whereas in some wild cervid populations the prion infection prevalence has been as high as 50% (Rivera et al. 2019). This tremendous disease burden is now driving a cervid population decline in some locations (DeVivo et al. 2017; Almberg et al. 2011). Transmission of CWD is mainly horizontal via a mucosal/oral route, although mother-to-offspring transmission has also been demonstrated prior to birth or through milk (Gilch et al. 2011; Kreeger et al. 2006; Beekes and McBride 2007; Safar et al. 2008; Sohn et al. 2020). Like BSE, CWD is transmissible to non-human primates (squirrel monkeys) (Marsh et al. 2005; Race et al. 2009a). Following oral inoculation, in deer clinical manifestations of CWD occur between 20 and 26 months later (Goni et al. 2015; Fox et al. 2006). CWD has also been shown to be transmissible to sheep, cattle, fallow deer, and several North American rodents (prairie voles, mice, and ferrets), which can scavenge on CWD carcasses (Hamir et al. 2005, 2006, 2011; Heisey et al. 2010; Kurt et al. 2011). Each of these animals can enter the human food chain directly or in the case of rodents by accidental inclusion in grain and forage. Large predators of cervids in the wild are not surprisingly preferentially killing incapacitated CWD-infected animals, raising the possibility of further cross-species spread (Krumm et al. 2010). So far, studies using transgenic mice expressing human PrP^{C} have failed to show transmission of CWD, suggesting there is a significant species barrier which is greater compared to the BSE to human barrier (Kong et al. 2005; Tamguney et al. 2006; Sandberg et al. 2010). On the other hand, two different strains of CWD have been identified with the likelihood that there are more (Angers et al. 2010). Whether any of these other strains for CWD have greater potential for human spread remains unknown. Furthermore, CWD prions have been found not only in the brain of infected deer but also in blood, muscle, feces, fat, urine, antler velvet, and saliva (Mathiason 2022; Gallardo and Delgado 2021; Safar et al. 2008; Angers et al. 2006; Mathiason et al. 2006, 2010; Race et al. 2009b; Haley et al. 2009; Tamguney et al. 2009; Angers et al. 2009). Therefore, the possibility of transmission to humans needs to be closely monitored. Studies using *in vitro* protein misfolding cyclic amplification (PMCA) showed that CWD PrP^{Res} can convert human PrP^{C} after the CWD prion strain was stabilized by passage with cervid PrP^{C}; this highlights the potential of CWD prions to infect humans (Barria et al. 2011). The risk posed to humans by CWD is difficult to estimate (Kong et al.

2005). The prevalence of CWD in free-range deer varies from up to 50% in some endemic areas to ~1% in states in which CWD has more recently been discovered (Mathiason 2022; Williams 2005; Sigurdson 2008). It is therefore certain that human exposure has occurred and continues to occur, either by direct contact in hunters and game processors, by consumption of venison, or by contact with products from cervids. Furthermore, the pre-clinical period of human prion infection via an oral route can be very long; in the case of kuru, an incubation period of 56 years was documented (Collinge et al. 2006). In contrast to the distribution of BSE-infected beef, which would be diluted in the food processing chain, it is more typical that only a few family members and friends consume venison from a CWD-infected animal, thus leading to a proportionally greater potential exposure. Human and other animal exposure to CWD may also occur from contaminated environmental sources; however, there are no data available to estimate the significance of such exposure. The CWD agent is extremely stable in the environment, where it readily binds to soil (Smith et al. 2011; Johnson et al. 2006; Saunders et al. 2010) and has even been detected in the water of CWD endemic areas (Nichols et al. 2009). Binding to certain types of soil has been shown to dramatically enhance CWD transmission; this infectivity remains stable over prolonged periods (Smith et al. 2011; Johnson et al. 2007; Kuznetsova et al. 2020). The likely exposure of humans to CWD-infected tissue is substantial. It is estimated that between 7,000 and 15,000 CWD-infected cervids are consumed by humans annually, an exposure that is increasing by ~20%/year (Mathiason 2022). In the United States, big game hunting (deer, elk, etc.) is a large ~26 billion industry pursued by ~9 million Americans (Mathiason 2022). A survey to assess potential CWD exposure in 17,372 US residents found that 67.4% of respondents had consumed venison (mainly obtained from the wild) and 18.5% hunted as a hobby (Abrams et al. 2011). A significant finding is that CWD is able to transmit with high efficacy nasally by aerosol among cervid PrP transgenic mice (Denkers et al. 2010). This represents the first documentation of prion spread via this respiratory route, although a subsequent study has shown that other prionoses may also have some limited ability to spread by aerosol (Haybaeck et al. 2011). Hence, if CWD were to cross the species barrier to humans, it would pose a major threat, likely far greater than vCJD, highlighting the need for ongoing surveillance and the need to develop better vaccination/immunomodulation approaches to prevent CWD transmission and uncontrolled spread (Nemani et al. 2020). The development and testing of such potential approaches is discussed below. Although there is no documented passage of CWD to humans, it is striking to note the recently reported significantly increased incidence of CJD in the United States from 1993 to 2014, concurrent with the CWD epidemic (Seitz et al. 2022). This is most likely related to increased surveillance of CJD; however, unrecognized zoonotic CWD also remains a possibility.

An important recent development is the appearance of prion-infected camels, with a new disease termed Camel Prion Disease (CPD) (Orge et al. 2021). Initially, this was recognized in dromedary camels (*Camelus dromedarius*) in Algeria in 2018 (Babelhadj et al. 2018). Dromedary camels account for ~94% of the world's camel population and are one of three species of camel. The other two species are the Bactrian camel (*Camelus bactrianus*) that is found in Central Asia and the wild

Bactrian camel (*Camelus ferus*) found in Northwest China and Mongolia (Orge et al. 2021). The initial cases were in a Saharan population in Ouargla (southeastern Algeria), which were identified on routine ante-mortem inspection when brought for slaughter at an abattoir (Babelhadj et al. 2018). A staggering 3.1% of the dromedary camels brought for slaughter had symptoms compatible with prion infection. Previously, it was thought that camelids, not being ruminants, were resistant to transmissible prion infection. Symptoms include weight loss, behavioral changes, and neurological findings such as ataxia, tremors, and hyperactivity. Because of the circumstances, the transmission is believed to be of infectious nature from an unknown source. It has been speculated that it could be related to the exportation of meat-and-bone meal from BSE affected countries, with subsequent entry into camel feed. An alternative explanation is that CPD originated from scrapie infected animals, since camels often gaze alongside sheep and goats. No cases have been reported of scrapie in Algeria; however, there is no scrapie surveillance program. In some of the animals, the diagnosis was confirmed by the presence of disease-specific PrP^{Res} in the brain and lymphoid tissue. Biochemically, this PrP^{CPD} showed differences with known BSE and scrapie, being less glycosylated compared to classical scrapie. PrP^{CPD} has a dominant monoglycosylated band and an apparent molecular weight slightly higher than found with PrP^{Sc} and much higher than found with BSE (Babelhadj et al. 2018). Another potential species population at risk for prion transmission are llamas. In all the Andean western part of South America up to the Equator, the meat of camelid llama (*Lama glama*) in captivity, and guanaco (*Lama guanicoe*) in the wild, is regularly consumed with no controls or surveillance for the presence of PrP^{Res} (Vila and Arzamendia 2022). Although anatomically different from susceptible ruminants; some South American camelids have been shown to express PrP with a sequence very similar to susceptible bovine species (Vermette et al. 2016). Since 2010, in remote areas of Brazil, there have been a few reports of atypical BSE in local abattoirs. A 2014 report of atypical BSE was in a Brazilian farm on the border with Bolivia in a location overlapping llama and guanaco ranges (Health WOfA 2014). This camelid population has the potential to develop a similar disease to CPD in North Africa or CWD in North America, a situation that requires careful monitoring. The emergence of CPD highlights the need for thorough surveillance of all animals that could enter human food chain. Two programs have been initiated to encourage surveillance of CPD: the CAMENET (Camel Middle East Network) and the EFRAN (Enhancing Research for African Network) (Orge et al. 2021). The CWD epidemic and the emergent CPD both highlight the importance of ongoing international surveillance for CJD and potential novel human prion diseases (Watson et al. 2021).

37.1 The Immune System and Prion Infection

Although PrP^{C} is predominately expressed in the central and peripheral nervous systems (Chesebro et al. 1985; Oesch et al. 1985), it is also expressed by many cells of the immune system. This includes T and B lymphocytes, natural killer (NK)

cells, platelets, monocytes, dendritic cells (DC), and follicular dendritic cells (FDC) (Aguzzi et al. 2013). However, expression level varies with the type of lymphoid cell involved. PrP^C levels are lower in B cells compared to monocytes, NK cells and particularly compared to T cells (Durig et al. 2000). There are also significant PrP^C level variations among T-cell subtypes (Durig et al. 2000). CD8+ cells have higher PrP^C expression compared to CD4+ cells, and the level of expression is at least 4.5 fold higher in CD25+ cells compared to CD25- cells (Isaacs et al. 2006), suggesting the diverse levels of PrP^C expression in cells of the immune system might have a potential regulatory role. Different PrP knock-out (KO) mice have not shown an overt neurological phenotype under normal conditions (Bueler et al. 1992; Manson et al. 1994); however, in classical neurological T-cell-mediated autoimmune diseases such as the experimental autoimmune encephalomyelitis (EAE) model, there is evidence of immune dysregulation (Onodera et al. 2014). A number of studies have shown that PrP KO mice have a more aggressive disease with particularly reduced clinical improvement during the chronic phase of the disease (Tsutsui et al. 2008; Hu et al. 2010; Gourdain et al. 2012). These observations have led to the hypothesis that PrP^C may have a neuroprotective and anti-inflammatory role, involved in immune quiescence, specifically protecting immune-privileged organs such as the brain (Onodera et al. 2014; Bakkebo et al. 2015). Due to the expression pattern of PrP^C and the lack of an immune response to a self-antigen under normal conditions, cells of the immune system can be active players involved in the peripheral asymptomatic replication of the prion agent and its ultimate access to the CNS, at which point this triggers neurodegenerative and clinical manifestations (Wisniewski and Goni 2016, 2018; Aguzzi et al. 2013). Although some anti-PrP antibodies have been shown to be neurotoxic, other can be neuroprotective (Wisniewski and Goni 2018; Ma and Ma 2020). Importantly, naturally occurring innocuous anti-PrP antibodies have been detected in patient samples without prion disease, suggesting it may be possible to develop safe and effective anti-PrP^{Res} immunotherapeutics (Senatore et al. 2020; Frontzek and Aguzzi 2020). Paradoxically, immune suppression with, for example, splenectomy or immunosuppressive drugs, increases the incubation period (Aucouturier et al. 2000), while non-specific immunostimulation has the opposite effect (Bremer et al. 2009). This incubation period, during which time the prion agent replicates peripherally, without producing any symptoms, is quite long, lasting many months in experimental animals and up to 56 years in documented human cases associated with cannibalistic exposure to the prion agent (Collinge et al. 2006). Regardless of the length of this peripheral incubation, this critical period would be ideal for various anti-prion therapeutic agents, which will not have to overcome the significant therapeutic access problem of the blood brain barrier. In particular, potential game-changing antibodies that are specific to infectious prion particles could be effectively utilized during this incubation period (Wisniewski and Goni 2015a, 2018; Ma and Ma 2020; Goni et al. 2017). Lymphatic organs such as the spleen, tonsil, lymph nodes, or gut associated lymphoid tissue (GALT) contain high concentrations of PrP^{Res} long before PrP^{Res} replication starts in the brain (Beekes and McBride 2007; Brown et al. 2000; Mabbott and MacPherson 2006). Cells found to be particularly important for peripheral

PrP^{Res} replication are the FDC and the migratory bone-marrow derived DC (Beekes and McBride 2007; Mabbott and MacPherson 2006; Kitamoto et al. 1991; Aucouturier et al. 2001; Langevin et al. 2010). DC from infected animals are capable of spreading the disease (Aucouturier et al. 2001; Langevin et al. 2010). Immunotherapeutic approaches which can overcome the self-tolerance of these immune cells will likely inhibit prion replication in the lymphorecticular system (LRS) and ultimately neuroinvasion; however, a delicate immunomodulation has to be accomplished in order to produce a qualitative immune response while avoiding potential auto-immune toxicity (Wisniewski and Goni 2016, 2018). A further consideration is that while in most prion diseases, infection and replication in the LRS shorten the incubation times and facilitate neuroinvasion, this does not appear to be the case in most BSE cases, in sCJD, and in some types of scrapie, such as the drowsy form of hamster scrapie (Bartz et al. 2005; Bessen et al. 2009; Siso et al. 2010). Hence, the potential beneficial effect of altering the immune response to PrP would have to be tailor-made and might require an immune response within the CNS as well as peripherally in some cases.

37.2 *In Vitro* Studies Using Anti-PrP Antibodies to Block Prion Propagation

A precise understanding of the molecular mechanisms and pathways involved in the PrP^{C} to PrP^{Sc} conversion remains to be fully elucidated; however, there is extensive evidence of the primal importance of "seeding" by aggregated PrP^{Sc} molecules acting as template for PrP^{C} binding and subsequent conversion to more PrP^{Sc} (Come et al. 1993; Prusiner 1982). This interaction is critically dependent on the correct stereochemistry, as supported by the existence of a species barrier for prion infection, related to variations in the primary sequence of PrP^{C} of differing species. Therefore, it is not surprising that antibodies that may alter or mask the key epitopes on PrP^{C} and/or PrP^{Sc}, involved during the mutual conformational complementarity involved in prion propagation, will be inhibitory for prion replication. This was initially shown in 1988 when an anti-PrP^{C} polyclonal antibody was used *ex vivo* on a prion inoculum prior to injection and a significant reduction in the infectivity titer was noted (Gabizon et al. 1988). Using scrapie infected cells, it was later shown that an anti-PrP mAb, 6H4 directed to residues 144–152, was able to clear infection *in vitro* (Enari et al. 2001). In the same year, Peretz et al. used a number of different PrP-specific Fab fragments for scrapie clearance in chronically infected N2a cells (Peretz et al. 2001). They found D13 (directed to residues 95–103) and D18 (directed to residues 132–156) to be the most effective at scrapie clearance (Peretz et al. 2001). Kim et al. generated a large panel of antibodies raised to either recombinant mouse PrP or purified mouse PrP^{Sc} in PrP knock-out mice and tested them therapeutically in a N2a scrapie infected cell line (Kim et al. 2004a, b). They found that all anti-PrP antibodies that were able to bind to PrP^{C} on the cell surface, as judged by flow cytometry, were able to inhibit prion infection. Another study using a panel of

anti-PrP monoclonal antibodies (mAbs) to different epitopes of PrP in scrapie infected N2a cells and found the most effective to be 6D11, which is directed to residues 95–105 (hence, similar to D13); however, antibodies directed to residues 130–140 and 143–155 were also quite effective (Pankiewicz et al. 2006). These various studies suggest that therapeutic antibodies need to have high affinities of binding to PrP^{C} and/or PrP^{Sc}, as well as targeting specific key PrP domains. Proof of principle that passive immunization could be effective *in vivo* was first shown using transgenic mice that produced an anti-PrP monoclonal antibody. These mice were resistant to disease following challenge with a mouse-adapted scrapie strain (Heppner et al. 2001a).

37.3 Therapeutic Targeting of Prions: The Challenge of Effectiveness Versus Toxicity

Several potential therapeutic non-mutually exclusive targets can be explored for preventing and/or treating prion infection and progression, which include: (1) blocking entry of orally acquired PrP^{Res} across the gut epithelial barrier and its nerve terminal ends, by dimeric secretory IgA with neutralizing anti-PrP^{Res} specificity; (2) promoting the elimination in the peripheral circulation/lymphoreticular tissue of cells carrying target PrP^{Res} on their members prior to passage across the blood-brain barrier (BBB) into the CNS; (3) blocking the conformational change that leads the PrP^{C} to PrP^{Sc} conversion either in the periphery, or in the CNS after neuroinvasion; (4) reducing the available PrP^{C} levels to minimize the substrate availability for PrP^{Res} conversion; (5) promoting PrP^{Res} clearance/elimination in the brain; and (6) neutralizing PrP^{Res} in the CNS to reduce toxicity and neuroinflammation. Several immune targeting options could be effective on pathway 1 or 2 at preventing infection/transmission from a peripheral source by neutralizing specifically PrP^{Res} with a low possibility of toxicity, as shown in a proof of concept mucosal immunization of white-tailed deer challenged with CWD (Goni et al. 2015). Targeting PrP^{Res} in the CNS introduces the BBB as a significant challenge, as any agent will have to be BBB penetrant. It has been shown in AD models that only about 0.1% of peripheral IgG crosses the BBB (Pardridge 2007; Pepinsky et al. 2011; Wisniewski and Goni 2015b); hence, some means of enhancing CNS delivery might be required (Poudel and Park 2022; Faresjo et al. 2021; Gonzalez-Mariscal et al. 2016). Intracranial administration of some anti-PrP monoclonal antibodies (that have no PrP^{C} or PrP^{Sc} specificity) was shown to induce neuronal apoptosis (Solforosi et al. 2004; Tayebi and Hawke 2006). Other studies using different anti-PrP antibodies did not show significant toxicity (Klohn et al. 2012; Xanthopoulos et al. 2013). Using a panel of anti-PrP antibodies, it was demonstrated that toxicity is likely PrP epitope dependent; with some antibodies showing no evidence of toxicity (Sonati et al. 2013). Generally, anti-PrP antibodies targeting the flexible tail of PrP^{C} are neuroprotective, versus antibodies that target the globular domain are neurotoxic (Frontzek and Aguzzi 2020; Sonati et al. 2013). Significantly, it has been recently reported that

naturally occurring, non-toxic anti-PrP antibodies have been detected in patient samples without prion disease, suggesting it may be possible to develop safe and effective anti-PrP^{Res} immunotherapeutics (Senatore et al. 2020). In addition, a study has shown that PrP^{C} can be a viable therapeutic target since genetic ablation of PrP^{C} expression in the presence of early spongiform pathology, resulting in reversal of this pathology without apparent toxicity (Mallucci et al. 2007; Verity and Mallucci 2011). Other studies have shown that raising an antibody immune response against protein secondary structure only present in oligomeric forms of pathological conformers can recognize the common denominator present not only in oligomeric Aβ and tau in AD models and human AD brains, but in oligomeric forms of scrapie, CWD, and human sCJD (Goni et al. 2017; Herline et al. 2018). For maximal effectiveness, without associated toxicity, it is likely that immunological therapeutic targeting within the CNS will need to be directed to the specific conformation of PrP^{Res} and/or to pathological oligomeric structures.

37.4 Passive Immunization for Prion Infection

The first evidence that an anti-PrP immunotherapy could provide some protection against prions *in vivo* was with the transgenic expression of μ chains of 6H4 in prion-infected mice(Heppner et al. 2001a). An initial passive anti-PrP immunization study using a more classical approach where antibodies were injected systemically into non-transgenic, CD1 mice it was shown that mAbs 8B4 (to mouse PrP residues 34–52) and 8H4 (to mouse PrP residues 175–185) given immediately after challenge with 139A scrapie by intraperitoneal (ip) injection (50 μg/week), resulted in a significant prolongation of the incubation period with 10% of the 8B4 treated animals remaining disease free in the group challenged with a lower dose of PrP^{Sc} (Sigurdsson et al. 2003). A similar study using higher doses (4000 μg/week ip) of either ICSM 18 (to mouse PrP residues 146–158) or ICSM 35 (to mouse residues 95–105), showed that prion infection from a peripheral source could be completely prevented if treatment was continued for 7 or 30 days immediately following PrP^{Sc} challenge or within 30 days (White et al. 2003). This approach could be used immediately following accidental exposure in humans to prevent future infection. Unfortunately, passive immunization was not found to be effective closer to the clinically symptomatic stages of prion infection (White et al. 2003). Another study used 6D11, which is directed to PrP residues 95–105, immediately after intraperitoneal challenge, producing a ~37% prolongation of the incubation period with a reduction in the severity of pathology (Pankiewicz et al. 2006; Sadowski et al. 2009). Song et al. (Song et al. 2008) demonstrated therapeutic efficacy with anti-PrP antibodies up to 120 days post inoculation, using direct intra-ventricular (i.v.) infusion, with a slight (~8%) prolongation of the incubation time using the Chandler scrapie strain. The same group tested one of these monoclonal antibodies (31C6 to mouse PrP 143–149) by peripheral injection into the tail veins at 120 days post inoculation (at about the onset of clinical disease) and found no significant survival

prolongation but a slight reduction in pathology(Ohsawa et al. 2013). The therapeutic effectiveness of passive immunization with monoclonal antibodies that specifically recognize PrP^{Sc} and not PrP^{C} remains to be tested. Our group has conducted studies generating antibodies that recognize the shared pathological structure of Aβ oligomers, pathological tau (in the form of paired helical filaments [PHF]) and PrP^{Sc} (Goni et al. 2016, 2017; Wisniewski and Goni 2015b; Herline et al. 2018; Drummond et al. 2018). These antibodies were generated using a polymerized peptide derived from a short version of the carboxyl terminus of the British amyloidosis (ABri) peptide, oligomerized using glutaraldehyde as a cross linker to form a stable population of oligomers, which we term pBri (Goni et al. 2010, 2013, 2017). This peptide is long enough to acquire a secondary structure mimicking the shared pathological conformation but short enough to avoid the development of tertiary structures that would compete with the intended target. More important, it lacks any sequence homology to any mammalian protein, including Aβ, tau, or PrP (Goni et al. 2010; Vidal et al. 1999; Rostagno et al. 2005); however, when polymerized and used as an immunogen, it can produce a humoral immune response to multiple oligomeric species. We have used this immunization strategy to also produce a family of mAbs that recognize multiple pathological conformers, including PrP^{Sc} (Goni et al. 2016, 2017). Such antibodies, which have enhanced specificity to oligomeric forms of PrP^{Sc} more than PrP^{C}, might have therapeutic potential in prion disease *in vivo*; however, this remains to be evaluated.

Recently, the results of the first human trial of passive immunization using anti-PrP antibody was reported (Mead et al. 2022). Six patients with CJD were included in a first-in-human, compassionate use clinical trial of PRN100, an anti-PrP monoclonal antibody (under a Specials License, with independent oversight) (Mead et al. 2022). PRN100 is a humanized version of the anti-PrP^{C} globular domain targeting antibody ICSM18. Repeated intravenous dosing of PRN100 was apparently well tolerated and was shown to produce a CSF level of 50 nM in four patients (Mead et al. 2022). Neuropathological examination was conducted in two of the treated subjects, and there was no evidence of neurotoxicity associated with PRN100 use. This milestone trial showed that intravenous passive immunization with an anti-PrP monoclonal antibody is safe and can lead to potentially therapeutic levels in the CSF. However, in this small trial, there was no evidence that this therapy altered the clinical course (Mead et al. 2022). Disease progression was certainly not stopped or reversed in any of the six patients; however, the MRC Prion Disease Rating Scale scores did indicate stabilization in three subjects covering a period when CSF drug concentrations reach the target dose. Given the small size of the trial, statistical power was not achieved. This proof-of-principle trial opens up the door to future more PrP^{Sc} targeted passive immunization trials that may have a higher chance of showing therapeutic efficacy.

Interestingly, targeting PrP^{C} with passive immunization using anti-PrP antibodies may have therapeutic applicability in other prion-like diseases, such as AD. A number of studies showed that amyloid β oligomers (Aβo) mediate their toxicity, in part, via binding to cellular prion protein (PrP^{C}) on the surface of neurons (Smith et al. 2019; Jarosz-Griffiths et al. 2016; Brody and Strittmatter 2018), with more

recent data suggesting that other oligomeric species including those of tau and α-synuclein also mediate toxicity via an interaction with PrP^C (Corbett et al. 2020; Rubenstein et al. 2017; Tang et al. 2020). Previously, it has been also reported that short-term treatment with an anti-PrP antibody, 6D11, in AD model APP/PS1 mice can dramatically reverse behavioral deficits without affecting the amyloid burden by blocking the Aβo/PrP^C interaction (Chung et al. 2010). As discussed above, the 6D11 antibody is also highly effective at treating prion infection in tissue culture and *in vivo* by blocking the interaction between PrP^C and PrP^{Sc} (Sadowski et al. 2009). It has also been shown that anti-PrP^C 6D11 blocks the Aβo binding site on PrP^C preventing the impairment in long-term potentiation caused by Aβo derived from AD brain extracts (Barry et al. 2011; Freir et al. 2011). Recently, our group has generated an anti-PrP antibody (TW1), which has an epitope on PrP that is similar to that of 6D11 (Boutajangout et al. 2021). We have shown that passive immunization of TW1 in a mouse model of AD with exclusive tau pathology, was therapeutically active, producing cognitive benefits and a reduction in pathology by blocking the tau oligomer to PrP^C interaction(Boutajangout et al. 2021). Hence, some anti-PrP antibodies can be used with passive immunization to ameliorate pathology related to oligomers in multiple prion-like disorders such as AD and Parkinson's disease. The recent results of the use of anti-PrP PRN100 in a clinical trial of CJD patients show that such a therapeutic trial could be done safely in other disease settings (Mead et al. 2022).

37.5 Active Vaccination for Prion Infection

A significant hurdle for the development of an active anti-PrP vaccine is overcoming T-cell tolerance to a self-antigen. For many years, the dogma was that to generate anti-PrP antibodies, it was required to use PrP KO mice. However, more recent studies indicate an immune response can be mounted naturally against some PrP epitopes without associated toxicity (Senatore et al. 2020). In addition, it has been documented that toward the clinical stages of prion infection, some anti-PrP antibodies are often generated (Sassa et al. 2010). The first *in vivo* studies of an active immunization like approach showed that challenge with a slow strain of PrP^{Sc} blocked the latter expression of a more virulent fast strain of PrP, mimicking vaccination with a live attenuated organism (Manuelidis 1998). We first demonstrated that active immunization with recombinant PrP delayed the onset of prion disease in wild-type mice; however, the therapeutic effect was very modest and eventually all the mice succumbed to the disease (Wisniewski et al. 2002). The limited therapeutic effect could be explained by the observation that the antibodies generated against prokaryotic PrP often do not have a high affinity towards the cell-surface, critical portions of PrP^C that are involved in binding and replication, and that the anti-PrP titers generated were low (Polymenidou et al. 2004). Various investigators tried to increase the immunogenicity of the PrP immunogen, by using dimers or PrP aggregates(Xanthopoulos et al. 2013; Polymenidou et al. 2004; Gilch et al. 2003); alternatively, heterologous PrP

peptides were used (Ishibashi et al. 2007). Another way to enhance immunogenicity was with strong adjuvants such as CpG oligodeoxynucelotides (ODNs) that are Toll-like 9 receptor agonists and stimulate T- and B-cell repertories against PrP (Rosset et al. 2004; Spinner et al. 2007). Interestingly, stimulation of immunity with CpG ODNs alone has been shown to ameliorate all AD-related pathology in multiple transgenic mouse models and in non-human primates (Scholtzova et al. 2014, 2017; Patel et al. 2021). Alternatively, repeated injections (biweekly) with PrP-absorbed Dynabeads, producing a multivalent immunogen, were able to induce a IgM PrP-specific humoral response that was associated with a slight (~11%) prolongation of incubation time after intraperitoneal prion challenge (Tayebi et al. 2009).

In addition to these PrP peptide or recombinant protein-based approaches, DNA vaccines have been used to enhance an immune response. In these studies, immunization with cDNA encoding for heterologous PrP fused to either a stimulatory T-cell stimulatory peptide (Alexandrenne et al. 2010) or a targeting protein that enhances antigen uptake and presentation via MHC class I has been used (Han et al. 2011). These DNA vaccines induce significant PrP-specific IgG, but their ability to prevent prion infection has not been well studied.

Active immunization has also been examined using PrP-displaying viral constructs. Virus-like particles (VLPs) are excellent immunogens, which typically trigger a strong humoral immune response. Studies using this approach to immunize wild-type mice, rats, and/or rabbits have demonstrated that this can stimulate high titers of anti-PrP antibodies that recognize PrP^{C} and/or PrP^{Sc} (Nikles et al. 2005; Handisurya et al. 2007). In addition, this approach has been used to transduce dendritic cells with adenovirus expressing human PrP, which was subsequently utilized to immunize wild-type mice. The mice developed anti-mouse PrP antibodies associated with a reduction in PrP^{Sc} accumulation in the spleen and a prolongation of the incubation period (Rosset et al. 2009).

Induction of a more PrP^{Sc} active immune response has also been tried. It has been suggested that three epitopes that are more exposed in PrP^{Sc} include: a YYR motif, a YML motif in β-sheet 1, and another YML within the rigid loop linking β-sheet 2 to a-helix 2. A vaccine based on these epitopes was able to induce a more PrP^{Sc}-specific sustained antibody response (Marciniuk et al. 2014; Taschuk et al. 2017); however, the effectiveness of this immunization approach remains unknown. Unfortunately, when other vaccine targeting the YYR disease-specific epitope (by intramuscular injection of a recombinant YYR-Lkt fusion protein with Emulsigen D) was testing in elk, which received a natural exposure to CWD via a contaminated environment, the vaccinated animals had a worse survival time (800 days) versus the controls (1,062 days) for unclear reasons (Wood et al. 2018).

37.6 Mucosal Active Immunization

A potentially ideal means of using immunomodulation to prevent prion transmission and infection is by utilizing active mucosal immunization. The most obvious reason for using this approach is that the gastro-intestinal tract is the major route of

entry for many prion diseases such as CWD, BSE, CPD, TME, and vCJD (Wisniewski and Goni 2018; Mathiason 2022; Gallardo and Delgado 2021). Mucosal immunization can be designed to induce primarily a humoral immune response with a neutralizing secretory IgA (sIgA) response in the gut that can prevent or inhibit entry of the prion agent into the body. sIgA is resistant to protease degradation thanks to the protective action of the secretory component (SC) attached to the dimeric form of the immunoglobulin; making it ideal to bind and neutralize any foreign agent along the whole passage of the gastro-intestinal tract. We have developed anti-prion vaccines with specific delivery systems that target gut associated tissue, the main site of entry of the prion agent (Wisniewski and Goni 2018; Goni et al. 2005, 2008, 2015), which can be fully protective for peripheral prion infection. We have expressed PrP in attenuated *Salmonella* strains, where one or more genes responsible for virulence have been deleted, as a live vector for oral vaccination (Goni et al. 2008, 2015; Boutajangout et al. 2009). Live attenuated strains of *Salmonella enterica* are very well characterized and have been used for many years as vaccines against salmonellosis in humans, as well as serving as a delivery system for the construction of multivalent vaccines with broad application in both human and veterinary medicine (Mastroeni et al. 2001; Moreno et al. 2010; Galen et al. 2021). A significant advantage for this system is that the safety of human administration of live attenuated *Salmonella* has been extensively confirmed in humans and animals, in whom it has been shown to be able to penetrate the gut mucosa and specifically deliver protein products to immune presenting cells in lymphoid follicles (Moreno et al. 2010; Galen et al. 2021; Tacket et al. 2000; Kirkpatrick et al. 2006; Roland and Brenneman 2013). A variety of animals have been effectively immunized by an oral route using live *Salmonella* to induce humoral mucosal responses (Galen et al. 2021; Roland and Brenneman 2013; Villarreal-Ramos et al. 1998; Chabalgoity et al. 2000). *Salmonella* targets M-cells, antigen sampling cells in the intestines, which importantly may also be critical for PrP^{Sc} uptake (Goni et al. 2008, 2015; Mabbott and MacPherson 2006; Heppner et al. 2001b; Sigurdsson and Wisniewski 2005). Therefore, this approach is more targeted than prior vaccination studies, providing a possible explanation for the improved efficacy (Wisniewski and Goni 2018; Goni et al. 2005, 2008, 2015; Boutajangout et al. 2009). The *Salmonella* vector can also express one or several repeating copies of PrP (which can be heterologous to enhance the immune response), producing and delivering a protein product that might simulate the three-dimensional sites critical for the PrP^{C} to PrP^{Sc} interaction (Wisniewski and Goni 2018; Goni et al. 2008, 2015). This approach takes into account that if tolerance is broken, the majority of the B-cell response will be devoted to producing dimeric secretory IgA in the mucosa with a more limited (in comparison to a conventional vaccination methodology) systemic IgG level, which will help to maintain an optimal level of anti-PrP systemic antibodies that can neutralize any PrP^{Sc} that breeches the gut barrier, with a low risk of autoimmune pathology. In a mucosal priming immunization, the genes of the V_H regions selected for recombination within the mesenteric lymphoid tissue are prone to produce a more neutralizing binding site (paratope), likely very distinct from a systemic humoral response. That same neutralizing paratope will be kept after the switch, not

only on the dimeric sIgA in the gut and other mucosa but also on the small number of concomitant systemic IgA and IgG, and some persistent IgM produced. Our past data using 139A scrapie prions in wild-type CD-1 mice indicate that in animals that have a significant anti-PrP mucosal IgA response and a systemic anti-PrP IgG response, full protection against oral challenge with the PrP^{Sc} is possible (Wisniewski and Goni 2018; Goni et al. 2005, 2008). Further refinement of mucosal immunization, aiming for greater specificity to critical epitopes expressed in PrP^{Sc} or that are involved in the conformational change to PrP^{Sc}, rather than high anti-PrP levels, is likely to lead to an effective means of preventing prion disease in animal and human populations at risk for prion exposure. This approach is being evaluated. Taschuk et al. developed a non-replicating human adenovirus expressing the rigid loop epitope of PrP (discussed above) fused with the rabies glycoprotein G and showed that oral immunization in white-tailed deer is able to induce both mucosal and systemic anti-PrP antibodies (Taschuk et al. 2017). Whether this approach will protect against prion challenges is not known.

We have shown that mucosal immunization is able to partially protect from CWD in white-tailed deer (Wisniewski and Goni 2018; Goni et al. 2015). CWD appears to be the most infectious prionosis to date, affecting free ranging and farmed ungulates (white-tailed deer, mule deer, elk, and moose), with significant zoonotic potential (Mathiason 2022; Williams 2005; Sigurdson 2008; Gilch et al. 2011; Aguzzi and Sigurdson 2004; Saunders et al. 2012; Aguilar-Calvo et al. 2015; Greenlee and Greenlee 2015; Waddell et al. 2017; Moreno and Telling 2017). Mucosal immunization in deer was performed by numerous oral inoculation, along with tonsil and rectal vaccination that were supplemented with polymerized recombinant deer PrP, which was produced by cross-linking recombinant PrP with glutaraldehyde as described for the short Bri peptide (Goni et al. 2010, 2015). This vaccination strategy was able to break mucosal immunological tolerance to PrP in deer, with production of gut and saliva IgA, as well as systemic IgM and IgG reactive to aggregated recombinant cervid PrP and also to PrP^{CWD}, which was associated with a significant prolongation of the incubation period and complete protection from clinical infection in one of the five vaccinated deer (Goni et al. 2015). These results indicated for the first time that specific antibody responses against the self-antigen PrP can be produced in the biological fluids (gut and plasma) of large cervid mammals naturally at risk for prion infection, in association with at least partial protection from CWD infection (Goni et al. 2015).

Nevertheless, many pitfalls arise from this proof-of-concept deer vaccination. We have since improved the immunogen with a number of modifications. We are now using a more efficient Salmonella delivery system and also 20–30 amino acid length PrP peptides to optimize the viability of the *Salmonella* delivery system up to three days at room temperature (Goni et al. 2019). Six different peptides in individual attenuated *Salmonella* covered most of the PrP molecule, helping to present single selected epitopes to the immune system to generate simultaneous immune responses by design. Each peptide was multi-species, incorporating single mutations that are privative of at least three different species. This strategy produced substantially higher titles of sIgA and IgG with only one priming and two boosts.

All the generated polyclonal responses recognized oligomeric PrP^{Sc} from different species, including human PrP^{CJD} (Goni et al. 2019). We hope to test this approach in large animal models in the future to test effectiveness for CWD.

37.7 Conclusions

None of the conformational neurodegenerative disorders have a highly effective therapy currently. Many studies using AD models have shown that immunotherapeutic approaches can reduce amyloid and tau-related pathology, which is associated with a cognitive rescue (Drummond and Wisniewski 2017); however, the majority of clinical trials in AD have resulted in failure (Kim et al. 2022; Pleen and Townley 2021; Reiss et al. 2020). On the other hand, some recent clinical trials in mild cognitive impairment and early AD subjects suggest that anti-Aβ immunotherapy targeting aggregated species or post-translationally modified Aβ clearly ameliorates AD biomarkers and may be associated with cognitive benefits (Cummings et al. 2021; Mintun et al. 2021; Budd Haeberlein et al. 2022). The prion diseases are much less common than AD; however, the past outbreak of vCJD originating from BSE, and the current epidemic of CWD and emergent CPD, with the potential of human transmission, highlights the importance of developing therapies for this group of disorders. The specific self-replicating ability of the pathological PrP^{Sc} to convert physiological PrP^{C} depends on features present in different parts of the protein. Extensive *in vitro* and *in vivo* data using prion infection models have shown that immunomodulation is effective at preventing infection. The recent clinical trial of PRN100 clearly demonstrates that an anti-PrP monoclonal can be given repeatedly intravenously, to achieve adequate CSF levels safely (Mead et al. 2022). Furthermore, the demonstration of naturally occurring anti-PrP antibodies in the human immunoglobulin repertoire suggests passive immunotherapy could be utilized safely (Senatore et al. 2020; Frontzek and Aguzzi 2020). Since many prion diseases have the mucosa of the alimentary tract as a point of entry, mucosal immunization may be particularly suitable for these forms of prion infection, with recent studies indicating that oral prion infection can be prevented by appropriate mucosal vaccination (Mathiason 2022; Goni et al. 2015, 2019). This approach may be particularly suitable to stem the current epidemic of CWD, as well as for CPD, with their associated specter of potential spread to large human populations. In the future, this approach could also be the basis of delaying the onset, or preventing the progression, of known familial prionoses and the treatment of sporadic CJD (if methods for pre-symptomatic diagnosis are developed). A developing promising immunomodulatory therapeutic approach is the specific targeting of the PrP^{Sc} conformation or the shared β-sheet-rich pathological conformation that is found in toxic oligomers, which are central to the pathogenesis of most neurodegenerative conditions.

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