Wen-Quan Zou · Pierluigi Gambetti
Editors

Prions and Diseases

Volume 2, Animals, Humans and the Environment

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Chapter 1
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. To date, a total of 34 L-type BSE and 27 H-type BSE have been detected worldwide by routine BSE testing in older cattle. The clinical phenotypes of atypical BSE forms are still undefined in field animals, although 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 a possible role in the origin of BSE epidemics. Further, the evidence that atypical BSEs are transmissible to mammals, including nonhuman primates, are issues that raise public health concerns.

Keywords Amyloid • Atypical BSE • Bovine amyloidotic spongiform encephalopathy • Bovine spongiform encephalopathy • Creutzfeldt–Jakob disease • H-type BSE • L-type BSE • Prion strains

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1.1 Introduction

The story of bovine spongiform encephalopathy (BSE) began in 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, BSE outbreak started.

1.2 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 since 1940s to increase protein content in animal diet, particularly in dairy herds. The preparation of protein-rich supplement followed a rendering process, whereby the slaughterhouse refuse (offal) were 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, preemptively slaughtered and destroyed, the number of animals was nearly four and a half million (Brown et al. 2001).

To forefront the outbreak, UK Government issued a series of preventive measures. In July 1988, started the prohibition to use and/or supply with ruminant-derived proteins ruminant feed, 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 brain, spinal cord, tonsil, thymus, spleen, and intestines, were excluded from the animal and human food chains. 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 cattle (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 of cattle were incinerated. In 2005, the OTM rule was replaced by mandatory BSE screening test of OTM cattle slaughtered for human consumption.

1.3 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 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 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 years the importation of MBM from the UK (Butler 1996). This in contrast with the French ban prohibiting UK meal 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, 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 a 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.

1.4 The Impact of 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 setup 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 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.

1.5 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, i.e., "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 USA maintained a 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 the 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 1.2 times.

In 2001, the active surveillance in Europe system snapshot the real occurrence of foodborne BSE, consistent with exposure during the period 1995–1996, in accordance with the estimated incubation period of 5 years. The reduction of BSE cases in several European countries during the following years, suggest 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 to 48 months for healthy slaughtered cattle and from 24 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.

1.6 The Detection of Atypical BSE Forms by Routine Testing

Since 1999, 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 Western blot test 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. 1.1a). L-type BSE was 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 USA, Canada, and in Japan (Table 1.1). 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).

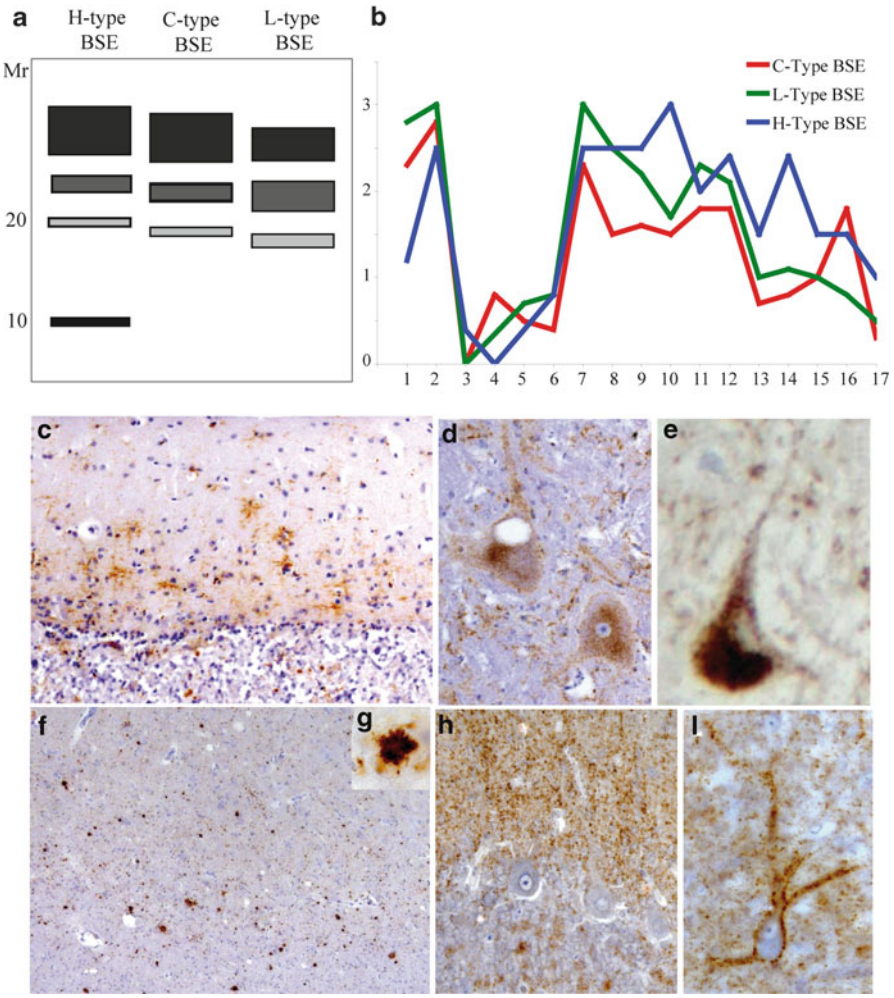

Fig. 1.1 *Biochemical features, lesion profile, and pathological phenotypes of classical and atypical BSE forms*. (**a**) Electrophoretic patterns of protease-resistant PrP in C-type and atypical BSE forms (H-type and L-type); (**b**) Histograms of the lesion load in H-type, C-type, and L-type BSEs; 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; and 17, Claustrum); numbers in ordinate denote vacuolation score (1 mild, 2 moderate, and 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**)

Table 1.1 Typical and atypical BSE cases detected worldwide from 1989 to date

	C-type			
Country	1989–2000	2001–2011	L-type	H-type
Austria	–	8	2	–
Belgium	19	114	–	–
Canada	–	18	1	1
Czech Republic	–	30	–	–
Denmark	1	15	1	–
Finland	–	1	–	–
France	95	775	13	14
Germany	7	406	1	1
Ireland	507	1,057	–	1
Israel	–	1	–	–
Italy	–	142	4	–
Japan	–	36	2	–
Luxembourg	1	2	–	–
Netherlands	8	79	2	1
Poland	–	69	8	2
Portugal	522	546	–	–
Slovakia	–	25	–	–
Slovenia	–	9	–	–
Spain	2	771	–	–
Sweden	–	1	–	1
Switzerland	366	98	–	1
UK (GB)	179,087	2,568	–	3
USA	–	2	–	2
Total	*180,615*	*5,457*	*34*	*27*

1.7 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 overreactivity 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 epidemic in the UK. The distribution and the score of vacuolar changes in different brain areas was examined by Scott and coworkers, who showed the highest lesion load in the medulla, midbrain, and thalamus, while 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, i.e., the solitary tract nucleus and the spinal tract nucleus of trigeminal nerve, allowing a 100% diagnostic specificity (Wells et al. 1989), in addition to 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 dystocia at 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 the recognition of these forms at slaughter inspection.

The neuropathological lesion profile of atypical BSE forms differed from C-BSE (Fig. 1.1b), 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. 1.1c, d). In H-type BSE, PrP immunohistochemistry disclosed a prevailing intraneuronal and intraglial pattern of deposition (Fig. 1.1e), 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. 1.1f–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).

1.8 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 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).

1.9 On the Origin of BSE

The enigma of 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 two cases per 1,000 (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 BSE epidemic started when the scrapie agent entered in the food chain crossing the sheep–cow species barrier (Fig. 1.2a).

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. 1.2b). 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 case 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).

Recently, a pathogenic E211K mutation has been reported in a cattle with H-type BSE, but the biological relevance of this finding is still unclear (Fig. 1.2c) (Richt and Hall 2008).

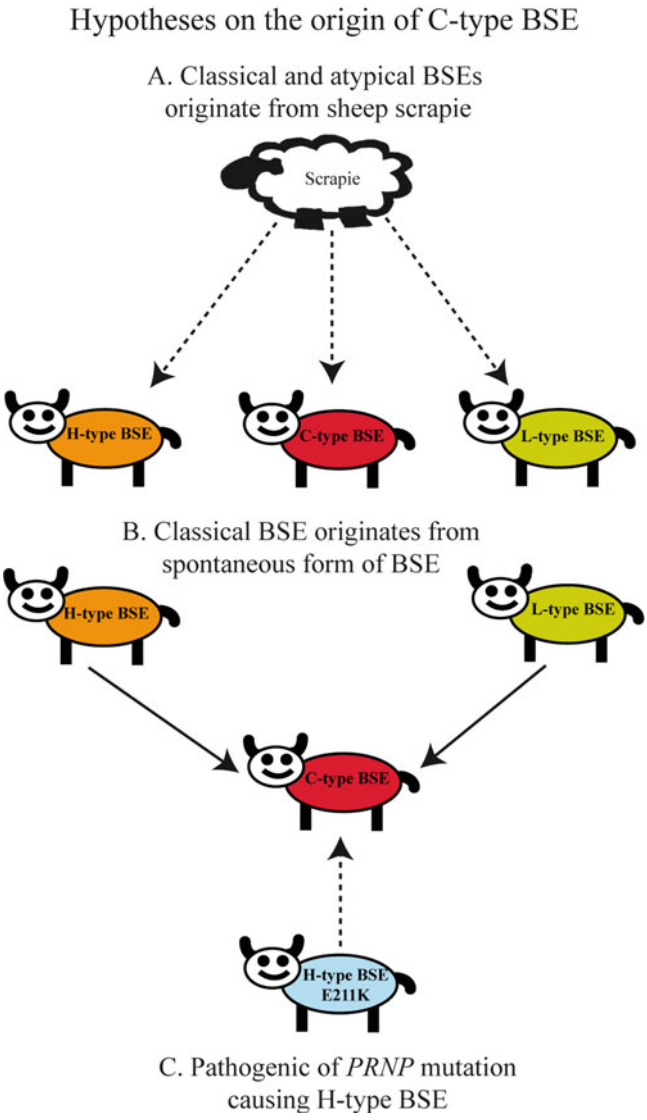

Fig. 1.2 Hypotheses on the origin of C-type BSE

1.10 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 nonhuman primates. Kong et al. (2008) showed that BASE was transmitted to TgHu mice overexpressing human PrP Met/Met at codon 129 (Tg40 mice), with an attack rate of 60%. 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 BSE. At 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 at 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 L-type BSE. Therefore, a zoonotic risk is potentially higher for 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 nonhuman 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 2
Classical and Atypical Scrapie in Sheep and Goats

Christine Fast and Martin H. Groschup

Abstract Scrapie is a naturally occuring transmissible spongiform encephalopathy (TSE) in sheep, goat and mufflons almost world-wide and is known for about 250 years. It is characterized 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 gene. Whereas classical scrapie is clearly a naturally occurring transmissible disease, atypical scrapie may also be caused by the spontaneous misfolding of 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 the characteristics of the most common scrapie strains.

Keywords Atypical scrapie • Classical scrapie • Pathological prion protein • Prions • Scrapie • TSE

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2.1 Overview

Scrapie is the most common name for the 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 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 and the immunodetection of pathological prion protein (PrP^{Sc}) depositions in the CNS primarily (OIE-Manual of Diagnostic Tests and Vaccines for Terrestrial Animals). PrP^{Sc} depositions can be revealed by immunohistochemical and biochemical methods (see Chap. 13). To date, two distinct scrapie types are known: classical and atypical scrapie.

2.2 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 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 the 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, a 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 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 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, a virus infection was the most commonly proposed theory, already formulated in 1938 (Cuille and Chelle 1938a, b). In 1954, the term of a "slow virus infection" was first time introduced (Sigurdsson 1954). However, already in 1966, an alternative to the virus origin was postulated as the causative agent, i.e., 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 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 new emerging form of TSE (Bruce et al. 2007). Atypical scrapie is distinguished from classical scrapie by clinical and epidemiological as well as by molecular and histopathological features. It is not rare compared to classical scrapie in most countries and found worldwide at a comparable incidence rate, which is indicative for a different, perhaps non-infectious aetiology (Fediaevsky et al. 2008).

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 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 in six natural cases so far (Wood et al. 1992a, b).

2.3 Geographical Distribution and Surveillance

Scrapie is endemic in almost all member states of the European Union (EU 27) as well as in Norway, Iceland and Switzerland. Brazil, Canada, Israel, Japan, Palestinian Autonomous Territories, Russia, Tajikistan and the USA reported scrapie cases (atypical and/or classical) in the last 6 years. Only individual atypical scrapie cases were documented on the Falkland Islands and New Zealand (Epstein et al. 2005, EU Commission, Kittelberger et al. 2010, World Animal Health Data Base (WAHID); http://web.oie.int/wahis/public.php?page=disease_timelines). According to the "World Livestock Disease Atlas 2011" (Anonymous 2011), scrapie ranks third worldwide as cause for sheep and goat losses.

An introduction of classical scrapie via imported sheep from the UK was suspected for 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 scrapie to date (Detwiler and Baylis 2003).

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 Manual). This is exemplified by the introduction of a harmonised active surveillance program for scrapie in sheep and goats throughout the EU in 2003. In the context of this program, animals over 18 months of age (fallen stock, emergency slaughter, as well as healthy slaughtered animals) were examined for TSE.

2.4 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 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). Similar results were obtained in sheep. The ovine Prnp consists of two alleles sA (short incubation period) and pA (prolonged incubation period), which are distinct primarily in the amino acid sequences encoded at codon

136 (Dickinson et al. 1968b; Hunter et al. 1996, 1997). Similar as in mice, the length of the incubation period is depending on the scrapie strain that is used (Foster and Dickinson 1988). Furthermore, in susceptible animals, effects on the incubation period can also result from polymorphisms at codons 154 and 171 (Hunter et al. 1996). Thus, the incubation period is determined at least by two factors: the genotype of the host and the agent strain.

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. 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 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 to 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 into a TSE genotype classification system (Dawson et al. 1998). This five group risk classification (Table 2.1) is the basis for breeding and scrapie eradication programs applied in the EU. The highest risk to develop scrapie carry VRQ/VRQ animals, the highest genetic resistance is associated to ARR/ARR sheep (Belt et al. 1995; Hunter et al. 1996; Hunter 1997). However, this classification is subject to restriction as, for example 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 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

Table 2.1 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	

carrying for example AC151RQ, AT137RQ, or ARQK176 (Acin et al. 2004; Thorgeirsdottir et al. 1999).

The classification system described above and in Table 2.1 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/AHQ, AHQ/ARQ and ARR/ARR, respectively. It has been shown that polymorphisms at codons 141 and 154 are linked to susceptibility. Genotype AF141RQ 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 29 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). At least five of them seem to be associated with TSE susceptibility (for detailed review see Vaccari et al. 2009):

- I142M haplotypes have a lengthened incubation period after experimental inoculations and are associated with increased resistance to classical scrapie under natural conditions (Goldmann et al. 1996; Barillet et al. 2009).
- 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) but have a comparable high risk associated with atypical scrapie (Moum et al. 2005; Arsac et al. 2007; Seuberlich et al. 2007).
- N146S/D polymorphisms encode low risk (some resistance) for scrapie infection but this genotype is confined to Damascus/Damascus crossbreed goats on Cyprus primarily (Papasavva-Stylianou et al. 2007).
- R211Q haplotypes have shown an increased resistance to classical scrapie in French case–control studies (Barillet et al. 2009).

– Q222K haplotype is associated with protection against classical scrapie in several breeds and countries, but heterozygous animals are reported to be infected (Acutis et al. 2006; Barillet et al. 2009; Vaccari et al. 2006).

At the time of writing, both haplotypes 146S/D and 222K are considered as candidate for TSE resistance breeding and eradication programs for goats.

In summary, the number of variables influencing the susceptibility to scrapie are high and depend not only on the genotype of the host and the infectious agent but also on individual flocks, breeds and geographical location, not to forget dose and route of inoculation effects.

2.5 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 ante mortem tests (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 scrapie see Hoinville 1996; Detwiler and Baylis 2003; Benestad et al. 2008).

2.5.1 Prevalence in the EU

A comprehensive overview on the prevalence of classical and atypical scrapie in the European Union is given by Fediaevsky et al. as well as by the European Commission (Fediaevsky et al. 2008, 2010; EFSA 2010).

Following the introduction of active surveillance programs for TSEs in sheep and goats in the EU in 2003, clearly defined epidemiological data were obtained for the first time. In this regard, the prevalence of classical and atypical scrapie showed different patterns with more variation seen in classical scrapie (Fediaevsky et al. 2008). In sheep, the overall prevalence of classical scrapie in the EU (excluding Cyprus) decreased from 2002 to 2009. The number of cases in fallen stock was significantly higher as compared to healthy slaughter animals (Fediaevsky et al. 2008). For example in 2009 in the EU, 27 1.158 sheep (of 331.027 tested) and 89 goats (of 117.868 tested) were TSE positive but only 14 cases of sheep and no goat scrapie case were detected in slaughter animals (EFSA 2010, European Commission). This data compilation excludes Cyprus and Slovenia which presented, at the time of writing, a very specific situation. It can be assumed that due to the genetic breeding programs for resistance to scrapie, the proportion of sheep carrying ARR alleles in the populations will increase and therefore the prevalence rates of classical scrapie will further

decrease (EFSA 2010). However, it has to be emphasised that the incidence rates of classical scrapie in geographical areas were non-uniform. Areas could be grouped into (1) countries with no cases detected at slaughterhouse, (2) countries with low prevalence rates and (3) countries with high prevalence rates at the slaughterhouse. This classification did not exclude areas with high rates in countries with low prevalence rates (EFSA 2010). Furthermore, 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 (Fediaevsky et al. 2008, 2010). In eight EU countries between 2007 and 2009, the incidence of atypical scrapie in healthy slaughtered sheep was similar 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). The prevalence seems to be stable within the EU and due to the breeding programs favouring the susceptible ARR genotype, it can be assumed that atypical scrapie will not be eliminated (Lühken et al. 2007). Furthermore, the same limitations of the active surveillance associated with classical scrapie are true for atypical scrapie. Other problems in estimating the exact prevalence of atypical scrapie include the age-dependent variations and the inconstant 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. In this reagrd of particular interest are recent results, which show infectivity in the lymphoreticular system of sheep infected with atypical scrapie (Andréoletti et al. 2011).

2.5.2 Transmission Routes in Scrapie

In the last centuries, a lengthy discussion about the mode of transmissions 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 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, 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). However, results of different studies indicate that an in utero infection prior to parturition does not occur (Hadlow et al. 1982; Andreoletti et al. 2002). The placenta and the amniotic fluid 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). Besides the placenta, amniotic fluid (Hoinville 1996), faeces (Terry et al. 2011) and milk (Konold et al. 2008; Lacroux et al. 2008; Maddison et al. 2009) 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 hamster 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 sheep models (Le Dur et al. 2005; Simmons et al. 2007, 2010). 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 programs 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). 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 (>1,000 sheep), overaverage 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).

2.5.3 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. There is a negative relationship between the genotype-encoded susceptibility in sheep and

the incubation period of the disease, i.e., sheep with VRQ homozygosity have shortest incubation periods (Detwiler and Baylis 2003; Ersdal et al. 2005; Ryder et al. 2004). 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 (average age 3.5 years). Although both sexes appear to be equally affected, disease manifestations in rams occur often at 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. Atypical cases are on average 6.5 years old (Benestad et al. 2008). 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. Most of the goats affected by classical scrapie are between 2 and 5 years old; 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; Fast and Groschup unpublished results). Atypical scrapie in goats was described in eight animals with an average age of 6.3 years (Colussi et al. 2008) and in two animals with 10, respectively, 12 years of age (Nentwig et al. 2007).

2.5.4 *Pathogenesis and Tissue Distribution of PrP^{Sc} and/or Infectivity*

The pathogenesis of TSEs is discussed separately in Chap. 4. Nevertheless, the most important facts are summarised here and in Fig. 2.1.

After oral uptake, it still remains enigma how the infectious agent overcomes the mucosal barrier of the gut (for detailed review see Mabbott and MacPherson 2006). 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 could be a possible route (Heppner et al. 2001). A 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 another option. 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). A first accumulation of PrP^{Sc} was seen in the

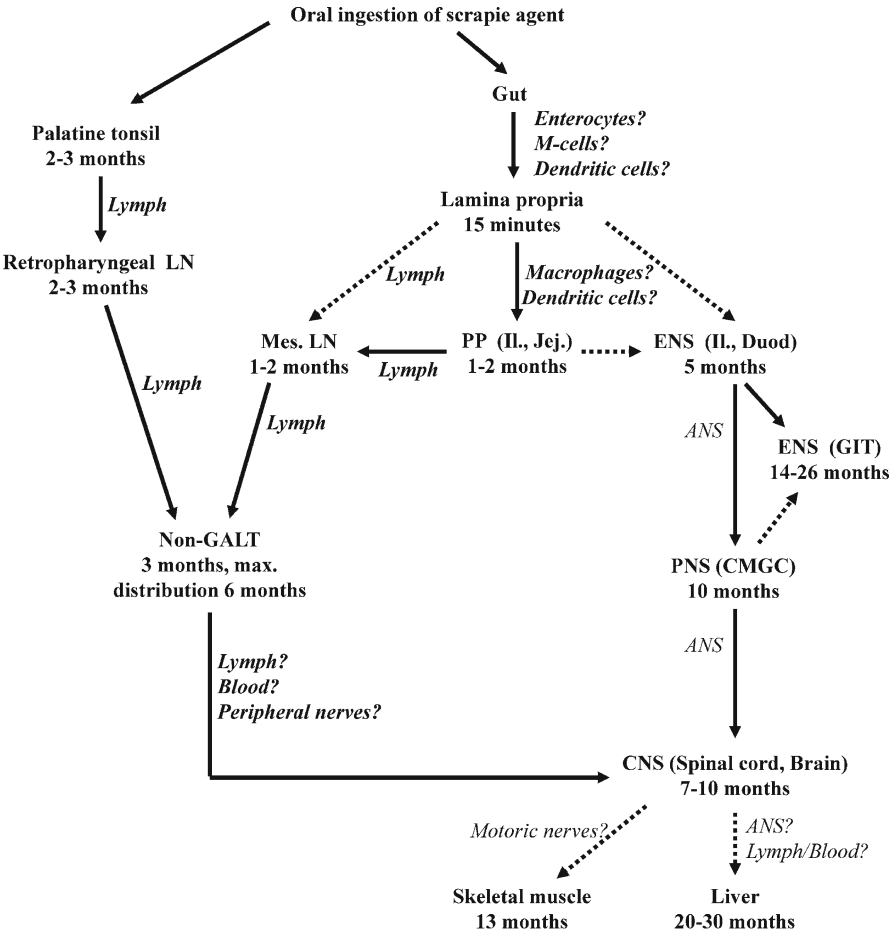

Fig. 2.1 Schematic illustration of the most common theories concerning the pathogenesis of scrapie (modified from van Keulen et al. 2002 and Sisó et al. 2010). The time periods stated are from different studies showing PrP^{Sc} accumulation by immunohistochemistry (Andreoletti et al. 2000, 2002, 2004; 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* gastrointestinal 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

gut-associated lymphoid tissues (GALT) of the tonsil and Peyer's patches 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 a replication and accumulation of de novo PrP^{Sc} was not seen before 1-month post-infection (Jeffrey et al. 2006).

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 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. 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). 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 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 PrP^{Sc} takes place (van Keulen et al. 2008).

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 was 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 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 the lower susceptibility of animals carrying this genotype. In such cases, PrP^{Sc} is mainly confined to the CNS (van Keulen et al. 1996).

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). 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 without any detectable PrP^{Sc} in the LRS (Jeffrey et al. 2002; Ligios et al. 2006; Konold et al. 2007a, b; González et al. 2009). Additionally, first results from ongoing 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; Simmons et al. 2007; Benestad et al. 2008; 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). Data on the tissue distribution of atypical scrapie in goats are not available to date.

2.6 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 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 in 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 goats are rare and most authors refer to scrapie 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 to 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 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. Tremor was hardly seen and—with exception of two British cases—alopecia due to pruritus is not occurring. Animals die unexpectedly or after a very acute progression phase. One goat was described with 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).

2.7 Diagnosis of Scrapie

The diagnosis of TSEs is discussed separately in Chap. 13. 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, it is recommend to include samples of the cerebellum as well (OIE). All samples with a reactive result in one of the rapid tests must be retested in the national reference laboratory using one of the OIE approved confirmatory methods (Matthews et al. 2004). 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.

2.7.1 Discriminatory Immunoblot

According to the EU-legislation (January 2005, EC regulation 36/2005), all confirmed TSE cases in small ruminants should be examined by a discriminatory testing to reveal BSE infections in sheep and goats. This includes discriminatory immunoblots (by defined immunoblot protocols) and mouse bioassay (strain typing) of any isolate with a BSE-like immunoblotting pattern (EFSA 2007).

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 or by using monoclonal antibodies binding to an epitope located on the ragged end of PK-cleaved PrP^{Sc}. One of these antibodies is, for example, mab P4, whose epitope WGQGGSH 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. 2.2 and 2.3). 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). 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 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 FLI test, PrP^{Sc} in a sample will be judged BSE-like and subjected to further mouse bioassaying (strain typing), if the sample is conform to the following three biochemical attributes (1) the glycoform ratio for the diglycosylated form is above 50%; (2) the antibody binding ratio P4/L42 has a lower value than 0.4 and (3) the molecular mass is by >0.5 kDa lower than that of the internal scrapie standard.

2.7.2 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

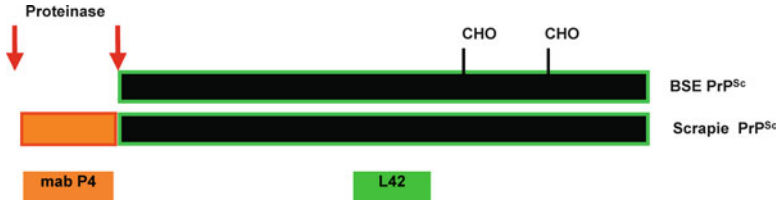

Fig. 2.2 Lack of detection of ovine and bovine BSE PrP^{Sc} by mab P4. As the PK cleavage sites vary between BSE and scrapie, mab P4 can be used to discriminate between these two TSE types. While BSE-related PrP^{Sc} is trimmed approximately to the amino acid 100 and the P4 epitope is therefore destroyed, 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

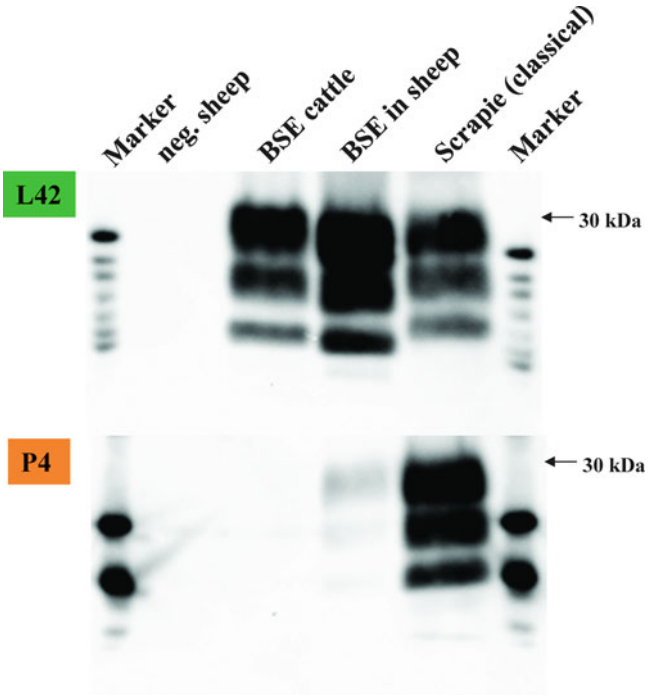

Fig. 2.3 Comparison of electrophoretic profiles and antibody labelling of PrP^{Sc} after proteinase K digestion, PTA precipitation and immunoblotting using mab L42 or mab P4. Both blots are loaded with the same quantities of precipitated PrP^{Sc} of each sample

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).

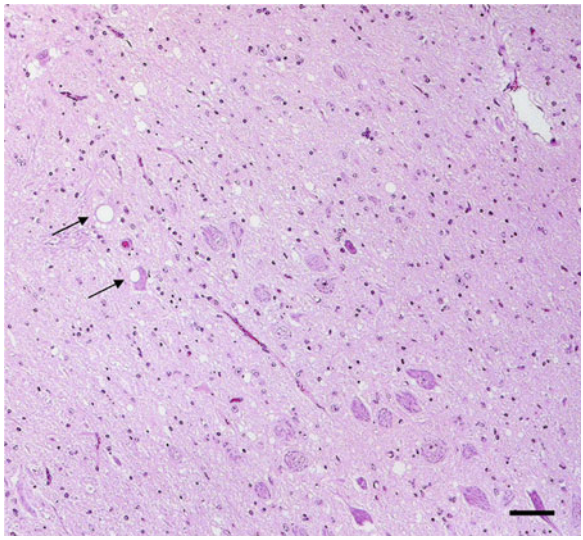

Fig. 2.4 Scrapie-infected goat with clear signs of spongiform encephalopathy in the brainstem at the level of the obex (dorsal motor nucleus of the vagus nerve). Vacuolation is detectable in the neuronal perikarya (*arrow*) and in the neuropil, H&E staining, bar 50 µm

Lesions are usually bilaterally symmetrical (Fraser 1993), especially at the brainstem at the level of the obex (Fig. 2.4) 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 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 detailed review see Jeffrey et al. 1995). Additional findings might be other signs of neuronal degeneration like 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

Table 2.2 Morphological types of PrP^{Sc} accumulation (Jeffrey and Gonzalez 2007)

Intracellular	Intraneuronal, Intramicroglial, 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

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).

2.7.3 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) 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 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 2.2). 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 some sheep and caprine TSEs (including ovine/caprine BSE) is possible by using the immunoreactivity of antibodies recognising different epitopes of PrP^{Sc} (epitope mapping). This method relies on the different protease cleavage sites for PrP^{Sc} in different cell types (the same principle as shown in Fig. 2.2). Both approaches may allow the definition of an immunohistochemical phenotype and the subsequent identification of the host and agent strain (for detailed review see Jeffrey and Gonzalez 2007).

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

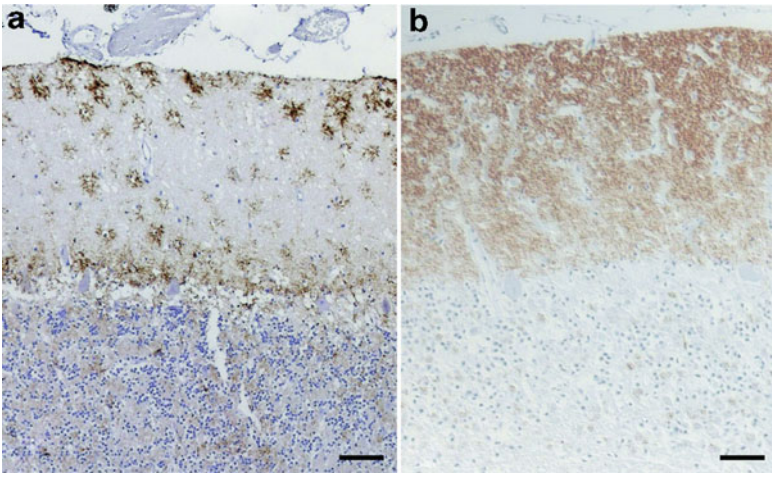

Fig. 2.5 Distinct differences in the PrP^{Sc} distribution pattern in the cerebellar cortices of sheep infected with (**a**) classical scrapie and (**b**) atypical scrapie, immunohistochemistry mab 6C2, bar 50 μm

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 detailed review see Benestad et al. 2008). The most pronounced immunostaining is usually detectable in the cerebellar (Fig. 2.5) 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 few morphological types (including fine granular, aggregates, plaque-like, linear and perineuronal) can be seen. An intraneuronal deposition staining has never been reported (Benestad et al. 2008; Moore et al. 2008).

2.8 Scrapie Agent Strains

The first reports on the existence of different scrapie strains date back to the 1960s (Fraser and Dickinson 1968, 1973) and more than 20 experimental TSE strains of scrapie were found to date (Bruce 2003). TSE strains are distinguished by their incubation period in a panel of inbred mouse lines, their vacuolation pattern (so-called "lesion-profile") and the PrP^{Sc} deposition pattern (Bruce and Fraser 1993; Bruce 2003) in the brains of these mice. Although this method allows the discrimination between BSE and scrapie as well as the differentiation between natural and experimental scrapie strains, not all scrapie isolates transmit to wild-type mice.

A panel of transgenic mice overexpressing ovine PrP^C as well as bank voles have been established as alternative models for strain characterisation. However, it has to be borne in mind that murine scrapie strains may also result from inter-species interaction and do not necessarily reflect the original ovine/caprine strain. Therefore, an improved characterisation of isolates from natural hosts (sheep and goats) was attempted in most recent studies (see Sect. 2.7.1 and 2.7.3). Investigations included the use of biochemical parameters of PrP^{Sc}, revealing differences in molecular masses, antibody binding affinities, glycosylation patterns and degree of resistance to tproteinase-K digestion. This way ovine/caprine BSE, CH1641, CH1641-like strains/isolates and atypical scrapie isolates could be distinguished. Additionally, differences seen in some classical scrapie isolates were indicative for the existence of more scrapie strains in sheep and goats (Stack et al. 2002; Buschmann et al. 2004, 2006; Gretzschel et al. 2005, 2006; Baron and Biacabe 2007; Benestad et al. 2008; Fragkiadaki et al. 2011; Fast and Groschup unpublished results).

Vacuolation profiles in the brain of the natural hosts, as revealed by histopathology, showed a high individual variability and can therefore not be used as strain typing method (Ligios et al. 2002; Begara-McGorum et al. 2002; Gonzalez et al. 2010a, b). The differences seen by IHC PrP^{Sc} deposition pattern allow the discrimination clearly between an infection with classical and atypical scrapie, CH1641 and ovine/caprine BSE. Moreover, the variations of the PrP^{Sc} depositions in the brain may reflect the strain diversity and perhaps even allow a discrimination of classical scrapie strain types (Jeffrey and Gonzalez 2007). Five different IHC phenotypes were found in a recent study on wild-type sheep from different flocks throughout Europe irrespective of the genotype and geographical origin (Gonzalez et al. 2010b). However, neither the influence of other genotypes nor further factors which might influence the IHC phenotype are completely understood to date.

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Chapter 3
Chronic Wasting Disease and the Development of Research Models

Glenn Telling

Abstract Chronic wasting disease (CWD) is a burgeoning epidemic prion disease of cervids. While its origins are mysterious, disease was first described in captive mule deer and was subsequently identified in free ranging, as well as captive Rocky Mountain elk, white-tailed deer, and most recently moose. As such, it is the only recognized prion disease of wild as well as captive animals. In addition to its expanding host range, disease continues to spread to new geographic areas. The unparalleled efficiency of prion transmission by a largely undefined mechanism, 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 it is not currently mandatory to have animals tested for CWD, it is likely that humans consume 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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3.1 Epidemiology

First identified in the late 1960s as a fatal wasting syndrome of mule deer (*Odocoileus hemionus hemionus*) in a northern Colorado research facility, CWD was recognized as a prion disease in 1978 by histopathological assessment (Williams and Young 1980, 1992). A retrospective study also revealed CWD infection of mule deer and black-tailed deer (*Odocoileus hemionus columbianus*) resident 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, 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). Most recently, CWD has occurred in wild (Baeten et al. 2007) and captive moose (*Alces alces shirasi*) (Kreeger et al. 2006) in the endemic region.

The prevalence of CWD varies across North America but can be as high as 30% in some areas of Colorado (Williams 2005). Based on hunter-harvested animal surveillance, the prevalence of CWD in the endemic area from 1996 to 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 during June 2006 to June 2009 continue to demonstrate 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). Long thought to be limited in the wild to the endemic area, since 2002 CWD has also emerged in free-ranging populations of white-tailed deer (*Odocoileus virginianus*) east of the Mississippi (Joly et al. 2003). Indeed, the spread of CWD in North America appears to be irrevocable. At the time of writing, CWD has been recognized in wild and/or farm-raised cervids from 18 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, 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 subclinically infected animals (Sohn et al. 2002; Kim et al. 2005). However, although most US states and Canadian provinces have introduced CWD surveillance programs,

its extent is variable, and ranges 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 postmortem analysis of CNS materials, and current evaluations almost certainly underestimate the true prevalence of disease. While testing for CWD in other countries has been minimal, limited active surveillance has, to date, not been detected CWD in Europe or Japan (Roels et al. 2004; Kataoka et al. 2005; Schettler et al. 2006).

CWD has a significant economic and wildlife conservation impact. The US Fish and Wildlife Service estimated in 2006, the last year for which survey reports are currently available, that total hunting expenditures totaled $22.9 billion, and an estimated 10.7 million hunters pursued big game, such as deer and elk. Within the first month of its diagnosis in free-ranging deer in 2002, the Wisconsin wildlife management agency spent ~$250,000 in control and public information efforts, and subsequently upward of $2.5 million a year for CWD control efforts. Saskatchewan has spent ~$30 million in eradication attempts in infected commercially operated game farms.

3.2 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 is characterized by extensive CNS and lymphoid tissue deposition of PrP^{Sc}, the latter being detectable early in disease (Sigurdson et al. 1999; Fox et al. 2006); however, again, CWD pathogenesis seems to vary between deer and elk with less PrP^{Sc} deposition in 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 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 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).

3.3 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, 2011; Tamguney et al. 2009) is consistent with the mechanism of contagious lateral transmission. The detection of CWD prions in elk antler velvet by transgenic 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 (*Odocoileus virginianus*) (Joly et al. 2003). Most recently, CWD has occurred in 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, in preliminary studies, muntjac deer *(Muntiacus reevesi)* (A. Young and R. Bessen personal communication; Nalls et al. 2011). While brain material from CWD-infected white-tailed deer and elk produced disease in 4 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).

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). Experimental transmission to other species has had mixed results.

Studies by Dr. Richard Marsh (University of Wisconsin) in the mid-1980s 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). Nontransgenic mice appear resistant to CWD infection (Browning et al. 2004).

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 interspecies transmission. The appearance of vCJD, following human exposure to BSE (Bruce et al. 1997; Hill et al. 1997), places the human species barrier to other animal prion diseases, particularly CWD, at the forefront of public health concerns. North American hunters harvest thousands of deer and elk each year, and it is not currently mandatory to haven these animals tested for CWD. He demonstration of CWD prions in skeletal muscle and fat of deer (Angers et al. 2006; Race et al. 2009a, b) therefore make it is 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 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 to 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 high priorities.

3.4 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 Q96, G96, A116 allele (QGA) was more frequently found in CWD-affected deer than the QSA allele (Johnson et al. 2003; O'Rourke et al. 2004). 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 132L allele protects against CWD.

3.5 Transgenic Mouse Models

While CWD is transmissible after intracerebral inoculation of mule deer with incubation periods 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 barrier, albeit of varying extent, 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 (Scott et al. 1989; Telling et al. 1994, 1995; Scott et al. 1997; Buschmann et al. 2000; Crozet et al. 2001; Vilotte et al. 2001; Castilla et al. 2003; Windl et al. 2005). Based on this concept, the last few years have witnessed the development of several transgenic mouse lines expressing either elk or deer PrP in which the species barrier to CWD has been eliminated. Prototype transgenic (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 transgenic 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), has 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; Green et al. 2008a, b; Meyerett et al. 2008; Kurt et al. 2009). 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; Angers et al. 2009; Race et al. 2009a, b). The availability of CWD-susceptible Tg mouse models has, for the first time, also provided a means of quantifying CWD infectivity by endpoint 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.

Transgenic mouse modeling also provided a means of assessing the role of PrP gene polymorphisms and species-specific amino acid differences on CWD pathogenesis. To more fully address the influence of the elk 132 polymorphism, transmissibility of CWD prions was assessed in transgenic 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 CerPrP-L132 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).

3.6 CWD Strains

Although original studies in transgenic 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 occurs 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 intrastudy 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 provides 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 coexistence 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 transgenic 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, 1 elk isolate from a total of 8 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.

3.7 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, ten times faster and more than 100-fold less expensive. Using these assays, quantification of RML prion titers about as low as those that can be determined by endpoint titration in mice is now possible. 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 reemerge 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 enzyme-linked immunosorbent assay (ELISA) using automated counting equipment (Elispot). Inclusion of RK13 cells stably transfected with 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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Chapter 4
Human Sporadic Prion Diseases

Pierluigi Gambetti and Silvio Notari

Abstract Sporadic prion diseases account for approximately 85% of all human prion diseases. They include sporadic Creutzfeldt–Jakob disease (sCJD), which affect over 90% of the cases, as well as sporadic fatal insomnia (sFI) and variably protease-sensitive prionopathy (VPSPr), which account for 1–2% and 3–4% of the cases, respectively. Sporadic CJD comprises five phenotypically distinct subtypes most of which are associated with distinct molecular features. The molecular signature of each subtype is determined by the pairing of the genotype at the methionine/valine polymorphic codon 129 of the prion protein (PrP) gene and either one of two disease-associated PrP (PrP^{Dis}) types characterized by distinct physico-chemical characteristics. VPSPr may include three similarly determined subtypes whereas no subtype is known in sFI. Currently, the most likely etiological-pathogenetic mechanism of sporadic prion diseases is the failure of the quality control complex permitting the presence of conformationally abnormal and pathogenic PrP variants capable of converting normal PrP into similarly pathogenic conformers. These conformers then propagate and damage the vulnerable tissues they populate. Essentially the same sequence of basic events may play out in other diseases forming amyloids such as Alzheimer's and other major neurodegenerative diseases as well as type 2 diabetes and amyloid A (AA).

Keywords Alzheimer disease • Amyloid A • Conformational diseases • Etiology • Pathogenesis • Prion protein • Propagation • Sporadic Creutzfeldt–Jakob disease • Sporadic fatal insomnia • Strain • Tauopathy • Toxicity • Type 2 diabetes • Variably protease-sensitive prionopathy

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4.1 Introduction

Human sporadic prion diseases have been known for almost a century old and yet they still are increasing as for the number of their types and subtypes (Table 4.1). They account for approximately 85% of all human prion diseases far exceeding the inherited prion diseases (approximately 15%) and the prion diseases acquired by infection (less than 1% of the total) (Gambetti et al. 2003). Sporadic prion diseases comprise Creutzfeldt–Jakob disease (sCJD), fatal insomnia (sFI), and variably protease-sensitive prionopathy (VPSPr). Sporadic CJD, first described in 1921, is exceedingly the most prevalent (over 90% of the cases) and the archetype of human prion diseases. Sporadic FI and VPSPr first reported in 1993 and 2008, respectively, combined account for 4–6% of the cases (Gambetti et al. 1993, 2008, 2011a, b).

All three types face the same uncertainties related to the etiology, pathogenesis, the characteristics of the abnormal prion protein associated with them (PrP^{Dis}), the classification and, ultimately, the early clinical diagnosis and treatment.

This chapter deals with these issues selectively and, when appropriate, comparatively, confronting the types of the human sporadic prion diseases. First, the basic characteristics of the three types and their subtypes will be briefly reviewed with emphasis on their distinguishing traits.

4.2 Individual Types and Subtypes

Sporadic Creutzfeldt–Jakob disease is still often perceived as one disease entity typically characterized by rapidly progressive dementia with onset after 60 years of age and less than 1-year duration. The triad of spongiform degeneration, astroglial reaction, and neuronal loss is the histopathologically hallmark. However, this characterization applies to the classical and most common subtype of sCJD, while the entire sCJD group is markedly heterogeneous. Phenotypic features such as prevalence, age at onset and duration, and type and topography of the lesions vary widely among subtypes. The characteristics of the PrP^{Dis} isoforms associated with sCJD also differ in a number of ways; they include different propensities to aggregate and co-occur in the same brain region, distinct chemical and physical features such as electrophoretic profile and stability, and capability of replicating in receptive hosts and in vitro (Parchi et al. 1999, 2009; Puoti et al. 1999; Cali et al. 2009; Bishop et al. 2010; Jones et al. 2011).

The phenotypic heterogeneity has been related to the variability of the PrP human genotype (PRNP), which is determined by the common methionine (M)/valine (V) polymorphism at codon 129, since it was observed that patients with different 129 genotypes had distinct phenotypes (Parchi et al. 1996; Gambetti et al. 2003). This initial heterogeneity is amplified by the presence in the affected brain of either one of two species of PrP^{Dis} identified as type 1 and 2 (Monari et al. 1994; Parchi et al. 1997). The PrP^{Dis} 1 and 2 species are distinguished primarily according to their main sites of cleavage by proteases that are located at residues 82 and 97, respectively,

Table 4.1 Classification of human sporadic prion diseases

Phenotype	Distribution[a,b]	Subtype	Distribution[b,c]	Onset/ duration[d]	Phenotypic features
sCJD	Over 90%	MM/MV1	58%	63.2/3.8	Typical CJD clinically and pathologically with fine spongiform degeneration (SD). Typical EEG (83%); "Synaptic" immunostaining pattern. Positive CSF tests and MRI
		VV1	4%	46.0/15.3	Early onset; no typical EEG; cerebellum spared; weak "synaptic" immunostaining.
		MM2	9%	60.3/15.7	No typical EEG; large and confluent vacuoles; coarse PrP immunostaining; cerebellum spared. Unhelpful CSF tests and positive MRI
		MV2	14%	60.3/17.0	Ataxia at onset; rarely typical EEG; no cerebellar atrophy but kuru plaques; plaque-like pattern of immunostaining. Positive CSF tests and MRI
		VV2	15%	60.3/6.6	As MV2 but no kuru plaques and cerebellar atrophy
sFI	1–2%	MM2	–	60.3/14.0	Severe neuronal loss and astrogliosis of thalamic nuclei with minimal or no astrogliosis. Variable but generally minimal PrP immunostaining. CSF tests and MRI generally negative. PET scanning positive
VPSPr	3–4%	MM	10%	64&78/ 41&50[e]	Fairly similar clinical signs in the three genotypes with variations in the psychiatric signs (psychosis or behavior and mood changes), speech deficit, and cognitive decline, often with prominent involvement of frontal lobe functions. These signs are followed by progressive motor impairment, especially parkinsonism and ataxia. There is minimal SD with vacuoles of intermediate size. PrP^{Dis} has different protease-resistance in the 3 genotypes. CSF and MRI unhelpful.
		MV	23%	72/45	
		VV	67%	65/23	

[a]Percentage of sporadic human prion diseases

[b]Based on 609 cases examined by the National Prion Disease Pathology Surveillance Center, Cleveland, OH, USA

[c]Percentage of the respective phenotype. However for simplicity the phenotypes of the MM, MV and VV cases with mixed types 1–2 PrP^{Dis} have not been included. They generally show the corresponding mixed phenotypes

[d]Average of onset (years) and duration (months)

[e]Only three cases of VPSPr-MM, of which 1 asyntomatic, have been described to date

and confer to the two PrP^{Dis} types different electrophoretic mobilities following protease digestion (Parchi et al. 2000). Of notice, PrP^{Dis} type 1 is found in over 90% of the patients with the 129MM genotype; conversely, PrP^{Dis} type 2 is present in over 80% 129MV and 129VV patients (Parchi et al. 1999; Gambetti et al. 2003). Therefore, the 129 genotype seems to partially control the PrP^{Dis} type.

Combined, all these findings prompted a new molecular classification of sCJD into six subtypes based on the pairing of the 129 genotype (M/M, M/V, and V/V) and PrP^{Dis} type (1 and 2) (Table 4.1). When the molecular subtypes were matched with sCJD clinical and histopathological features, good and consistent fits were observed with previously known and newly identified phenotypes allowing for the identification of five sCJD subtypes. This classification rationalizes to a great extent the phenotypic heterogeneity hinging it to the molecular diversity of the sCJD subtypes. For example, disease duration is relatively short in sCJDMM1 and sCJDVV2 and three to four times longer in the other subtypes. Similarly, age at onset is significantly earlier in the sCJDVV1 than in all the other subtypes. The preferential involvement of the cerebellum at the clinical and histopathological levels is an exclusive feature of sCJDMV2 and sCJDVV2, yet the cerebellar histopathology is distinguishable between these two subtypes. The subtypes also show remarkable variability in their prevalences which are 59% for sCJDMM1/MV1, 14–15% each for sCJDVV2 and sCJDMV2, 9% for sCJDMM2 and down to 4% for sCJDVV1, the rarest of all subtypes. This variability does not reflect the prevalences of the three 129 genotypes in the general Caucasian population which is 49% MV, 43% MM and 8% VV (Zimmermann et al. 1999).

Sporadic fatal insomnia (sFI) is essentially the phenocopy of fatal familial insomnia (FFI), a familial prion disease linked to the mutation D178N and genotype 129 M. Hence sFI is considered the sporadic form of FFI. Sporadic FI differs clinically from sCJD because of the frequent association with severe and distinctive insomnia along with dysfunction of the autonomic system while the histopathology consists of severe loss of neurons and astrogliosis of specific nuclei of the thalamus, which has prompted also the label of sCJDMM2 thalamic variant. In contrast, spongiform degeneration (SD) is minimal or lacking. Although sFI, like sCJDMM2, is almost invariably associated with the M/M 129 genotype and the PrP^{Dis} type 2, its phenotype and PrP^{Dis} characteristics are distinct from those of sCJDMM2. Furthermore, sFI is very rare accounting for only approximately 1% of all sporadic prion diseases (Gambetti, P., unpublished data).

Variably protease-sensitive prionopathy, like sCJD, affects all three 129 genotypes, but it basically differs from sCJD in a number of ways. The disease duration for the three genotypes combined is on average 2 years compared to 8 months for all sCJD subtypes; other clinical features and diagnostic tests also are different. A major disparity between VPSPr and sCJD regards the prevalence of the three 129 genotypes. In VPSPr, the 129MM, 129MV and 129VV genotypes account for 10%, 23%, and 67% of all cases, respectively, almost the opposite of sCJD where the 129MM is the most represented one (~70%). While the histopathology and PrP immunostaining patterns, although distinguishable, are not basically different from those of sCJD, the PrP^{Dis} electrophoretic profile is very distinctive; it forms a ladder of at least 7

bands (if PrP antibodies to the N- and C-termini are used) which display mobilities encompassing 27 and 7 kDa and lacks the diglycosylated PrP^{Dis} form. Furthermore, unlike sCJD, the heterogeneity among the three 129 genotypes of VPSPr is limited to, and primarily affects the physico-chemical characteristics of PrP^{Dis} rather than the clinical and histopathological phenotypes. The reported prevalence of VPSPr of approximately 3% is low. However, it might be higher as VPSPr has been only recently identified. Furthermore, its atypical dementia along with non-prion disease-like clinical presentation makes it unlikely that VPSPr cases are examined at autopsy.

4.3 Etiology and Pathogenesis

At variance with inherited prion diseases, which are associated with a mutated PrP, and prion diseases acquired by infection, which are initiated by exposure to exogenous infectious PrP^{Dis}, the etiology of the sporadic forms of prion disease remains matter of conjecture. Several cellular biological scenarios that are commonly considered include (Moreno-Gonzalez and Soto 2011): (1) somatic mutations in the PrP gene leading to the expression of an unstable, variant PrP in the mutated cell; (2) inadequate stringency or failure of the apparatus that controls misfolded proteins, often called quality control system, in vetting the properly conformed PrP species (Sanders and Nagy 2000; Ellgaard and Helenius 2001a, b; Sitia and Braakman 2003; Ulloa-Aguirre et al. 2004); (3) errors in posttranslational modifications including PrP misrouting which may expose PrP to an inappropriate environment; and (4) failure of the cell to control $iPrP^{C}$, an insoluble and partially protease-resistant PrP^{C} isoform (also called silent prion), normally present in the brain, which is considered to be intermediate between PrP^{C} and PrP^{Sc} (Yuan et al. 2006); iPrP would spontaneously convert to PrP^{Sc} (Moreno-Gonzalez and Soto 2011; Zou, Chap. 15). The failure of the quality control complex, of which the loss of control on $iPrP^{C}$ might be part, seems the most plausible of all these possible mechanisms for at least three reasons: (1) it is directly involved in guiding proper folding of proteins, (2) it is thought to be negatively affected by aging, the major risk factor in sporadic prion and other conformational diseases, and (3) may be especially vulnerable in neurons (Neef et al. 2011; Nunziante et al. 2011). All these etiological mechanisms result in the uncontrolled presence of misfolded PrP, which currently seems to be the first firmly established event in the pathogenesis of all forms of prion disease. Furthermore, all these mechanisms are compatible with the occurrence of the initial misfolding event in a limited brain region, even in a single cell, from which the templated misfolding process spreads out (Makarava et al. 2011).

Propagation of PrP^{Dis} by templating PrP^{C} is a well-established process in prion diseases and it is thought to be the basic molecular mechanism of prion propagation. Experimentally, propagation has been shown to occur along nerves and trans-synaptically in centripetal and centrifugal directions (Seelig et al. 2011; Bessen et al. 2010; DeJoia et al. 2006; Bartz et al. 2003). However, how propagation occurs in the central nervous system and whether propagation takes place inside the axons,

at the surface of the axon or through the cells immediately surrounding the axon remains to be determined (Heikenwalder et al. 2007). Nonetheless, PrP^{Dis} propagation is a robust process as in the final stages of sporadic prion diseases PrP^{Dis} can be demonstrated virtually throughout the brain albeit in widely different amounts (Parchi et al. 1999). Furthermore, if indeed the initial PrP^{C} to PrP^{Dis} conversion takes place in a limited brain region and then spreads to other regions impairing cell function and structure, this process must be not random but fairly stereotyped for each sporadic prion disease subtype since as mentioned previously, clinical and histopathological phenotypes associated with the individual sCJD subtypes and sFI are quite consistent (Parchi et al. 1999).

The mechanisms and timing by which propagating PrP^{Dis} impairs function and structure of brain in sCJD and in all the other forms of prion disease are unclear. It has recently been proposed that infectious and pathogenic or toxic PrP^{Dis} are two distinct PrP^{Dis} populations (Sandberg et al. 2011; Collinge and Clarke 2007; Harris and True 2006a, b). This proposal is based on the experimental observation that when mice with three different PrP^{C} expression levels are inoculated with scrapie prions, infectivity reaches the same plateau in all the mice albeit at different times. Only after the plateau phase is reached, clinical signs are detected with a timing directly related to the PrP^{C} expression level. This important observation raises a number of questions. The use of the first appearance of clinical signs as the indication of the beginning of the "toxic" phase raises the question of whether and for how long tissue damage precedes the symptomatic phase of the disease. Astroglial reaction, a common sign of tissue damage, was detected in prion-infected mice at a time interval after inoculation that was half that of the appearance of clinical signs (Tamgüney et al. 2009).

Although data on infectivity and toxicity of PrP^{Dis} in human prion diseases are limited and crude, available data from two studies are consistent with a more complex correlation between prion propagation and tissue damage. Both studies were performed on subjects suffering from FFI, which, as previously mentioned, is a phenocopy of sFI and, therefore, data from one disease are likely to apply to the other. In the first study, brain regional metabolism was examined by positron emission tomography (PET) (Cortelli et al. 2006). The study was carried out from the time the subjects were free of clinical signs to the terminal stage of the disease. Metabolism remained normal between 63 and 21 months before the clinical onset. Significant hypometabolism was first detected in the thalamus 13 months before the clinical onset. Thereafter, hypometabolism spread out to involve a number of regions at the time and after the patient became symptomatic. These data indicates that in FFI the degenerative process begins in the thalamus between 13 and 21 months before the clinical signs appear, and continues to spread to other brain regions thereafter.

In the second study, amount and distribution of protease-resistant PrP^{Dis} ($resPrP^{Dis}$) were examined post mortem in FFI-affected subjects with different disease durations spanning from 7 to 33 months (Parchi et al. 1995). Three distinct patterns of $resPrP^{Dis}$ regional accumulation as function of disease duration were observed: (1) significant amount of $resPrP^{Dis}$ and no increase regardless of the disease duration (in thalamus and brain stem); (2) initial smaller amount of $resPrP^{Dis}$ that progressively increased

and reached a plateau in cases with 18–33-month duration (in basal ganglia and limbic cortex); and (3) minimal or no initial amount of resPrP^{Dis} and major progressive build up which continued even with the longest duration (in cerebral neocortex). Combined, these pre-mortem and post-mortem data suggest that formation of resPrP^{Dis} may reach a plateau in brain regions that are affected first. However, this event might occur after rather than before initial the tissue damage has been inflicted. Furthermore, concomitantly to reaching a plateau in early-affected regions, resPrP^{Dis} propagates to, and increases in, other regions subsequently affected. In conclusion, the three phases—propagation, saturation and tissue damage—might be distinct but overlapping phases of the same general pathogenetic process.

The mechanisms, relationships, and timings of the lesions of the triad—SD, neuronal loss and astrogliosis—with the occasional presence of PrP amyloid plaques, also remain matter of speculation (Soto and Satani 2011). PrP^{C} to PrP^{Dis} conversion, at least initially, is thought to occur at the level of cell membranes, most likely at the plasma membranes of cell body and processes of neurons including synaptic membranes but likely also in membranes of glial cells (Jeffrey et al. 2011). Indeed the PrP^{Dis} immunostaining in sCJDMM1, the most common subtype of sCJD, co-distributes with synaptic markers while in sCJDMV2 and sCJDVV2 PrP^{Dis} appears to align with the neuronal plasma membrane at the level of the body and proximal processes (Parchi et al. 1996). Therefore, PrP^{Dis} might exert a toxic effect on cell membranes altering their permeability and causing intracellular edema resulting in SD (Kovacs and Budka 2008). The preferential localization of vacuoles in neuronal and glial processes including synapses supports this conclusion (Gonatas et al. 1964; Bignami and Forno 1970). Furthermore, intracerebral administration of ouabain, a well-known inhibitor of Na, K-ATPase, has been experimentally shown to cause an SD similar to that of Creutzfeldt–Jakob disease (CJD) as well as distinctive electroencephalographic pattern of sCJD (Bignami and Palladini 1966; Cornog et al. 1967).

As for the neuronal loss and astrogliosis, many mechanisms may account for these two lesions in prion diseases. Neuronal loss might result from direct injury caused by PrP^{Dis} deposition. However, regional neuronal loss may be very severe in the absence of SD in some prion diseases such as sFI, suggesting that some PrP^{Dis} species have more direct and harsher toxic effect on neurons. Astrogliosis also is a common reaction to nervous tissue injury (Rodríguez and Verkhratsky 2011; Heneka et al. 2010).

4.4 Phenotypic Heterogeneity and Prion Strains

The heterogeneity of human sporadic prion diseases suggests that each of the distinct phenotypes is associated with a distinct prion strain (Table 4.2). This notion is supported by transmissibility studies of the sCJD subtypes to Tg mice expressing human PrP^{C} with the three 129 genotypes (Bishop et al. 2010). This study confirms that sCJDMM1 and sCJDMV1 may share indistinguishable prion strains, and that

Table 4.2 Correlation between human sporadic prion diseases and proposed strains

Strain	Phenotype[a]
M1	sCJD(MM/MV1)
V1	sCJD(VV1)
M2	sCJD(MM2)
V2	sCJD(VV2)
MV2	sCJD(MV2)
sFI	sFI
VPSPr[b]	VPSPr(MM)(MV)(VV)

[a]The phenotypes associated with cases with mixed PrP^{Dis} types 1 and 2 have not been included for simplicity

[b]Currently it has not been determined whether distinct strains are associated with each the 129 genotypes

all the other sCJD subtypes—sCJDMM2, sCJDVV2 and sCJDMV2—have transmissibility characteristics, such as incubation period, type and distribution of the histological lesions and immunostaining patterns, that are distinct. The finding that sCJDVV1 could not be transmitted also is distinctive. Preliminary transmissibility data lead to similar conclusions for the PrP^{Dis} associated with sFI (Moda et al. 2012, Kong, Gambetti et al., unpublished data). Furthermore, detailed comparative analyses using one- and two-dimensional electrophoresis under various conditions, have uncovered major difference between PrP^{Sc} associated with sFI, an MM2 prion disease, and sCJDMM2 (Notari et al. 2008; Pan et al. 2001; Cracco, Gambetti et al. unpublished data). The data further support the conclusion that the PrP^{Dis} associated with sFI has the characteristics of a distinct strain. Similarly, the specific physicochemical characteristics of the PrP^{Dis} associated with VPSPr also seemingly qualify it as prion strain although transmission studies still in progress are needed to confirm this conclusion. The finding that at least seven prion strains may be associated with human sporadic prion diseases raises the question of how such a number of strains forming "spontaneously" in the same species can be accommodated within current concepts of strain formation (Collinge 2012; Weissmann et al. 2011).

The complexity of the strain issue stems from the number, diversity and dynamic characteristics of the variables at play in strain formation. The basic element of "strainess" is thought to be the conformations of the prion protein, which, although theoretically infinite, are limited by the energy requirements as well as environmental and metabolic constraints, which permit only a limited number of stable conformations. (Weissmann et al. 2011; Surewicz and Apostol 2011). While they are forming, prion strains can diversify owing to several factors including the primary structure of the converting PrP and the biological environment where the conversion takes place. PrP non-obligatory posttranslational modifications such as glycosylation might also add to the heterogeneity (Weissmann et al. 2011). A disease-related prion strain is currently envisioned as a set of conformations in hierarchical order with a dominant and less well represented components, and the label of quasi-species has been suggested for

this ensemble (Collinge 2012; Weissmann et al. 2011). This strain complex is the result of, and maintained by, the selection process imposed by the biological environment. Hence, shifts in the strain hierarchical order may occur according to the environment (or PrP^{C} characteristics, such as glycosylation, in a particular environment) resulting in the surge to prominence of minor components causing a shifting or mutation of the strain (Collinge 2012; Weissmann et al. 2011).

Most of these strain general features applies easily to human sporadic prion diseases. The different amino acid sequence as determined by the 129 methionine/valine polymorphism would easily explain the related dominance of the type 1 or 2 major strain species, which seem to fit the definition of quasi-species as they include a number of sub-strains. Strong similarities between strains associated with genotypically distinct PrP^{Dis}—like in sCJDMM1 and sCJDMV1—might result from similar biological environments of the site(s) where the initial conversion takes place. Conversely, the apparent dissimilarity between strains associated with PrP^{Dis} that are both genotypically identical and belong to the same type—like sCJDMM2 and sFI—might result from strain shifting due to different sites of the initial conversion, for example cerebral cortex in sCJDMM2 and thalamus in sFI, and/or the ensuing different propagation route of PrP^{Dis} from the distinct sites of initial conversion.

Additional features that need to be accommodated within the strain concept are the quite different prevalences of the various types and subtypes human sporadic prion diseases and, therefore, of the strains associated with them, as well as the consistency of these prevalences. Subtype prevalences have remained essentially the same over the years and in different geographic areas when variations due to improved detectability are discounted. Therefore, despite the numerous variables involved, formation of major pathogenic strains in human sporadic prion diseases appears to be not a random but a stereotypic process. This suggests that site of the initial formation of PrP^{Dis}, its propagation pathways and pathogenicity are relatively constant in each phenotype of human sporadic prion diseases.

4.5 Are Prion Principles Applicable to Other Protein Conformational Diseases?

For many years, non-prion neurodegenerative diseases have quietly been achieving some of the same milestones that prion diseases had to achieve (among controversy), in order to qualify as protein conformational rather than viral diseases. Currently, Alzheimer's disease (AD), tauopathies, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease as well as other amyloidoses, such as type 2 diabetes and amyloid A (AA), have been shown to share the basic principles and pathogenic mechanisms of classical prion diseases (Table 4.3) (Aguzzi 2009; Münch et al. 2011; Mougenot et al. 2011; Cui et al. 2008; Lundmark et al. 2002; Morales et al. 2011; Ren et al. 2009; Soto, personal communication). Thus, the pathogenic proteins associated with the each of these diseases follow a template-directed amplification process to self-replicate and to propagate from initial site(s) of formation

Table 4.3 Protein conformation diseases with possible prion-like pathogenesis[a]

Phenotype	Protein	*Trans*-cellular aggregate movement in culture	Aggregate propagation in vivo
Alzheimer's disease	Amyloid-β	Yes	Yes
Parkinson's disease	α-synuclein	Yes	Probable
Tauopathies	Tau	Yes	Yes
Amyloid A(AA) amyloidosis	Serum AA	Yes	Probable
Huntington's disease	Polyglutamine (PolyQ)	Yes	n.d
ALS	SOD1, TDP-43, et al.	Possible	n.d.
Type II diabetes	IAPP	n.d.	n.d.

[a]Moreno-Gonzalez and Soto 2011; Polymenidou and Cleveland 2011; Frost and Diamond 2010; Aguzzi 2009

to other regions causing the disease. In AD, tauopathies, Parkinson's disease, AA amyloidosis, and type 2 diabetes disease transmission has been achieved replicating to various extent the phenotype, or just some of the typical histological lesions, either in mice which do not spontaneously develop the disease or by significantly predating the onset and accelerating the course of an existing disease process. In some instances transmission has revealed striking features. Clinical and pathological changes of AD have been reported to be transmitted by blood transfusions from affected to unaffected mice (Soto, personal communication); disease transmission has also been achieved following intraperitoneal inoculation of AD brain homogenate from affected transgenic mouse models of AD to their unaffected counterparts (Eisele et al. 2010). These findings indicate that Aß, the pathogenic protein of AD, like prions, can be replicated outside the brain and propagate to the brain from peripheral locations. Furthermore, the intraperitoneal inoculated mice developed Aß deposits different in type and location from those present in the brains of the mice that received intracerebral inoculations suggesting that propagation in non-brain environments favored the emergence of a distinct Aß strain, an archetypical property of prion strains (Eisele et al. 2010). Amyloidosis A has been transmitted to receptive mice by oral administration (Westermark and Westermark 2009). Although the species barrier for AA amyloidosis is not known, this finding suggests that like variant CJD, AA amyloidosis can be acquired by ingestion of protein A amyloid, which is known to occur in cattle and other animals supplying food production (Yoshida et al. 2009).

However, several aspects such as species barrier, strain existence and characteristics, including diversity and adaptation, remain unexplored. Furthermore, it has been pointed out that in Parkinson's disease, ALS, Huntington disease, and tauopathies only the histological lesions have been reproduced in the host upon inoculation rather than the complete disease; and that unlike PrP^{Dis}, the disease-associated pathogenic proteins of most non-prion neurodegenerative diseases do not show

the classical features of an infectious agent such as human to human transmission and spreading within communities causing epidemics (Aguzzi 2009).

Scrapie-infected transgenic mice expressing anchorless PrP at normal levels form prominent PrP amyloid aggregates but remain asymptomatic (Chesebro et al. 2005). Therefore, the absence of the anchor appears to be associated with a prion condition different from classical prion diseases and more like those non-prion conformational neurodegenerative diseases, which are not considered true infectious diseases (Aguzzi 2009). These findings also imply that the same protein, i.e., PrP^C, can adopt abnormal conformations that carry quite different capabilities of disease transmission and toxicity. Preliminary data on transmissibility of VPSPr to transgenic mice expressing human PrP^C also are consistent with this conclusion (Gambetti et al., unpublished data).

Combined, most data on transmissibility of prion and non-prion diseases supports the notion that all these disease share the basic pathogenetic stages of self-replication and propagation, which, in some instances, appear strikingly similar in prion and non-prion diseases. The differences between the classical prion diseases and the other conformational diseases seem mostly related to the efficiency of the transmissibility process; they might be justified by variations in critical features of the individual native proteins such as cell topology as well as the pathogenic conformations and type of aggregates that their corresponding pathogenic isoforms can engender.

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Chapter 5
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, 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

5.1 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

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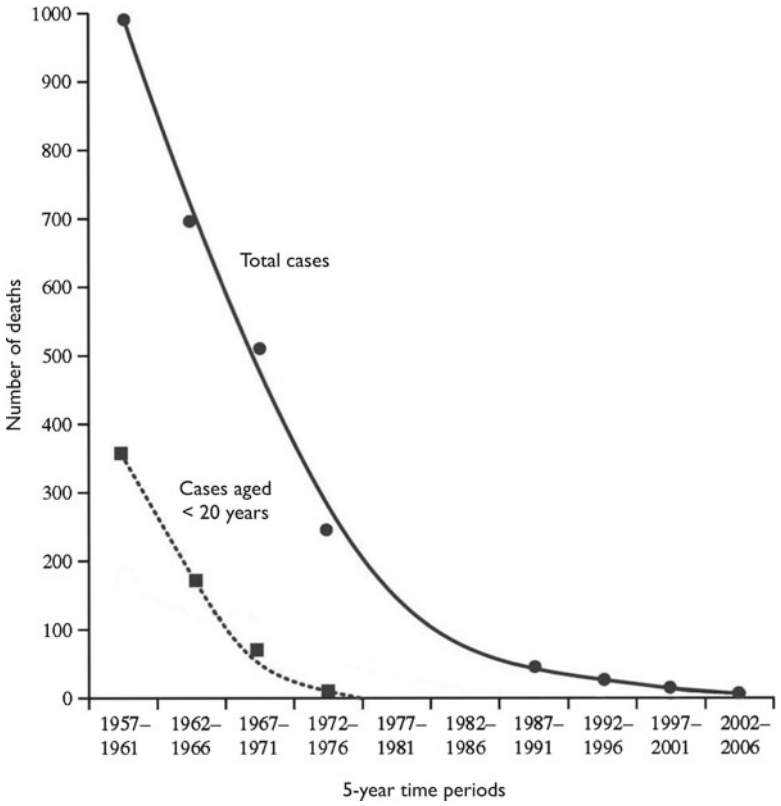

Fig. 5.1 Kuru mortality 1957–2006. Only one case has been identified during the most recent period (2007–2011). (Modified with permission from Alpers MP (2008) The epidemiology of kuru: monitoring the epidemic from its peak to its end. Philos Trans R soc B 363:3707–3713)

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 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. 5.1) (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 latest, and possibly the last) in 2009. Four were male and four were female, all 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.

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 early 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 Bovine Spongiform Encephalopathy (BSE) (Cervenáková et al. 1998).

5.2 Creutzfeldt–Jakob Disease

Environmentally 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.

5.3 Iatrogenic CJD

Iatrogenic CJD has very recently been reviewed and brought up to date through the end of 2011 (Tables 5.1 and 5.2) (Brown et al. 2012). Selected historical references are included in the following account, together with a few more recent references; however, most national and regional surveillance teams have either used internet web sites to provide updated numbers, or have not made the information available to the general public.

5.4 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

Table 5.1 Global distribution of cases of iatrogenic CreutzfeldtJakob disease

	Surgical procedures				Medical procedures		
	Dura Mater Grafts	Surgical instruments	EEG needle	Corneal transplants[a]	Growth hormone[b]	Gonadotropin	Packed red cells[c]
Argentina	1						
Austria	3				1		
Australia	5					4	
Brazil					2		
Canada	4						
Croatia	1						
France	13	1			119		
Germany	10			1			
Ireland					1		
Italy	9						
Japan	142						
Netherlands	5				2		
New Zealand	2				6		
South Korea	2						
Qatar					1		
South Africa	1						
Spain	14						
Switzerland	3		2				
Thailand	1						
UK	8	3			65		3
USA	4			1	29		
Totals	228	4	2	2	226	4	3

[a]Additional possible single cases following corneal transplant or keratoplasty (not included in table) in Japan, the UK, and the USA

[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 additional asymptomatic but infected red cell recipient died of an unrelated illness; another asymptomatic infected hemophilia patient who had been exposed to potentially contaminated Factor VIII also died of an unrelated illness (neither is included in the table)

Table 5.2 Clinical features of environmentally-acquired Creutzfeldt–Jakob Disease according to the source and route of infection

Source of infection	Number	Agent entry presentation	Mean incubation period (range)	Usual clinical presentation
Corneal transplant[a]	2	Optic nerve	18 months, 27 years	Dementia/cerebellar
Stereotactic EEG	2	Intracerebral	16 months, 20 months	Dementia/cerebellar
Neurosurgery	4	Intracerebral	21 months (18–28 months)	Visual/dementia/cerebellar
Dura mater graft	228	Cerebral surface	12 years (16 months–30 years)	Cerebellar (visual/dementia)
Growth hormone[b]	226	Hematogenous (?)	17 years (5–42 years)[c]	Cerebellar
Gonadotrophin	4	Hematogenous (?)	13.5 years (12–16 years)	Cerebellar
BSE-infected tissue (1°vCJD cases)	224	Oral	12–15 years	Psychiatric/sensory
RBC transfusion[e] (2° vCJD cases)	3	Hematogenous	6.5, 7.8, 8.3 years	Psychiatric/cerebellar

[a]Additional possible case in Japan

[b]Additional possible single cases in Australia, Scandinavia, and New Zealand

[c] 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

[d]Estimate based on epidemiologic data for BSE and vCJD (dates of infection for primary cases of vCJD are unknown)

[e]One additional asymptomatic but infected red cell recipient died of an unrelated illness; another asymptomatic infected hemophilia patient who had been exposed to potentially contaminated Factor VIII also died of an unrelated illness (neither is included in the table)

into a 55 year-old woman. The autopsy later revealed a diagnosis of CJD. 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 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.

Only two other instances of corneal transplant transmission of CJD have occurred in the 40-odd years since this case was reported, and neither can be considered definite. In one case, CJD developed 16 months after a corneal transplant, but the cause of death in the donor was not established; in the other case, both donor and recipient died of neuropathologically verified CJD, but the interval between transplant infection and clinical signs was 30 years. It seems likely that donor deferral criteria based on an ever-increasing diagnostic awareness have been largely responsible for the absence of additional cases.

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, especially in the UK, where 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.

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

The decade began quietly enough, although well before the first intimation of trouble in the growth hormone sector, the possibility of risk was already under study in Alan Dickinson's laboratory in Edinburgh, based on his appreciation of the fact that the pituitary was closely associated with the brain and thus likely to be infectious. 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 diagnosis was not established until 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 grand total of 226 cases from 1985 through the end of 2011 (Fig. 5.2). Case numbers for the three principally affected countries were: 29 (USA), 65 (UK), and 119 (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

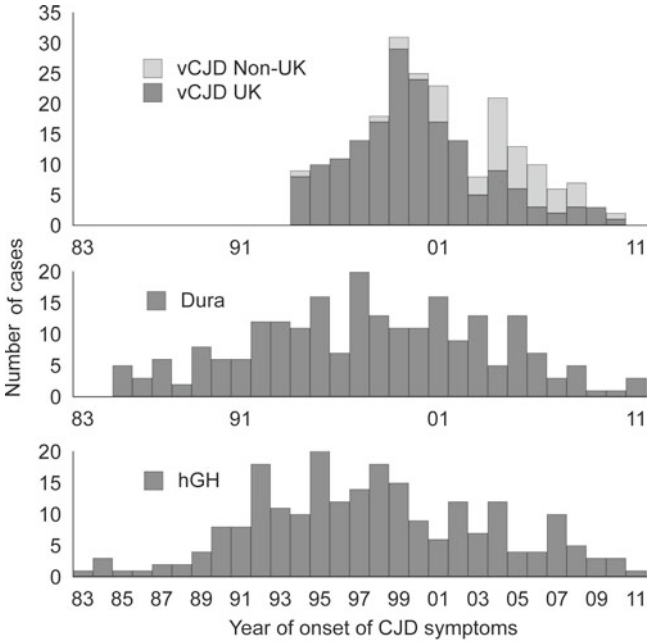

Fig. 5.2 Incidence of iatrogenic CJD due to contaminated cadaveric human growth hormone and dura mater, and of vCJD due to ingestion of BSE-contaminated tissues, 1982–2010

contamination due to both sourcing and processing deficiencies ((Abrams et al. 2011; National Creutzfeldt–Jakob Disease Research Surveillance Unit 2009), and unpublished data).

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 5.2). The incubation period, estimated from the mid-point of what was usually a several-year course of treatment, was approximately 17 years, but again like kuru, could extend out to 30 years and beyond—the current record for the longest incubation period from any cause of iatrogenic disease is 42 years in a recently diagnosed U.S. 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. 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 two inoculated monkeys, consistent with the occurrence of low-dose random contamination (Gibbs et al. 1993).

5.6 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. 5.2). As with the growth hormone contamination, recognition of the source of 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 new cases of iatrogenic disease from this source.

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 two-thirds of the 228 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.

5.7 The 1990s: BSE and vCJD

The following first-hand account of how BSE was discovered is described by Dr. Raymond Bradley (personal communication):

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 transmissible spongiform encephalopathy 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 bovine spongiform encephalopathy (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. 5.3), 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

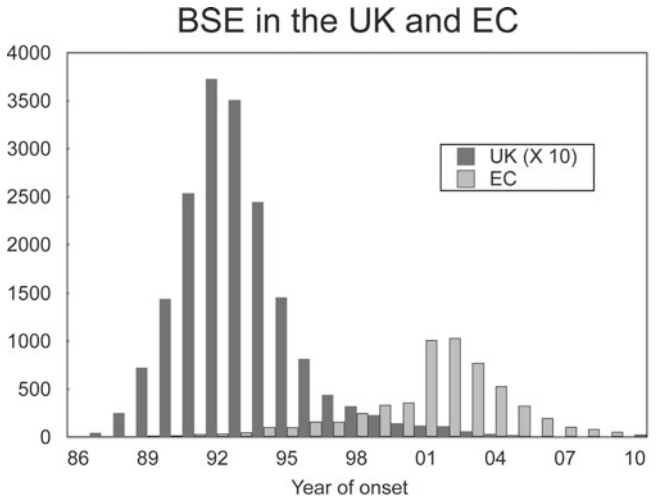

Fig. 5.3 Incidence of BSE in the UK and non-UK European Community, 1986–2010. Note that UK case totals are ten times the vertical axis numbers

tissue (dead or alive) imported from the UK, or from simultaneous endogenous mini-epidemics of BSE due to widespread similar changes in rendering practices.

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 20 years later.)

The author remembers having been contacted by Prof. Robert Will in late 1995 about the neuropathology of a few young adult cases of sporadic CJD in the NIH collection, and the subsequent 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 later described by Richard Rhodes (Rhodes 1997):

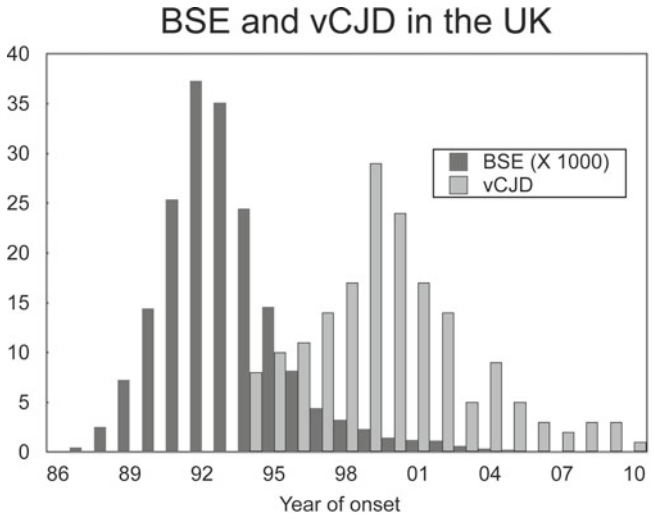

Fig. 5.4 Incidence of BSE and vCJD in the UK, 1986–2010. Note that the BSE case totals are 1,000 times the vertical axis numbers

> "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 global total of vCJD through 2011 stands at 224 cases.

The incidence curves of BSE and vCJD in the UK can be used to estimate the average incubation period for vCJD (Fig. 5.4). 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 upwards. Cattle can therefore be presumed to have first been infected towards 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 distinctive clinical characteristic of vCJD is its presentation in the form of behavioral or sensory abnormalities, rather than the dementia/cerebellar/visual syndrome typical of sporadic CJD (Will and Ward 2004) (Table 5.2). 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: all symptomatic cases have had a methionine–methionine coding genotype at polymorphic codon 129 of the *PRNP* gene; and 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 "florid" plaques—globular accumulations of misfolded "prion" protein (PrP^{TSE}) surrounded by a halo of vacuoles.

5.8 The Millennium: Denouement

The era of iatrogenic CJD and BSE-induced variant CJD is rapidly passing into history, but as with most outbreaks of infectious disease, there are always at least a few cases that trail out beyond the expected dates of extinction. In 2011, single new cases of dura mater-related CJD occurred in Austria, Korea, and the Netherlands, and one new case of hGH-related CJD occurred in the UK. In 2012, two new cases of vCJD were identified in France.

With respect to vCJD, the "end" has also been complicated by the recent 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 2006), as well as an inapparent infection in a recipient of plasma-derived Factor VIII (Peden et al. 2010). However, there are several reasons for hoping that further transmissions will not occur. The incubation periods of the three symptomatic cases were 6.5, 7.8, and 8.3 years (Fig. 5.5), (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 10 years (Hewitt et al. 2006), (updated by Prof. RG Will, personal communication). Also, leukodepletion of red cells was instituted in the UK in 1999 and none of 13 recipients of leukodepleted red cells from these donors has developed disease after intervals of 5–10 years. 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.

One other possible cause of future cases needs to be mentioned: the dreaded "second wave" of cases with long incubation periods due to codon 129 genotypes other than methionine–methionine. This is certainly not an unreasonable concern in view of the tendency towards prolonged incubation periods associated with alternative genotypes in both kuru and hGH-related forms of CJD. In each outbreak, however, the alternative genotypes began to appear well before the methionine–methionine cases had been exhausted, and that has not happened with vCJD infections. We are near

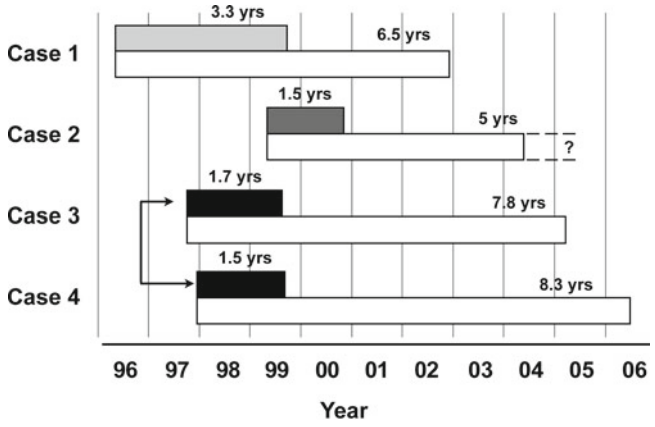

Fig. 5.5 Graph of intervals between transfusions and disease in four instances of secondary vCJD infections transmitted via packed red blood cells from donors who later died of vCJD. *Upper bars* of each pair represent donors and *lower bars* represent recipients. The second recipient died from a non-vCJD 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

the end of the outbreak and not a single symptomatic case of vCJD has occurred in a heterozygote or valine homozygote. The caveat to this observation is 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 the appendices removed from two homozygous valine individuals in a large UK "blinded" prevalence study reported in 2004 (Ironside et al. 2006). As these two individuals are anonymous, there is no possibility of ever knowing their ultimate fate. A more recent analysis of immunohistochemical tests performed on over 32,000 appendix samples removed between 1995 and 1999 yielded 16 positives, and an estimate of the 'carrier rate' of vCJD infection in the UK of approximately 1 per 2000. No information about the codon 129 status of the positive individuals was given (UK Advisory Committee on Dangerous Pathogens, 2012).

It has been obvious for many years that the most effective means to prevent further environmentally acquired cases of CJD would be a reliable laboratory test to detect pre- or subclinical infection. Around the turn of the century, nearly a dozen different laboratories were working to develop such a 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 a high sample throughput modification (QuIC test) of the PrP^{TSE} amplification technique that promises, finally, to produce a practical blood screening test (Orrú et al. 2011). Until that happens, we will need to continue to depend on the other two means of prevention—recognition and deferral of high-risk donors, and decontamination of instruments and therapeutic products—while maintaining a vigilant attitude towards as yet unidentified future sources of environmental infection.

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Chapter 6
Prions in the Environment

Shannon L. Bartelt-Hunt, Jason C. Bartz, and Samuel E. Saunders

Abstract Scrapie and chronic wasting disease (CWD) 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 and are present throughout the diseased host. 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. Highly sensitive and accurate detection of prion infectivity in the environment is not currently possible, severely hampering informed management of disease. A more thorough understanding of the interaction of prions with the environment in combination with robust detection methods may lead to means to reduce or eliminating prion disease in free-range and captive animal populations as well as mitigating the risk of zoonotic prion transmission.

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

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6.1 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). Recent experimental and epidemiological work suggests that soil may play a role in natural prion transmission (Saunders et al. 2008a, 2012a, b; Smith et al. 2011). 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).

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). Thus, long-term survival of prions in soil is possible and could explain the long-term environmental persistence of prions (Saunders et al. 2008a). 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 addition, the disposal of mortalities during BSE outbreaks, both in the past and potential future disposal events, serves as another environmental source of prions with the potential to infect humans. Therefore, it is clear that prions pose a significant environmental concern.

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 recently performed by Gough and Maddison (2010). Prion shedding can occur many months prior to clinical manifestation of the disease (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 (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).

Prions shed into the environment will interact with soil (Fig. 6.1). Given the close contact that animals, especially ruminants, have with soil through many routine behaviors, including ingestion of soil via feeding and mineral supplementation, there is significant opportunity for transmission of prions via soil (Saunders et al. 2008a, 2012a, b; Smith et al. 2011). No experimental work to date has directly investigated soil-mediated CWD or scrapie transmission in the natural hosts of these diseases.

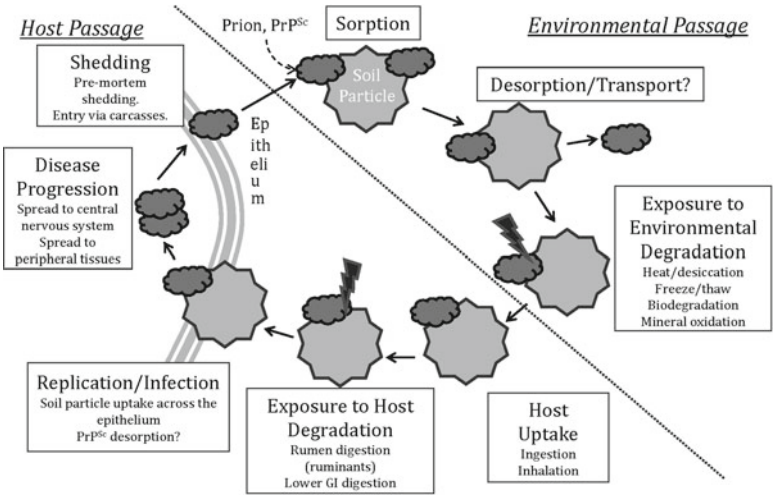

Fig. 6.1 Conceptual Model for soil-mediated prion transmission. From Saunders et al. (2012b)

Nevertheless, there is now ample evidence from studies in rodents that soil-mediated transmission is a viable and likely significant mechanism of natural prion transmission. Replication of soil-bound hamster and CWD prions (seeding conversion of PrP^{c} to PrP^{Sc}) has been demonstrated (Saunders et al. 2011b), and soil-bound hamster prions are infectious via oral (Johnson et al. 2007) and intracerebral routes (Saunders et al. 2012a).

6.2 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 6.1). 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 6.1). 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 2,000 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 was 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

Table 6.1 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), 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), 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), 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)

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

immobilized on the soil surface, forming potent reservoirs for efficient transmission. In contrast, prions contacting sandy soils may be more readily transported below the surface and diluted by surface or groundwater.

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 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 6.1) (Cooke et al. 2007; Maddison et al. 2010a; Saunders et al. 2010; Jacobson et al. 2009).

6.3 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). Three 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 et al. (2009, 2010) observed minimal HY TME PrP^{Sc} migration in columns packed with five different soils. 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. Macropore colloid-facilitated transport could quickly move prions into groundwater or surface waters and therefore warrants further study.

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 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 6.1) (Cooke et al. 2007; Maddison et al. 2010a; Saunders et al. 2010; Jacobson et al. 2009).

6.4 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 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, 2011c), although prions bound to soil organic matter may be more susceptible than prions bound to other surfaces (Saunders et al. 2011c). Preliminary data indicate that binding to soil decreases prion resistance to heat desiccation irrespective of soil type (authors' unpublished data), which suggests soils that retain moisture could favor prion persistence.

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). 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. (2006) found no significant decrease in PrP^{Sc} after 16-day incubation in mesophilic anaerobic sludge and observed PrP^{Sc} solids association. 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). 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 landspreading of biosolids.

6.5 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 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 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 datasets were limited.

Recently, 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). Results for the other deer habitat factors analyzed, which were distance to riparian habitat, location near wintering concentration areas, and landownership (private/public), were less conclusive. The results of this study are somewhat difficult

to interpret as clear goodness-of-fit parameters were not presented. Nevertheless, this study not only supports a link between soil and CWD transmission but also implicates a specific soil factor, clay content, with increased local prion incidence.

6.6 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). A recent study reported 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 QUIC (quake-induced conversion) method (Atarashi et al. 2007, 2008), which uses recPrP as a substrate instead of uninfected brain homogenate, might be 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.

6.7 Conclusion

As prion diseases, and CWD in particular, continue to spread geographically and disease residence times in cervid populations and habitats increases, 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 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). In addition, reliable methods for detecting and quantifying infectious prions in the soil environment are clearly required. Although detection of prions in natural soil samples has not yet been reported, use of protein misfolding cyclic amplification (PMCA) or similar methods appears to be the most promising avenue (Saunders et al. 2012a). PMCA has been used successfully with soil-bound prions (Saunders et al. 2011a, b, c).

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, 2011c). 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 7
The Spectrum of Tau Pathology in Human Prion Disease

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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 cooccurrence of a primary 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; and (3) neurofibrillary degeneration 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 degeneration in several subcortical, allocortical, and neocortical regions is consistently associated with certain *PRNP* mutations in Gerstmann–Sträussler–Scheinker disease or PrP cerebral amyloid angiopathy. 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.

Keywords Alzheimer disease • Argyrophilic grain disease • Cerebral amyloid angiopathy • Corticobasal degeneration • Creutzfeldt–Jakob disease • Dementia with Lewy bodies • Fatal familial insomnia • Gerstmann–Sträussler–Scheinker disease • Glycogen synthase kinase 3β • Neurodegenerative disease • Neurofibrillary tangle • Prion protein • Prion protein gene • Progressive supranuclear palsy • Proteinase K

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List of Abbreviations

AD	Alzheimer disease
AGD	Argyrophilic grain disease
CAA	Cerebral amyloid angiopathy
CBD	Corticobasal degeneration
CJD	Creutzfeldt–Jakob disease
DLB	Dementia with Lewy bodies
FFI	Fatal familial insomnia
gCJD	Genetic CJD
GSK3β	Glycogen synthase kinase 3β
GSS	Gerstmann–Sträussler–Scheinker disease
iCJD	iatrogenic CJD
NDD	Neurodegenerative disease
NFT	Neurofibrillary tangle
PD	Parkinson's disease
PK	Proteinase K
PRNP	Prion protein gene
PrP	Prion protein
PSP	Progressive supranuclear palsy
sCJD	Sporadic CJD
vCJD	Variant CJD

7.1 Overview of Tauopathies

Prion diseases belong to the group of neurodegenerative diseases (NDDs) that are characterized by progressive loss of neurons. A prerequisite to understand the relevance of tau pathology in prion diseases is knowledge of the spectrum of NDDs including tauopathies.

7.1.1 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 protein (FUS) (Kovacs et al. 2010). Variability in NDDs is reflected by distinct distribution 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).

7.1.2 Modifications of Tau Protein

Tau is a microtubule-associated protein encoded by a single gene (*MAPT*). The *Tau* (*MAPT*) gene 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 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. These include (1) major bands at 60, 64, and 68 kDa [e.g., in Alzheimer disease (AD)]; (2) bands at 64 and 68 kDa [e.g., in corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and argyrophilic grain disease (AGD)]; (3) bands at 60 and 64 kDa (e.g., in Pick's disease); and (4) a minor band at 72 kDa that usually associates with the first pattern (Lee et al. 2001). It is also important to distinguish different isofoms 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). 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. In summary, 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).

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 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.

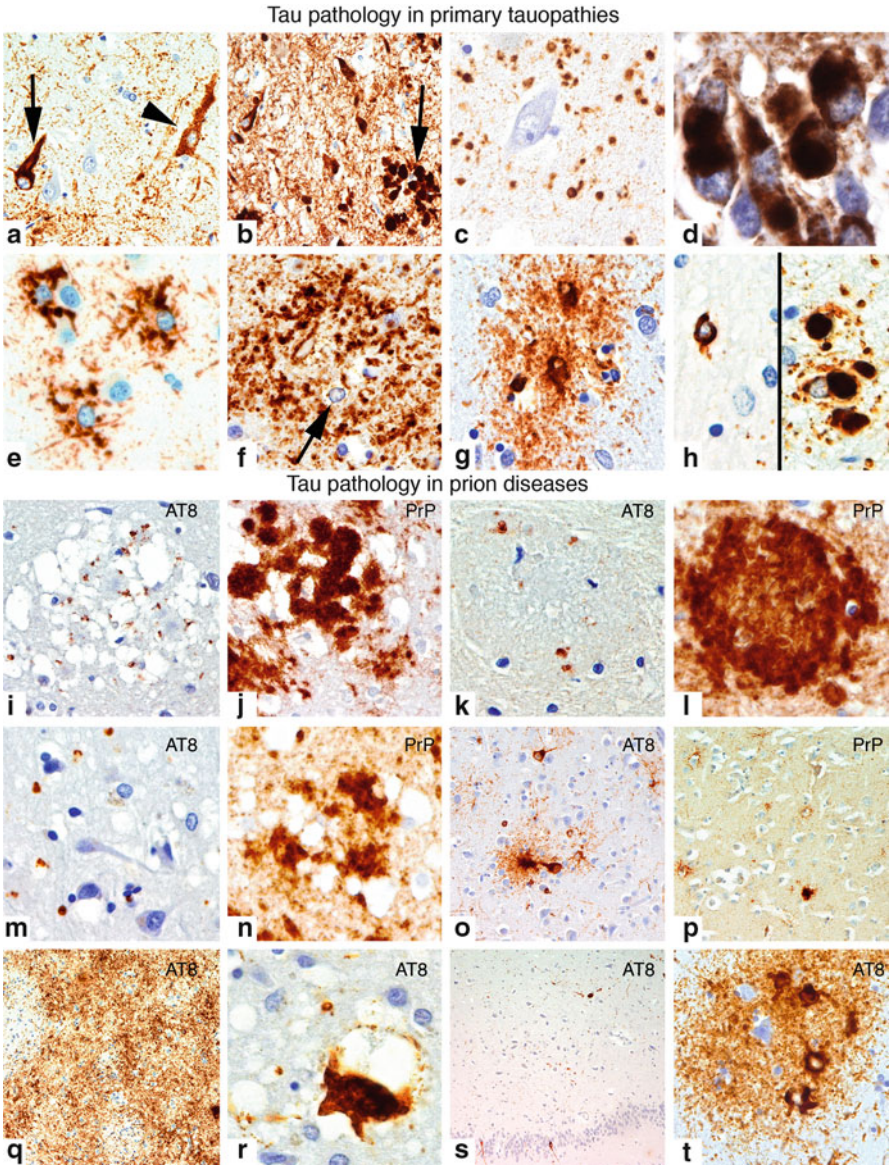

Fig. 7.1 Overview of tau pathology in primary tauopathies and prion diseases. **a**: Neurofibrillary tangle (indicated by an *arrow*) and diffuse cytoplasmic neuronal immunoreactivity (indicated by an *arrowhead*) in Alzheimer's disease hippocampus sample. **b**: Dystrophic neurites (indicated by an *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 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 supranuclear paly and white matter tauopathy with globular glial inclusions, respectively.

7.1.3 *Immunomorphology of Pathological Tau Deposition in Primary 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. 7.1a–h) (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

Fig. 7.1 (continued) **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 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) immu noreactive 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 genetic CJD (E200K mutation). **t**: Tau immunopositive astrogliopathy in the amygdala in genetic CJD (V203I mutation)

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 neurofibrillary tangle, which is argyrophilic (i.e., detected by silver stains) and ubiquitin immunoreactive (Bancher et al. 1989a, b). 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 certain anatomical pathways of the appearance of tau immunoreactive lesions can be recognized. This was originally described for the neurofibrillary degeneration 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.

7.1.4 Spectrum of Tau Pathology in Other Conditions

Pathological tau may be present in normal aging or nonneurodegenerative disorders (summarized in Goedert et al. 2006; Kovacs et al. 2010; Kovacs and Budka 2009b). In several conditions age-associated neurofibrillary degeneration is observed. In dementia with Lewy bodies (LBD), a primary α-synucleinopathy, a wide range of tau pathology may be detected. Chronic traumatic encephalopathy or postencephalitic parkinsonism also represent distinct tauopathies. Tau pathology is known to accompany cerebral amyloidoses or some storage diseases (i.e., Niemann–Pick type C).

7.1.5 How Is Tau Pathology in Prion Diseases to be Characterized?

Association of a tauopathy with other diseases requires analysis of the following aspects:

- Is it within the frame of age-associated neurofibrillary degeneration?
- Is it compatible with a well-established 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?

7.2 Tau Pathology in Human Prion Diseases

Human prion diseases may be classified according to the etiology as idiopathic (sporadic) Creutzfeldt–Jakob disease (sCJD), acquired (iatrogenic CJD—iCJD and variant CJD—vCJD), or genetic (familial and hereditary) CJD (gCJD), fatal familial insomnia (FFI), or Gerstmann–Sträussler–Scheinker disease (GSS). These disorders differ in brain pathology: spongiform encephalopathy in CJD; thalamic degeneration in FFI; and brain amyloidosis in 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.

7.2.1 Tau Pathology in Sporadic CJD

There is a paucity of data on systematic evaluation of phospho-tau immunoreactivity in different anatomical regions in sCJD. In addition to case reports of concomitant AD and sCJD, argyrophilic grains were reported in a single elderly sCJD patient (although not confirmed with phospho-tau immunopositivity) (Kawashima et al. 1999). In our cohort we observed one case combining CBD with sCJD (*unpublished observation*). A recent study evaluated phospho-tau immunostaining in a large cohort of sCJD patients but was restricted to the frontal cortex and cerebellum and focused only on the comparison of tau pathology with PrP immunostaining (Reiniger et al. 2011).

According to the literature and our experience, a concomitant 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 recent study (Reiniger et al. 2011) as well as our experience indicates 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).

2. Coexistence of AD-related and CJD pathology
This is observed in all larger CJD series, as both conditions preferentially occur in the elderly; however, tau pathology as well as 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 than 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 has been suggested (Tsuchiya et al. 2004): the first when AD patients develop CJD in the late stage of disease and a second form when sCJD patients 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), neurofibrillary degeneration was also reported corresponding to stage II according to Braak and Braak in a 76-year-old patient (Head et al. 2010).

7.2.2 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, and 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 neurofibrillary tangles 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 as 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, a tauopathy seems to be a regular component of the neuropathology of vCJD.

7.2.3 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). There accumulation of phospho-tau, α-synuclein, and Aβ was frequent, while TDP-43 immunoreactivity was never present. However, 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 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 *neurofibrillary degeneration 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 features of an *unclassifiable tauopathy* that did not fulfill criteria of established sporadic tauopathy entities (Kovacs et al. 2011b). This could be further subdivided in two major types (a) cases with neurofibrillary tangles, diffuse cytoplasmic tau immunoreactivity (pretangle-like), and threads in the basal ganglia, brainstem (substantia nigra, dorsal raphe nucleus, and locus coeruleus) 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 neurofibrillary tangles. 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 neurofibrillary tangles and prominent diffuse neuronal granular cytoplasmic immunoreactivity not only in CA4, CA3, and CA2 subregions and dentate gyrus, but also in the CA1 subregion and subiculum, without or with scant neurofibrillary degeneration 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 neurofibrillary tangles in the noradrenergic locus coeruleus. In these cases, however, further biochemical evaluation of tau protein was not available.

An unusual pattern of tauopathy was described in the *R208H gCJD* reminiscent of the type B of unusual tauopathy described above in E200K gCJD: few NFTs and neurons 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) exhibited features of an unusual tauopathy associated with a peculiar tau-astrogliopathy described in nonprion diseased elderly demented patients (Kovacs et al. 2011a).

Neurofibrillary degeneration was 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 neurofibrillary degeneration 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 important since here PrP deposition is only mild as compared to other prion diseases.

7.2.4 Tau Pathology in GSS

GSS is a form of brain (PrP) amyloidosis characterized by the appearance of (multicentric) amyloid plaques in the brain (Ghetti et al. 1995). The biochemical hallmark of GSS 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). As atypical cases, at least four stop codon mutations in the *PRNP* feature PrP cerebral amyloid angiopathy (PrP-CAA) (Ghetti et al. 2011). 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 GSS cases. It is characterized by tau-immunoreactive dystrophic neurites surrounding the PrP amlyoid plaques and neurofibrillary degeneration. However, not all GSS mutations are associated consistently with neurofibrillary degeneration.

The following mutations inconsistently show NFTs or other type of tau pathology (those 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 neurofibrillary tangles in the hippocampus, frontal cortex, and temporal cortex (Jansen et al. 2011b).

In contrast, neurofibrillary degeneration has been reported in the following mutations with GSS phenotype: G131V-129M, S132I-129M, H187R-129V, F198S-129V, D202N-129V, Q217R-129V, Y218N-129V, Q227X-129V, furthermore in Y145X-129M, Y160X-129M, and Y226X-129V mutations predominantly 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.

7.3 Concluding Remarks

7.3.1 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 recent 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 differs between mutations, as suggested by recent in vitro observations (Wang et al. 2008). Although there are several components of the tau–PrP 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). All together these studies indicate a complex interaction of tau and PrP.

7.3.2 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 is the best performing surrogate laboratory marker, total tau protein presents comparable levels of sensitivity and specificity (reviewed in Quadrio et al. 2011). Interestingly, a high rate of 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). Although evaluation of phospho-tau in CJD is less helpful, or still needs to be evaluated in all etiological forms of prion disease, an interesting future test seems to be the ratio of total tau to phospho-tau, particularly in the context of evaluating atypical AD patients.

7.3.3 Synopsis: 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 cooccurrence of a primary tauopathy with CJD, tau pathology presents in the following patterns (summarized in Figs. 7.1i–t and 7.2):

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.
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. *Neurofibrillary degeneration*, which can be further grouped as follows:
   (a) Neurofibrillary degeneration 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) Neurofibrillary degeneration restricted to the medial temporal lobe but not following 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).

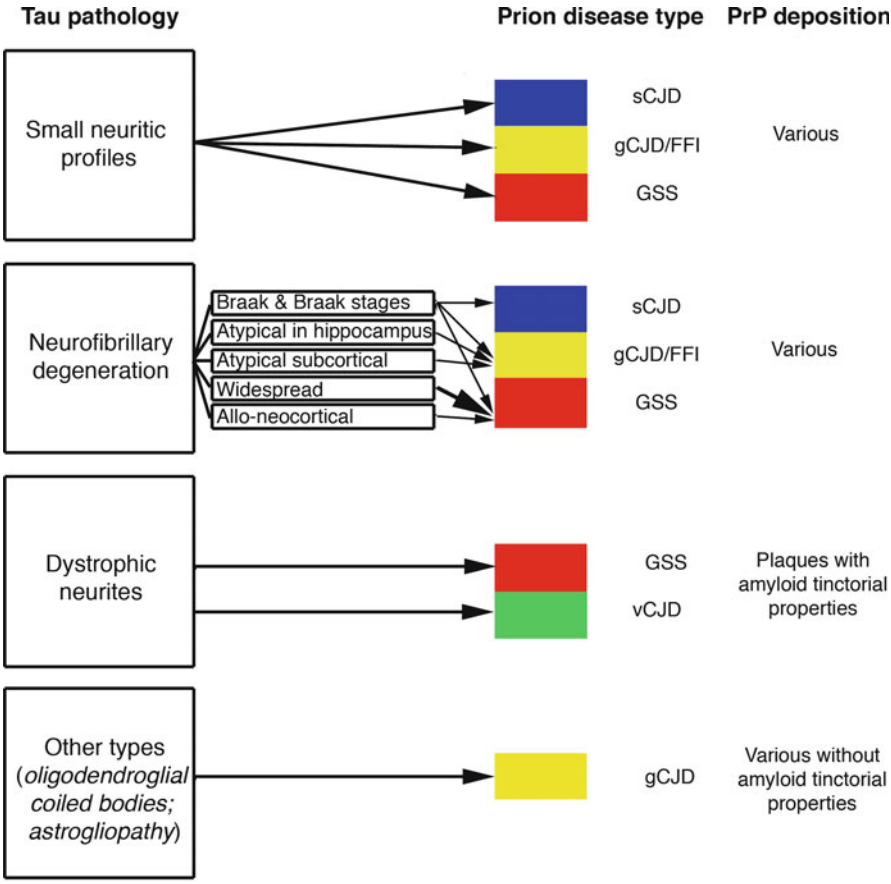

Fig. 7.2 Stratification of tau pathology according to morphology, prion disease type, and PrP immunoreactivity (see text for details)

(c) Neurofibrillary degeneration 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 neurofibrillary degeneration in several subcortical, allocortical, 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) Neurofibrillary tangles 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.

7.3.4 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 full anatomical spectrum of tau pathology in sCJD? Recent studies on a considerable number of cases focused only on the frontal cortex and cerebellum (Reiniger et al. 2011).
2. What is the relation between tau pathology and PrP deposition? Although small neuritic profiles correlate with the PrP load, the relation of further morphologies with PrP requires more studies (in particular in gCJD).
3. What further factors influence the appearance of tau pathology? In particular (1) why do GSS cases with various mutations, all by definition with prominent amyloidosis, considerably differ with regard to neurofibrillary degeneration? and (2) why does gCJD with the same single mutation (i.e., E200K) associate with clearly distinct spectrum of tau pathologies, including subcortical and hippocampus predominant forms, while other cases show only small neuritic profiles?

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Chapter 8
Risk of Transmission of Creutzfeldt–Jakob Disease by Blood Transfusion

Alexander H. Peden, Mark W. Head, and James W. Ironside

Abstract Early epidemiological studies on sporadic Creutzfeldt–Jakob disease did not identify blood transfusion as a risk factor for the disease. However, the emergence of variant Creutzfeldt–Jakob disease (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. 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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8.1 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 appears lacking to date. The emergence of variant Creutzfeldt–Jakob disease (vCJD) in the UK 15 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.

8.2 Experimental Evidence for Prion Disease Transmission by Blood Transfusion

8.2.1 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 and 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).

8.2.2 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 recently been particularly well served by the development of an experimental blood transfusion paradigm using the BSE agent experimentally transmitted to sheep.

8.2.3 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 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, reliable and perhaps more relevant 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 263K model 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) and to investigate partitioning during plasma

protein manufacture (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 scrapie strain hamster 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 phase, primarily in buffy coat and plasma, with lower levels in platelets and no infectivity detectable in red blood cells (Cervenakova et al. 2003).

These experiments demonstrated 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.

8.2.4 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) 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) was shown to transmit disease when further macaques were exposed orally or intravenously (Herzog et al. 2004). Reported use of primate models to directly mimic transfusion practice has been surprisingly limited in scope and has recently been complicated by the finding of 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).

8.2.5 Sheep Models

To date, only in 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 time points) 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). Interestingly, efficient transfusion transmission is not a property restricted to the BSE agent. Similar transmission rates (43%) were seen in parallel 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 a different sheep scrapie model system in which transfusion-mediated transmission rates approach 100% (Andreoletti et al. 2012; Lacroux et al. 2012). The results using this model system demonstrate a marked discrepancy between prion titres in sheep blood as defined by i.c. challenge of susceptible (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).

Each of the above rodent, primate and sheep experimental systems is at one or more removes from the events they seek to model, and 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 10IU/ml of blood, whereas this has now been revised downwards to less than 1IU/unit of blood (~400ml) 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.

8.3 Evidence for vCJD Transmission by Blood Transfusion and Plasma

8.3.1 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 (TMER), a collaboration of the National CJD Research & Surveillance Unit (NCJDRSU) and the UK Blood Services (Hewitt et al. 2006; http://www.cjd.ed.ac.uk/TMER/TMER.htm). Figure 8.1 summarises information on the time of the relevant transfusions and the

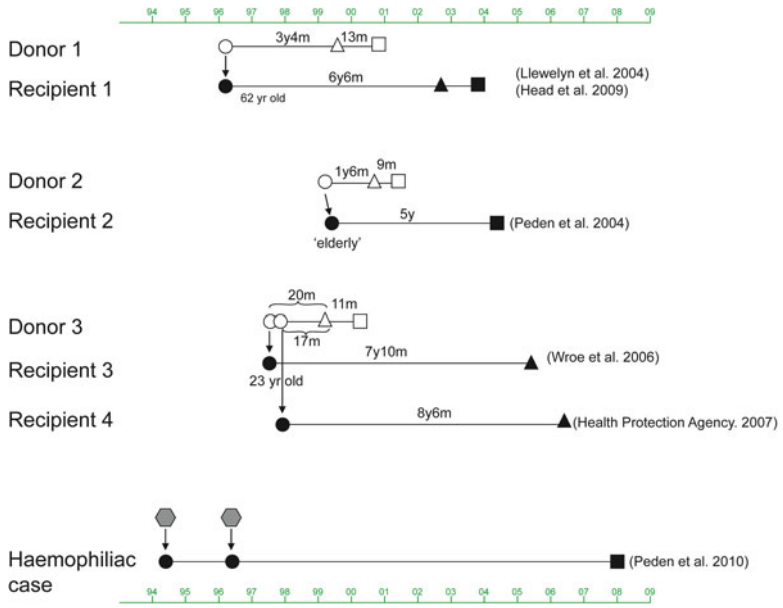

Fig. 8.1 Time lines for the donors and recipients of blood in the four known cases of blood transfusion-associated vCJD infection. The interval between donation/transfusion (*circles*) and death (*square*) or vCJD disease onset (*triangles*) are represented by *lines* drawn to a scale indicated at the *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

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 there have been no secondary vCJD cases in patients receiving leucodepleted blood.

The clinical reports of recipients 1, 3 and 4 were typical for vCJD and genotype analysis showed 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. The neuropathological findings for recipients 1 and 3 were typical for vCJD (Head et al. 2009; Wroe et al. 2006) (Fig. 8.2a–d). In both of these recipients, Western blotting analysis of brain homogenate following treatment with proteinase K revealed the 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 methionine/valine (M/V) heterozygous at *PRNP* codon 129 (Fig. 8.1) (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/Western blotting (NaPTA/WB) (Fig. 8.2g), paraffin embedded tissue blotting (PET) 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 129M/V individuals might be either susceptible to vCJD or capable of incubating this disease.

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).

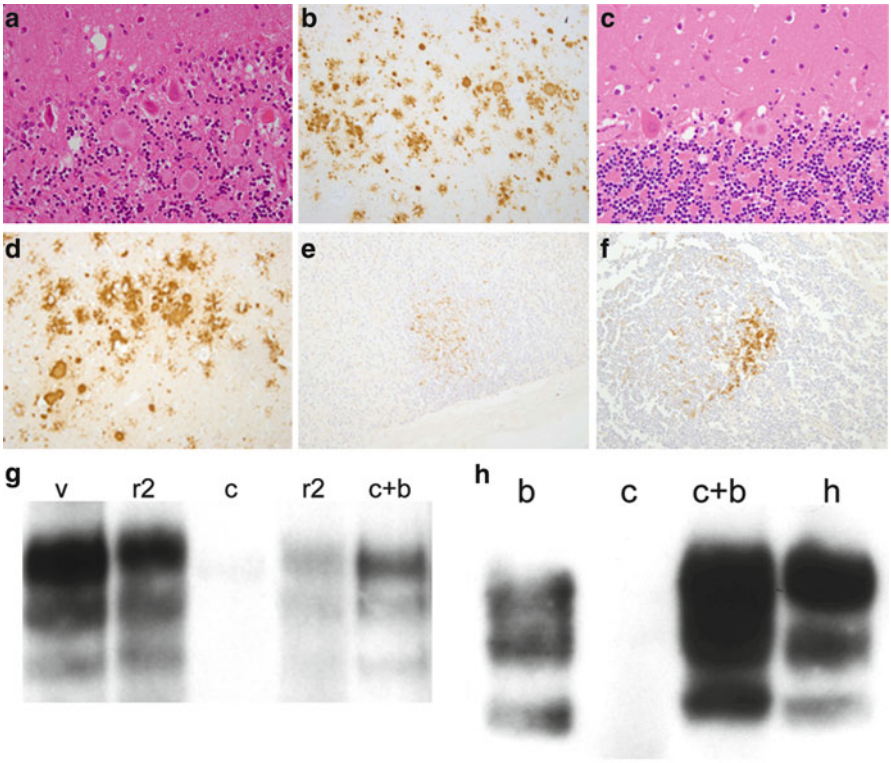

Fig. 8.2 Pathology and biochemistry of autopsy tissues from symptomatic and asymptomatic vCJD infected individuals following blood transfusion and plasma product administration. Haematoxylin and eosin stained sections of the cerebellum from blood donor 1 (A) and the corresponding blood transfusion recipient 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

8.3.2 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. 8.2h). 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 9,025 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).

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

8.4.1 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 (http://www.nibsc.ac.uk/spotlight/cjd_resource_centre/cjd_tests.aspx).

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 1:2,000 based on retrospective screening of archived tonsil specimens in England (Health Protection Agency 2012). 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.

8.4.2 *Approaches to Sensitive Detection of PrP^{Sc}*

A wide variety of approaches have been taken to the development of blood tests for vCJD. We have reviewed these recently (Peden et al. 2008) 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. Despite considerable scientific and commercial interest none of these conventional approaches have, as yet, delivered a prototype assay for the detection of PrP^{Sc} in human blood.

8.4.3 *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 to 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 Western blotting (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 the experimental hamster 263K scrapie model, serial PMCA has been able to distinguish between bloods from infected and uninfected hamsters at the clinical phase (Castilla et al. 2005) and during the asymptomatic preclinical phase (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), medical (Jones et al. 2007) and veterinary (Thorne and Terry 2008) 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 (Castilla et al. 2005; Saa et al. 2006; Thorne and Terry 2008). Our own approach (in collaboration with the Scottish National Blood Transfusion Service) has been to collect PrP^{Sc} from plasma, perform serial PMCA using out-dated human platelet extracts as substrate, followed by detection using conformation-dependent immunoassay (Jones et al. 2009). Other configurations of the PMCA methodology aimed at human blood testing also appear promising (Tattum et al. 2010).

A third generation 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. 2007; Atarashi et al. 2008). RT-QuIC is already under evaluation as a clinical diagnostic using cerebrospinal fluid from suspected cases of sporadic CJD (Atarashi et al. 2011; McGuire et al. 2012). Problems with relatively inefficient detection of vCJD brain and CSF samples (Peden et al. 2012) and with inhibitors of QuIC in plasma appear to have been overcome by a further modification of the methodology (termed e-QuIC) that incorporates PrP^{Sc}-specific antibody immunoprecipitation, a chimeric recombinant PrP substrate and a reaction buffer replacement step (Orru et al. 2011). e-QuIC is reported to be able to detect a 10^{14}-fold dilution of vCJD brain or 2 ag/ml of vCJD PrP^{res} making it the most sensitive assay yet reported as judged by limit of detection (LoD) of human CJD brain. However, the method has not yet been tested on clinical vCJD blood specimens and relevant controls. A prospective blood test with a somewhat higher LoD 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 assay failed to detect PrP^{Sc} in blood taken from sCJD patients ($n=27$).

Table 8.1 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).
Leucodepletion of all blood components (1999).
Importation of clinical fresh frozen plasma for patients born after January 1996 (2004). Extended to all patients under the age of 16 by 2005.
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 tissues products and alternatives throughout the NHS.

8.4.4 Future Perspectives

The above assays, in addition to a further blood test under development by Prionics AG, all have a considerable distance 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. At present, e-QuIC (Orru et al. 2011) and the assay of Edgeworth et al. (2011) appear most promising. The serial format of PMCA involved in achieving the appropriate analytical sensitivity makes assays such as sPMCA/CDI better suited to development as a confirmatory blood test. It is tempting to speculate that these technologies could be in implementable forms within the next 2 or 3 years. However, the track record in this area indicates that this is far from certain and the potential benefits of implementation of any test will need to be weighed carefully against the costs and potential consequences.

8.5 Conclusions

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 taken to reduce the risks of vCJD transmission by blood and blood products in the UK are summarised in Table 8.1. It remains to be seen whether any further measures will be implemented, for example the introduction of “prion filters”. The cases of transfusion-associated vCJD infection all occurred prior to the full introduction of leucodepletion in the UK. The most recent data from sheep models indicate that

whilest leucodepletion alone does not prevent disease transmission completely (McCutcheon et al. 2011), it does have a pronounced effect, and that it is the leucoreduction component of combined leucodepletion/prion reduction filters that is responsible the prion removal (Lacroux et al. 2012). Both of these sheep studies also show that all blood components may be considered potential vectors for prion transmission (McCutcheon et al. 2011; Lacroux et al. 2012). These findings reinforce the need for multiple control measures to reduce the risk of vCJD transmission by blood and blood products and raise the possibility that future cases of transfusion-associated vCJD may be identified in recipients of blood products other than non-leucodepleted red blood cell concentrates. In view of the uncertainties over the prevalence of asymptomatic vCJD infection in the UK, it seems likely that these control measures will continue to be required, perhaps until an effective test for asymptomatic vCJD infection is available.

The evidence for the transmission of other forms of CJD by blood transfusion is far less clear-cut: although data from experimental models indicate that different strains of prions can be transmitted by blood, the epidemiological evidence in humans to support these findings is largely absent. The recent contention that blood transfusion may be a risk factor for sCJD (Puopolo et al. 2011) has renewed interest in this field of research and will hopefully generate new research that is better designed to answer this problem than many of the previously published studies. Until these new studies are concluded, continued surveillance and analysis of risk factors for all forms of human prion disease is required.

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Chapter 9
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 amino acid sequence of the normal host prion protein (PrP^{C}), the available pool of exogenous abnormal and infectious prion protein (PrP^{Sc}), and the ability to establish a subclinical infection are all important determinants of prion species barriers. Mechanistically, maintenance of species barriers to prion infection is likely dependent upon the conformational diversity of the PrP^{Sc} molecules in an infectious inoculum and the potential for conformational compatibility between the exogenous PrP^{Sc} and endogenous host PrP^{C}. However, the lack of high resolution structural information for PrP^{Sc}, the potential for host factors and posttranslational modifications to PrP^{C} to influence species barriers, and the fact that the amino acids important in prion species barriers differ between species, makes it difficult to predict prion species barriers based on PrP^{C} sequence alone. In vivo or in vitro experimentation in relevant models of infection remains the only way to determine species barriers to prion infection.

Keywords Prion • Transmissible spongiform encephalopathy • Species barriers • Prion protein • Scrapie • PrP

Abbreviations

BSE	Bovine spongiform encephalopathy
CJD	Creutzfeldt–Jakob disease
CNS	Central nervous system

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GPI	Glycophosphatidylinositol
MBM	Meat and bone meal
NMR	Nuclear magnetic resonance
Prnp	Prion protein gene
PrP	Prion protein
PrP^{C}	PrP cellular
PrP^{Sc}	PrP scrapie
sCJD	Sporadic Creutzfeldt–Jakob disease
Sinc	Scrapie incubation time gene
TME	Transmissible mink encephalopathy
TSE	Transmissible spongiform encephalopathy
vCJD	Variant Creutzfeldt–Jakob disease

9.1 Introduction

Prion diseases, also known as transmissible spongiform encephalopathies or TSEs, can be transmitted both within and across species. Intraspecies transmission of TSE diseases 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. 2009b), 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 TSE infectivity therefore correlates with the presence of detectable levels of infectivity in non-CNS tissues.

Regardless of which tissues are positive for TSE infectivity, interspecies transmission of prions is much more difficult than intraspecies transmission. Species barriers in prion diseases are defined by the difficulty that prions from one species have in triggering TSE infection in a second species. As long as low prion 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 1986 (Wells et al. 1987; Wilesmith et al. 1988). 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 variant CJD (vCJD) was identified in young people in the UK 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 (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 prion diseases in nonruminant species and concerns that CWD has the potential to do the same, makes understanding the mechanisms underlying species barriers to TSE infection critical.

9.2 Prion Protein and TSE Species Barriers

Species barriers to TSE infection were initially defined based primarily upon the experimental inoculation of different types of TSE agent into multiple mammalian species including mice, hamsters, ferrets, and mink. For example, transmissible mink encephalopathy (TME) can be transmitted to hamsters but not mice (Marsh et al. 1969). Suffolk sheep scrapie can infect both mice and mink, while Cheviot sheep scrapie infects 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

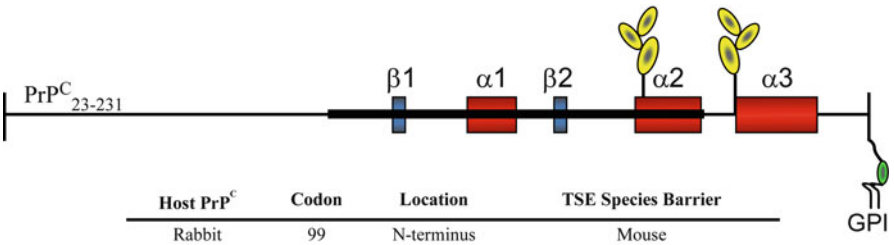
with TSE disease (Bolton et al. 1982). Soon after its discovery, it was determined that PrP was a normal host protein (Basler et al. 1986; Locht et al. 1986) which was both soluble and protease sensitive (Bendheim et al. 1988). During prion disease pathogenesis, normal PrP (termed PrP^{C} for PrP cellular) is refolded into an abnormally aggregated, protease-resistant, and infectious form known as PrP^{Sc} (for PrP scrapie) which accumulates, eventually causing a TSE disease. Comparison of PrP^{C} molecules from different mammalian species demonstrated that, while the *Prnp* gene is highly conserved, its amino acid sequence can vary by as much as 20% (Wopfner et al. 1999). This provided a potential molecular basis for TSE 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 or not infection and disease could occur.

9.3 Role of PrP Amino Acid Sequence

9.3.1 Region of PrP Involved in Species Barriers

In order to determine whether or not the sequence of PrP^{C} was a determinant of TSE 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 TSE 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). These experiments clearly showed that the amino acid sequence of the host PrP^{C} molecule was a major determinant of species barriers in TSE diseases. They also provided an explanation for why earlier studies had implicated the *Sinc* gene in TSE species barriers: the gene for PrP (*Prnp*) 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 to 189 (Fig. 9.1) (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 homologous (Wopfner et al. 1999) and there are only three amino acid differences between the two species in the region from codon 108 to 189, suggesting that one or more of these residues were contributing to the mouse–hamster TSE species barrier (Kocisko et al. 1995).



Host PrP^C	Codon	Location	TSE Species Barrier
Rabbit	99	N-terminus	Mouse
Mouse	101	N-terminus	Human (vCJD), Sheep, Hamster
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)
Vole	169	β2-α2 loop	Bovine (BSE)
Sheep	171	β2-α2 loop	Bovine (BSE)
Rabbit	173	α2	Mouse
Ferret	179	α2	Mink (TME)
Rabbit	214	α3	Mouse
Ferret	224	C-terminus	Mink (TME)

Fig. 9.1 *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 black line* indicates the region of PrP^C where most of the amino acid residues important in TSE 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

9.3.2 Influence of Single Amino Acid Residues

The influence of these three amino acid residue differences at codons 138, 154, and 169 on the species-specific formation of mouse PrP^{Sc} was analyzed in vitro using mouse neuroblastoma cells infected with the mouse scrapie strain RML (Priola and Chesebro 1995). These cells express mouse PrP^C and generate both mouse PrP^{Sc} and mouse scrapie infectivity. When mouse PrP^C expressing a unique antibody epitope tag is expressed in scrapie-infected 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 TSE species barrier, it was determined that a single hamster-specific residue at codon 138 in mouse PrP^C prevented the production of mouse PrP^{Sc} in cells (Priola and Chesebro 1995). The other mutations at codons 154 and 169 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 TSE species barriers to infection could be dependent upon relatively minor differences in sequence between the endogenous host PrP^{C} and exogenous PrP^{Sc} molecules.

Some types of prions, including BSE and sCJD, have never been successfully used to persistently infect cells in vitro. This limits the usefulness of cell-based systems in defining and understanding the mechanisms underlying TSE species barriers. Fortunately, in addition to both natural and transgenic models of prion disease, there are cell-free systems that are not restricted by prion species (Bossers et al. 1997; Castilla et al. 2005, 2008; Eiden et al. 2011; Kocisko et al. 1994, 1995; Raymond et al. 1997) which 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 TSE infectivity across species barriers differ depending upon the species (Fig. 9.1). For example, species-specific formation of hamster PrP^{Sc} has been mapped to codon 155 in vitro (Priola et al. 2001) and the same residue has been implicated in species barriers in 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). 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 TSE infection (Gibbs and Gajdusek 1973), multiple amino acid residues appear to be important for PrP^{Sc} formation (Vorberg et al. 2003).

Depending upon the species, resistance to BSE is associated with different amino acids in PrP^{C} (Fig. 9.1). 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 (Goldmann et al. 1994; Raymond et al. 1997). 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 TSE species barriers reside in different regions of PrP^{C} (Fig. 9.1). Thus, it would appear that there is no single structural component of PrP^{C} which is absolutely associated with species barriers to TSE infection.

9.3.3 Effect of **Prnp** *Heterozygosity*

Heterozygosity at the *Prnp* gene may also influence TSE 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 molecules in a dominant-negative fashion, slowing down or preventing clinical disease. In this manner, heterozygosity at the *Prnp* allele may contribute to the maintenance of TSE species barriers.

9.4 Influence of PrP Posttsranslational Modifications

PrP^C is posttranslationally modified by glycosylation at two N-linked glycosylation sites as well as by the addition of a glycophosphatidylinositol (GPI) membrane anchor (Caughey et al. 1989; Haraguchi et al. 1989; Stahl et al. 1987). The GPI anchor appears to have little or no effect on the species-specific formation of abnormal prion protein (Priola and Lawson 2001). However, PrP^C glycosylation can influence the binding between heterologous PrP^C and PrP^{Sc} molecules in a species-specific manner (Priola and Lawson 2001). At a molecular level, less efficient binding of heterologous PrP^C and PrP^{Sc} molecules would result in the production of less PrP^{Sc}. In vivo, this would likely contribute to the prolonged disease incubation times which are the hallmark of prion disease species barriers.

9.5 Non-PrP Host Factors

There are several examples in transgenic mice where, despite the fact that the host PrP^C amino acid sequence is identical to the incoming PrP^{Sc} amino acid sequence, species barriers to infection were maintained. For example, transgenic mice expressing human PrP^C can be more resistant to infection with vCJD than wild-type mice but more susceptible to infection with sporadic CJD (Bishop et al. 2006; Hill et al. 1997). 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 still suggest that host factors other than PrP might play a role in species barriers to prion infection. However, no such factor has yet been identified and thus the role of non-PrP host factors in transmission of prions across species remains unclear.

9.6 Molecular Mechanisms of TSE Species Barriers

9.6.1 Mechanism of PrP^{Sc} Formation Across Species

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 also influence this process suggests that it is the tertiary structure of PrP, and not its primary structure, which is ultimately 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 TSE infection are controlled at the level of PrP conformation. 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. 9.2a).

Interspecies transmission of prions can occur when the host PrP^{C} and the exogenous infectious PrP^{Sc} are heterologous, but the amino acid 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/or its subsequent conversion to PrP^{Sc} occurs efficiently enough that PrP^{Sc} can "replicate" to pathogenic levels (Fig. 9.2b). Thus, the differences in PrP^{C} conformation are insufficient to cause a species barrier to infection.

Interspecies transmission of prions would not occur when the host PrP^{C} and the exogenous infectious PrP^{Sc} are heterologous, but the amino acid differences do reside within critical regions of the PrP molecule. In this case, the amino acid differences 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. 9.2c). Thus, PrP^{Sc} would be unable to "replicate" itself very efficiently (if at all) and would not accumulate to sufficient levels to trigger disease in the new host, i.e., a prion disease species barrier would exist.

9.6.2 Structural Regions of PrP^{C} Implicated in Species Barriers

If PrP conformation is the driving force behind species-specific formation of PrP^{Sc}, species barriers to prion infection cannot be predicted based upon the primary sequence of PrP^{C} alone. Unfortunately, PrP^{Sc} conformation cannot be used to predict prion species barriers either as there are no high resolution PrP^{Sc} structures available (Moore et al. 2009). However, 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

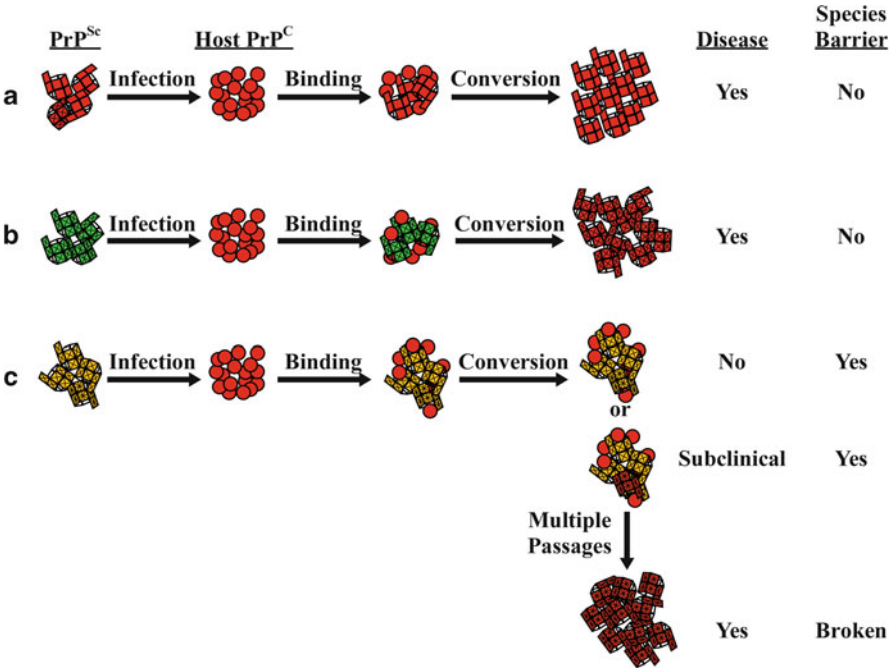


Fig. 9.2 *Molecular mechanism of prion disease species barriers*. *Red* indicates PrP molecules derived from the host species with aggregates of *squares* representing PrP^{Sc} and *circles* representing PrP^{C}. The degree of hatching within the *squares* represents different PrP^{Sc} conformations. 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, i.e., there is a species barrier to infection unless there is a small, conformationally divergent fraction of PrP^{Sc} which can trigger new PrP^{Sc} formation. A subclinical infection would then be established which, given continued passage through the host species, could eventually lead to clinical disease and a species barrier to prion infection being broken

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. 9.1).

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 is important 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. 9.1). When amino acid residues that have been experimentally shown to have a major influence on species-specific PrP^{Sc} formation are superimposed onto the structure of PrP^C, the vast majority of them reside within the disordered loop/turn regions (Fig. 9.1) suggesting that these are the critical structures. Thus, conformational variability within these loop regions between different species of PrP molecules may influence prion species barriers (Moore et al. 2009).

Polymorphisms within the disordered loop/turn structure which 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), bovine (Lopez et al. 2000), and human (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. 9.1). 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 TSE 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) (Fig. 9.1), 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 antiparallel steric zippers, the stability of which may determine TSE species barriers (Apostol et al.

2011). 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.

9.6.3 Effect of Variable PrP^{Sc} Conformation

Differences in PrP^{Sc} conformation may also help to explain the early observation that the range of TSE strains in the infectious inoculum is one determinant of whether or not a TSE 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). Prion strains are defined by PrP^{Sc} molecules with the same 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 would effectively have the same influence on species barriers as differences in the primary sequence of PrP^{C}.

9.7 Breaching TSE Species Barriers

Even if a species barrier is not crossed during primary passage into a new host species, the presence of multiple TSE strains in the infectious inoculum coupled with the potential for subclinical infection (i.e., prion replication but no disease) might eventually lead to a breach of the barrier to infection. 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). Similarly, the species tropism of BSE can be changed by passage through sheep. Sheep-passaged BSE can infect transgenic mice expressing human PrP^{C} (Plinston et al. 2011) while BSE from cattle cannot (Plinston et al. 2011; Tamguney et al. 2009a). All of these experiments show that crossing species barriers can change the tropism of the infectious agent leading to the unpredictable emergence of prions with distinct species tropisms which can differ from that of the original inoculum.

The existence of PrP^{Sc} molecules with the same PrP sequence but different conformations can also help to explain adaptation across even a strong species barrier. A minor fraction of the exogenous PrP^{Sc} could be conformationally compatible with the endogenous host PrP^{C} resulting in the generation of low levels of infectious PrP^{Sc} which now has the sequence of the host PrP^{C} molecule (Fig. 9.2c). This new

PrP^{Sc} begins to accumulate over the lifetime of the infected host but does not reach levels sufficient to cause disease (i.e., subclinical infection). 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}. This effectively decreases the time it takes for PrP^{Sc} to reach pathogenic levels until eventually it causes disease within the lifetime of the host (Fig. 9.2c). The tropism of the final prion agent would likely reflect the minor fraction of PrP^{Sc} that was eventually able to amplify efficiently enough over multiple passages to cause disease. Thus, as long as infectivity 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.

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Chapter 10
Transgenic Mice Modelling

Abigail B. Diack, Rona Wilson, Enrico Cancellotti, Barry Bradford, Matthew Bishop, and Jean C. Manson

Abstract Although the prion protein (PrP) was discovered in the early 1980s, there is still a considerable 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

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10.1 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. There are currently over 60 different transgenic mouse models constructed to assess the role of PrP in health and disease. This review concentrates 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. In particular, it will focus on models in which 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.

10.2 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 (Bueler et al. 1992; Manson et al. 1994a). 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. 1994b; 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. 1994a). This demonstrated, as had a number of previous experiments with mice overexpressing the *Prnp* gene, that the expression levels of PrP altered incubation time, with overexpression in general shortening incubation periods and reduced expression leading to longer incubation periods (Westaway et al. 1991; Scott et al. 1989).

Early mouse studies revealed that susceptibility to disease and incubation period could be influenced by the PrP genotype. The first transgenic mouse studies by 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 incubation period 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 incubation periods; the greater the similarity between PrP sequences the greater their susceptibility to disease and the shorter the incubation period. 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 bovine *Prnp* gene in a 129Ola background inoculated with bovine spongiform encephalopathy (BSE) have a longer incubation period 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 incubation periods 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 to modify disease incubation period on cross species transmission of BSE to mice (Manolakou et al. 2001).

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 also that transmission rates were higher and incubation periods 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 10.1).

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 GSS (Gerstmann–Sträussler–Scheinker disease) produces disease in 288 days with 100% susceptibility, suggesting

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

[2] Sporadic CJD is subclassified via the codon 129 genotype of the host and typed by biochemical properties

Table 10.1 Primary inoculation of TSE strains in three transgenic mouse lines

	HuMM		HuMV		HuVV	
Strain of agent	IP	TSE pathology[a]	IP	TSE pathology	IP	TSE pathology
vCJD	>401	11/17	>600	11/16	–	1/16
sCJD (M1CJD)	446	29/29	457–475	31/32	588–603	29/34
sCJD (M2CJD)	–	0/16	–	2/18	–	3/17
sCJD (V1CJD)	–	2/16	557	9/14	568	7/14
sCJD (V2CJD)	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	–	–	–	–	–	–

[a]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) and BSE data from Bishop et al. (2006)

the importance of the proline to leucine change in determining susceptibility (Manson et al. 1999). More unexpected however was that when these mice were inoculated with hamster-passaged scrapie (263K) or a pooled natural scrapie strain (SSBP/1), the incubation period 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 incubation period in 101LL mice compared with wild-type mice despite being of the same species (Manson et al. 1999; Barron et al. 2001). These studies suggests 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, while G1 and G2 PrP appeared mainly on the cell surface similar to wild-type PrP (Cancellotti et al. 2005). Intracerebral (i.c.) inoculation of several agents into these mice demonstrated that glycosylation of host PrP was not essential for establishing infection

within a host or transmitting infectivity to a new host. When these mice were inoculated intracerebrally with two mouse-passaged agents (ME7, 79A), G3 mice were only susceptible to 79A and exhibited a significantly longer incubation period than G1 or G2 mice. G2 mice were susceptible to both agents but show increased incubation period with 79A, an extended study also showed an increased incubation period with the 301C strain. In comparison, G1 mice were only susceptible to 79A (Tuzi et al. 2008). 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 (Tuzi et al. 2008; Cancellotti et al. 2010).

10.3 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 or through blood as has been the case with vCJD (Bruce et al. 1997; Peden et al. 2004; Llewelyn et al. 2004). Thus the periphery plays an important role in the disease pathogenesis. However, the route and mode of spread of the agent from the periphery to the CNS of the host is still unclear.

Following peripheral transmission of TSE, there is an early accumulation of disease associated PrP (PrP^{Sc}) in tissues of the lymphoreticular system (LRS) such as the spleen and lymph nodes, before the disease spreads to the CNS (Muramoto et al. 1993). It has been proposed that host cellular PrP (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 incubation period were observed between Schwann cell PrP knockout mice and controls. Thus, while 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.

10.4 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 and incubation period length of sCJD and acquired CJD (Collinge et al. 1991; Palmer et al. 1991). 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 176 UK vCJD cases have occurred so far (http://www.cjd.ed.ac.uk/, http://www.oie.int/en).

In comparison to 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 has revealed that human-to-human transmission of vCJD is possible with all genotypes having the potential to be affected. The results from the mouse studies suggest that MV and VV genotypes may have a longer incubation period or may not develop clinical disease (Bishop et al.

2006) indeed there may be significant levels of subclinical vCJD disease in the human population in all three genotypes.

There are ongoing concerns that recently identified animal TSE diseases such as atypical scrapie and H-type and L-type BSE could be transmissible to humans, in particular sheep and/or goat BSE and chronic wasting disease (CWD). Using tg650 mice which express sixfold human PrP (129MM) and are fully susceptible to vCJD, Beringue et al. (2008) showed that classical BSE transmits relatively inefficiently (4/25 mice), while L-type BSE shows 100% transmissibility and H-type BSE does not transmit at all to this mouse model. 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, no other genotype of mice were affected (Plinston et al. 2011) (Table 10.1). 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. Chronic wasting disease in comparison has failed to transmit clinically and pathologically to mice overexpressing human PrP: 129MM Tg35, 129MM Tg45 and 129VV Tg152 (expressing two, four and sixfold, respectively), which are susceptible to both human and BSE prions (Sandberg et al. 2010) and gene targeted humanised mouse models: HuMM, HuMV and HuVV (Wilson et al. 2012).

10.5 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 incubation period of disease, PrP^{Sc} biochemical profile as assessed by Western blot and the amount and distribution pattern of vacuoles and PrP^{Sc} deposition in the brain (Bruce et al. 1999; Ritchie et al. 2009; Dickinson et al. 1968). 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, 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. Clinicopathological phenotypes of sCJD can be subgrouped according to host codon 129 genotype and the biochemical characteristics of PrP^{Sc} (Parchi et al. 1999; Brown et al. 1994b; 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 mice and four distinct strains emerged: $M1^{CJD}$ (MM1, MV1), $M2^{CJD}$ (MM2), $V1^{CJD}$ (VV1) and $V2^{CJD}$ (MV2 and VV2) (Table 10.1, Fig. 10.1). MM1 and MV1 ($M1^{CJD}$) isolates showed identical transmission characteristics based upon incubation periods, vacuolation profiles, western blot profile and PrP^{Sc} deposition patterns. MV2 and VV2 ($V2^{CJD}$) isolates showed similar characteristics, while 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 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 10.1) 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. (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 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).

10.6 Mechanisms of Neurodegeneration

At the clinical end point of TSE disease, there is characteristic vacuolation, PrP^{Sc} deposition and neuronal loss in various areas of the brain. The targeting of these pathologies may be modulated by both strain and host factors. The mechanisms that

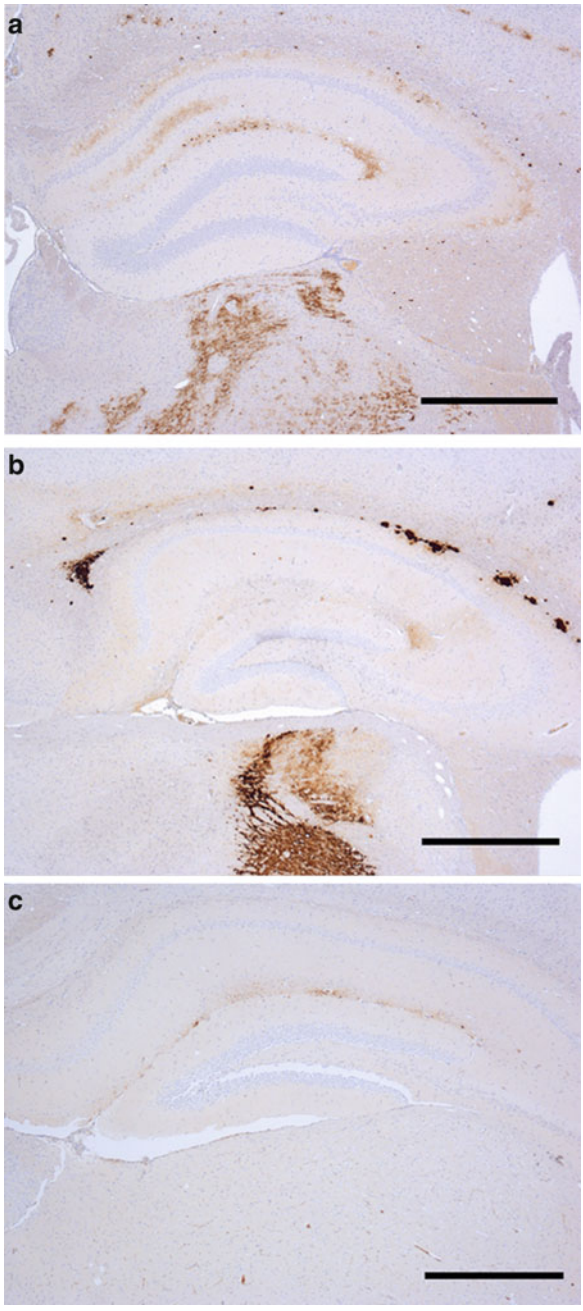

Fig. 10.1 Comparison of immunocytochemistry for PrP in transgenic mice expressing the human 129MM genotype (HuMM) challenged with (**a**) $M1^{CJD}$ (MV1), (**b**) $V2^{CJD}$ (MV2) and (**c**)$V1^{CJD}$ (VV1). V2CJD did not transmit to HuMM mice. Immunocytochemistry with ant-PrP antibody (6H4) was performed on histological sections of the mouse brains. Representative sections are shown through the hippocampus/thalamus (magnification: 2.5×)

initiate the cascade leading to neuronal loss are unknown. It is not known whether PrP^{Sc} is neurotoxic or if loss of function of PrP^{C} plays a role in rendering neurons susceptible to degeneration. Gliosis is evident early in the pre-clinical phase of

disease, but the role of the non-neuronal cells in the disease process is not known. 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 (Ilieva et al. 2009). Disease incubation period in the prion diseases is related to the amount of total PrP^C in the brain (Manson et al. 1994b; Scott et al. 1989). 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 (Race et al. 1995) 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. 1994a). Moreover, further evidence for cell-autonomous processes was provided using a model in which PrP^C expression was removed from neurons at a specific time point during the course of disease. The disease process appeared to be blocked by the removal of neuronal PrP (Mallucci et al. 2003) 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 only (Raeber et al. 1997) experienced neurodegeneration (Jeffrey et al. 2004) and succumbed to TSE disease. 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; Kercher et al. 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).

In order to address cell-autonomous versus non-autonomous mechanisms of neurodegeneration, we have produced a model in which we can induce the removal of PrP^C in a spatial–temporal manner. We combined gene targeted *LoxP* flanked *Prnp* controlled by the endogenous murine promoter (Tuzi et al. 2004) and tamoxifen-inducible neuronal expressed Cre recombinase transgenes to allow timed control of removal of PrP^C from neuronal populations. We tested this model using the well-characterised ME7 strain of agent to determine the effect of tamoxifen-induced neuronal PrP^C knockout on TSE disease. We have demonstrated a non-cell-autonomous mechanism of neurodegeneration which is associated with greatly extended incubation times of disease but does not prevent pathogenesis, clinical disease or death (Bradford submitted).

10.7 Conclusions

Transgenic mouse models have made a major contribution to our understanding of TSEs, particularly in the assessment of zoonotic potential and modelling intraspecies transmission where the host species may be large animals or humans. Using gene targeted or knockout mice to understand the pathogenesis of TSE disease allows us to unravel the mechanisms of prion replication and the infective process while also providing a model for other neurodegenerative protein misfolding diseases. As new techniques in transgenic production are implemented in these studies, 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 11
Transgenic Mouse Models in Prion Transmission Studies

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Abstract Prion diseases are a unique group of mostly transmissible neurodegenerative diseases where the ubiquitous cellular prion protein (PrP^C) plays a central role. Numerous transgenic mouse models have been instrumental in dissecting the roles of PrP^C and other factors in the replication, pathogenesis, and transmission of the prion agents. This chapter summarizes the seminal roles of transgenic mouse models in prion transmission studies with an emphasis on the contributions of PrP primary sequence and prion strains to prion transmission barriers and non-PrP factors in prion pathogenesis.

Keywords Prion protein • Prion strain • Transgenic mice • Transmission barrier

11.1 Prion Diseases and Prion Protein (PrP)

Prion diseases, also named transmissible spongiform encephalopathies (TSEs), are a growing family of fatal neurodegenerative diseases that strike humans and many animals (Prusiner 1998; Kong et al. 2004). The prevailing "protein-only" hypothesis postulates that the transmissible pathogenic agent in prion diseases is protein in nature, self-replicating, and requiring no nucleic acids (Prusiner 1982). Numerous experiments have demonstrated that the cellular prion protein (PrP^C) is essential for both prion replication and prion pathogenesis, and that a misfolded PrP aggregate form named PrP^{Sc} is the main (maybe only) essential component of the infectious prion agents, which replicates through PrP^{Sc}-templated conversion of PrP^C.

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PrP^C is a ubiquitously expressed, cell surface glycoprotein that is glycosylphosphatidylinositol (GPI)-anchored to the outer layer of the cell membrane and highly expressed in the nervous systems, lymphoid tissues, and skeletal muscles. It has also been implicated in several diseases (such as Alzheimer disease, muscle diseases, and cancer) and in many physiological processes (such as neuronal differentiation and neuroprotection, cell adhesion, T cell development and function, and stem cell differentiation) (reviewed by Biasini et al. 2012; Zomosa-Signoret et al. 2008). PrP is encoded by a single copy gene that has numerous polymorphic variants in both humans and animals; some of the polymorphisms are known to modulate prion susceptibility.

Multiple prion "strains" have been observed in humans and animals, characterized by distinctive clinical symptoms, incubation period, histopathology, and specific type and topology of PrP^{Sc} in a specific host species. The basis of prion strains is still not fully established (Collinge and Clarke 2007; Telling 2011), but it is believed that prion strain is encoded in the conformations of PrP^{Sc} (Prusiner 1991; Peretz et al. 2002; Legname et al. 2006), may contain multiple "quasi-species" (Collinge and Clarke 2007), and can undergo mutation and selection (Li et al. 2010). When a prion strain is transmitted to a different host species, the so-called prion transmission "species barrier" is often observed, which manifests as either undetectable infection or variable and prolonged incubation period (including less than 100% transmission) during first passage in contrast to the shortened and less variable incubation period in subsequent passages in the same species. Such barriers could exist even when a prion strain is passaged in animals of the same species but of different PrP genotype or of slightly different genetic background, so the general term "prion transmission barrier" will be used in this chapter.

Transgenic (Tg) mouse models have played many critical roles in prion research and this topic has been reviewed extensively (Baron 2002; Groschup and Buschmann 2008; Telling 2011; Wadsworth et al. 2010; Weissmann and Flechsig 2003). This chapter is not intended to provide an exhaustive review of all prion-related studies involving Tg mice. Instead, I summarize the types of Tg mice used, describe several Tg mouse models and the critical roles they have played in dissecting the factors influencing prion transmission barriers, with a special emphasis on the multifaceted impact of the primary PrP sequences of the prion agent and the host animal. The contributions of host PrP expression level and prion strain to the prion transmission barrier as well as other genes that may participate in prion pathogenesis will also be discussed.

11.2 Creation of Tg Mice

Tg mouse models have played pivotal roles in our understanding of prion diseases, such as establishing the central roles of cellular PrP in both prion replication and prion pathogenesis, modeling various sporadic, genetic and acquired human prion diseased, understanding the basis of prion strains and transmission barriers, uncovering the normal functions of cellular PrP, and evaluating other genes potentially

involved in prion pathogenesis. The Tg mouse models for prion studies are usually created by one of three methods: targeted PrP gene knockout via gene-targeting technology in embryonic stem cells, targeted PrP gene replacement (knock-in) in embryonic stem cells, and conventional transgenesis via chromosomal integration of a wild-type or mutated PrP gene from the same or different species after pronuclear injecting of a PrP transgene DNA construct into individual fertilized eggs (see Gama Sosa et al. 2010 for a general review on various Tg mouse techniques). The few reported PrP knock-in mouse models (Kitamoto et al. 2002; Moore et al. 2005; Jackson et al. 2009) have the unique advantage of ensuring nearly authentic level and tissue profile of the Tg PrP expression, but they will not allow variation of the level and profile of transgene PrP expression. The conventional Tg technology is more versatile for a number of reasons. It can generate from one transgene construct many unique Tg mouse lines with different level and tissue profile of transgene PrP expression because the chromosomal transgene integration site is relatively random and the copy number of the integrated transgene can vary hugely from one line to another. It also allows the use of specific promoters that confer tissue/cell specific or developmentally regulated transgene expression to more precisely control the location, timing, and amount as well as other regulations (such as response to a specific stimulus or chemical). As discussed in detail below, the PrP knockout mice and to a lesser extent, the PrP knock-in mice have played significant and unique roles in prion research, whereas most of the Tg mouse models for prion studies were established with the conventional transgenesis technique.

11.3 Influence of Donor (Prion) and Host PrP Primary Sequences

One of the most important contributions of Tg mouse models is demonstrating the requirement of PrP^C in prion replication and pathogenesis. PrP knockout mice had been generated by targeted gene knockout technology in several laboratories (Büeler et al. 1992; Manson et al. 1994a; Sakaguchi et al. 1995; Moore et al. 1995; Rossi et al. 2008), which revealed that PrP^C is not essential for viability and the PrP knockout mice with undisturbed doppel expression appear almost completely normal (Büeler et al. 1992, Manson et al. 1994a). Prion transmission experiments in these PrP knockout mice invariably failed to lead to infection (Büeler et al. 1993; Prusiner et al. 1993; Manson et al. 1994a; Sailer et al. 1994; Sakaguchi et al. 1995) and reintroduction of functional PrP expression via a transgene restored the susceptibility (Prusiner et al. 1993), proving unequivocally that PrP^C expression is required for prion replication. The evidence for the requirement of host PrP^C expression in prion pathogenesis mainly came from neurografting (Brandner et al. 1996) and conditional knockout experiments (Mallucci et al. 2007). When brain tissues from PrP-overexpressing mice were grafted to the brain of PrP knockout mice, after intracerebral prion inoculation, the prion pathology was found to be limited to the grafted PrP-expressing tissues; in contrast, there was little prion pathology in the

other brain regions despite the presence of significant amount of PrP^{Sc} (Brandner et al. 1996). In addition, when the neurografted mice were challenged by intraperitoneal prion inoculation, no pathology was detected in the grafted tissue, suggesting the requirement of extracerebral PrP^{C} expression for neuroinvasion of peripherally inoculated prions (Brandner et al. 1996). These results indicate convincingly that PrP^{C} expression is essential for prion pathogenesis. This conclusion is reinforced by more recent experiments (Mallucci et al. 2002, 2003, 2007) using the Cre–Lox conditional knockout system (reviewed by Feil 2007). A Tg mouse line (NFH-Cre/tg37) was created by breeding the tg37 line expressing a PrP transgene flanked by loxP sites with the NFH-Cre mice expressing the Cre DNA recombinase gene under the developmentally regulated NFH promoter, which initiates Cre expression in neurons 9–10 weeks after birth to knock out neuronal PrP transgene expression (Mallucci et al. 2002). When the tg37 mice were inoculated with the RML prion strain at 1 week of age, the animals started to display behavior deficits and early spongiosis in the brain at 9 weeks and reached terminal stage at 14 weeks. In similarly prion-inoculated NFH-Cre/tg37 mice, the animals displayed behavior deficits and early spongiosis in the brain at 9 weeks of age as expected, but soon after the spongiosis is reversed and behavior deficits disappeared (Mallucci et al. 2003, 2007). These pivotal experiments not only demonstrate the requirement for host neuronal PrP^{C} expression in prion pathogenesis but also prove in principle that prion pathology can be reversed at early stages through knocking out neuronal PrP expression, suggesting that reducing neuronal PrP expression is an effective therapeutic strategy for prion diseases.

Tg mouse models are also instrumental in establishing that the primary PrP sequences of the prion donor and recipient host animals are critical factors for the strength of prion transmission barrier. Earlier prion transmission studies were often carried out in nontransgenic wild-type rodents (mice, hamsters, and guinea pigs), where highly variable transmissions were often observed depending on the source of the prion inoculum. Wild-type mice are usually poor hosts to prions from humans and other species. In 1989, the Prusiner group reported the first Tg mice expressing hamster PrP, which were readily infected by hamster prions (Scott et al. 1989). In 1994, the Prusiner group showed that Tg mice expressing chimeric human–mouse PrP [Tg(MHu2M)] in the presence of endogenous mouse PrP are highly susceptible to three human CJD isolates (Telling et al. 1994). They also showed that Tg(HuPrP) mice expressing human PrP in the presence of endogenous mouse PrP were poorly susceptible to the CJD isolates (Telling et al. 1994), but ablation of the mouse PrP gene made the Tg(HuPrP) mice highly susceptible to CJD prions (Telling et al. 1995), indicating that replacement of mouse PrP with human PrP in Tg mice breaks the resistance to human prions and the endogenous mouse PrP^{C} interferes with the propagation of CJD prions. Subsequent creation and testing of Tg mice expressing PrP from other species (cattle, sheep/goat, cervids, pig) in many laboratories further confirmed that Tg expression of PrP from a certain species in mice in the absence of endogenous mouse PrP renders the mice susceptible to homologous prions (e.g., Buschmann et al. 2000; Vilotte et al. 2001; Browning et al. 2004; Kong et al. 2005), establishing the central role of PrP primary sequence in prion transmission barriers. However, there appear to be a few exceptions. For example, bank vole is highly susceptible to prions from humans

(Nonno et al. 2006) and many other species despite significant differences in their primary PrP sequences. It appears likely that bank vole PrP^{C} is inherently prone to adopting misfolded PrP^{Sc} conformations, which makes the homology with PrP^{Sc} less important. This notion is supported by the observation that Tg mice overexpressing wild-type bank vole PrP (TgBVPrP) develop spontaneous prion disease that is transmissible to both wild-type mice and Tg(BVPrP) (Watts et al. 2012).

Tg mice expressing polymorphic or modified PrPs showcased how even seemingly small changes in the primary PrP structure of the host animal can dramatically influence its susceptibility to specific prion strains. The PrP codon 129M/V polymorphism is common in human populations, and it is known to have a huge impact on the susceptibility to not only exogenous prion infections but also to the risk of developing sporadic CJD (reviewed by Kong et al. 2004). One example is variant CJD (vCJD) caused by BSE infection in humans. All the 200-plus clinical cases of vCJD detected so far are homozygous for Met at the PrP codon 129. This dramatic preference of classic BSE prion for the human PrP-129MM genotype is confirmed by transmission of BSE prion in Tg mice expressing human PrP-129M, human PrP-129V, or human PrP-129MV, although these Tg mouse studies also suggest that individuals with PrP-129VV or PrP-129MV genotypes may not be completely resistant to the BSE prion (Asante et al. 2002, 2006; Wadsworth et al. 2004). Tg mice carrying the single amino acid P101L mutation associated with the inheritable human Gerstmann–Sträussler–Scheinker syndrome (GSS) cases also altered the incubation times for various prion strains (Manson et al. 1999). In addition, the so-called heterozygous inhibition has been observed in Tg mouse models expressing two types of PrP^{C} (Hizume et al. 2009; Kobayashi et al. 2009).

Moreover, Tg mice expressing modified PrP revealed a structure region critical for cross-species prion transmission barrier. The β2–α2 loop region of PrP^{C} is less conserved among different species, which can be roughly classified into two groups: those with a rigid β2–α2 loop and those with a disordered β2–α2 loop. Using transgenic mouse models expressing PrP with rigid or disordered β2–α2 loop, Christina Sigurdson and her colleagues found that the transmission barrier is generally weaker when the transgenic host and the prion inoculum contain PrPs with the same type of β2–α2 loop and stronger when their β2–α2 loops do not match (Sigurdson et al. 2010).

11.4 Contribution of Host PrP Expression Level

The host PrP^{C} expression levels also affect prion transmission barrier: higher PrP^{C} levels usually correspond to lower transmission barriers, which is not unexpected given that host PrP^{C} is the substrate for PrP^{Sc} replication. The first experimental evidence for this principle came from the Prusiner lab, where the wild-type Syrian hamster PrP (SHaPrP) was expressed at various levels in the Tg(SHaPrP) mouse lines and the incubation period of a hamster prion strain was found to be inversely proportional to the level of SHaPrP in the mouse brain (Scott et al. 1989; Prusiner et al. 1990). Similar results were later obtained using prion strains from other

species and Tg mice expressing homologous PrP from those species. Westaway et al. (1991) found that Tg mice overexpressing the mouse PrP gene exhibit reduced incubation time when challenged with the Chandler scrapie isolate. Also, the hemizygous PrP knockout mice (*Prnp*$^{+/o}$) expressing half the amount of PrP of wild-type mice showed delayed onset of disease (Prusiner et al. 1993; Büeler et al. 1994; Manson et al. 1994b), whereas the Tga20 mice overexpressing the mouse PrP gene had a much shorter incubation time (Fischer et al. 1996). The increased susceptibility of Tg mice overexpressing PrP from a species is therefore used to shorten the incubation time of prions from the same species to save time and cost in laboratory studies. Moreover, Tg mice overexpressing PrP have also been utilized to detect weak cross-species prion transmissions. For example, the classical BSE strain led to detectable but poor infection in Tg mice overexpressing HuPrP (Scott et al. 1999; Asante et al. 2002, 2006; Wadsworth et al. 2004), but it failed to infect the knock-in mice that express HuPrP at wild-type mouse level (Bishop et al. 2006). We also found that both the classical BSE strain and the atypical H-type BSE strain resulted in poor but detectable infection in the Tg40h mice expressing HuPrP-129M at 2× wild-type level, but not in the Tg40 mice expressing HuPrP-129M at 1× wild-type level (Q Kong et al. unpublished data). The Prusiner group (Tremblay et al. 1998) reported the dramatic impact of host PrP-expressing levels on prion transmission using tetracycline-regulated Tg mice (reviewed by Stieger et al. 2009). The Tg mice normally express very high levels of mouse PrP and became ataxic only 50 days after inoculation with the RML prions, but suppression of PrP^{C} expression by >90% with oral doxycycline completely prevented clinical prion disease despite the presence of low levels of PrP^{Sc}. These findings prove that the transgenic PrP expression level has a dramatic impact on prion transmission barrier and this factor must be taken into account when Tg mouse models are used to evaluate prion transmission species barriers. Furthermore, reduction of host PrP expression levels in the brain appears to be a viable strategy to treat prion diseases as evidenced by RNA interference experiments in mice (Kong 2006; Pfeifer et al. 2006; White et al. 2008).

11.5 Effect of Prion Strain

The first transgenic evidence for the influence of prion strain on transmission barrier was reported by Peretz et al. (2002). They showed that transmission of the hamster scrapie strain Sc237 in the Tg(MH2M) mice expressing chimeric hamster/mouse PrP gene was poor and led to a new prion strain with changed conformational stability and disease phenotype; in contrast, transmission of the DY (drowsy) hamster prion strain in the same Tg(MH2M) mice had no barrier and the resulting prion maintained the characteristics of the original DY strain. More recently, Jean Manson's group reclassified several distinct human sporadic CJD isolates belonging to the six subtypes of sporadic CJD (sCJD) (MM1, MM2, MV1, MV2, VV1, and VV2) as described by Parchi et al. (1996) using their knock-in mouse models expressing HuPrP-129M, HuPrP-129V or both HuPrP-129M and HuPrP-129V (Bishop et al. 2010). They found that these six

sCJD subtypes actually represent four human prion strains that are different in transmissibility, incubation time, histological lesion profile, and types and deposition patterns of PrP^{Sc} (Bishop et al. 2010). For example, the sCJDMM2 subtype (the $M2^{CJD}$ strain) has zero to very poor transmission in all three knock-in mouse models, whereas the MM1/MV1 subtypes (the $M1^{CJD}$ strain) are at least moderately transmissible in all mouse models. Strain-dependent barriers for prion transmission are also observed with prion strains from mouse and cattle in Tg mouse models expressing respective PrPs.

11.6 Non-PrP Factors Involved in Prion Diseases

Many non-PrP proteins are suspected to play a role in prion pathogenesis. They either colocalize with PrP^{C}, participate in/are associated with Alzheimer's disease, are elevated during prion disease, or function in PrP-mediated signaling, PrP glycosylation, PrP^{C} processing or protein maintenance. Tg mice where the respective gene of the suspected protein is knocked out or overexpressed are effective tools to assess the involvement of such candidate genes. Tamgüney et al. (2008) presented a comprehensive study using this strategy to examine 20 such candidate genes, including amyloid precursor protein (APP), amyloid-beta precursor-like protein (APLP2), human ApoE (ε3 and ε4 alleles), interleukin-10, interleukin-1 receptor type I (IL-1R1), tumor necrosis factor (TNF-α), transforming growth factor-β1 (TGF-β1), chemokine (C-C motif) receptor 2 (CCR2), chemokine (C-C motif) receptor 5 (CCR5), methionine sulfoxide reductase A and B (MsrA and MsrB), human SOD-1, human Hsp70, mannosdie-b1,4-N-acetylglucosaminyltransferase III (Mgat3), caveolin-1, Fyn kinase, receptor protein tyrosine phosphatase-α (RPTPα), doppel (Dpl), and CD9. However, most genes examined appear to have no significant impact and only ablation of APP or IL-1R1 and overexpression of human SOD-1 were found to slightly increase the incubation time (13–19 %). Other PrP-interacting proteins have also been studied. Plasminogen was reported to bind and cleave PrP^{C} (Kornblatt et al. 2003). However, knocking out the mouse plasminogen gene has no major effect on the survival of scrapie-infected mice (Salmona et al. 2005). The 36-kDa/67-kDa laminin receptor (LRP/LR) was implicated as the cell surface receptor for both PrP^{C} and PrP^{Sc} (Gauczynski et al. 2001, 2006; Hundt et al. 2001) and its expression is required for PrP^{Sc} propagation in scrapie-infected neuronal cells (Leucht et al. 2003). Expression of a mutant LRP interfered with PrP^{Sc} propagation in cultured neuronal cells (Vana and Weiss 2006) and significantly prolonged the incubation time and reduced PrP^{Sc} accumulation in Tg mice (Pflanz et al. 2009), suggesting that LRP facilitates PrP^{Sc} replication in vivo.

Factors affecting the clearance of PrP^{Sc} also influence prion pathogenesis. Knocking out Mfge8 (milk fat globule epidermal growth factor 8, which mediates engulfment of apoptotic bodies by phagocytes) was found to result in accelerated prion disease, excessive PrP^{Sc} accumulation and increased prion titers (Kranich et al. 2010). Safar et al. (2000) also reported a linear correlation between the protease-sensitive PrP^{Sc} fraction and the length of incubation time and suggested

that different incubation times of various prion strains may arise predominantly from distinct rates of PrP^{Sc} clearance. It can be inferred that a species with a powerful PrP^{Sc} clearance mechanism will be more resistant to prion infection. On the other hand, the effectiveness of the PrP^{Sc} clearance mechanism of an animal could be prion strain dependent and influenced by the age and other physiological or pathological factors. This consideration could partially explain the age-dependent nature of the onset of prion diseases.

More recently, sustained translational repression by phosphorylated translation initiation factor eIF2α is also implicated in prion pathogenesis, because in prion-inoculated mice, transgenic overexpression of an eIF2α-specific phosphatase significantly prolonged survival, whereas treatment with an inhibitor of dephosphorylation of phosphorylated eIF2α shortened survival (Moreno et al. 2012).

11.7 Conclusions

The numerous transgenic mouse models have been essential to our current knowledge on the nature and replication of the prion agents and prion strains, other factors/genes involved in prion pathogenesis, as well as the basis of prion transmission barrier. Development and application of novel transgenic mouse models, complemented by cell culture models of prion replication and cell-free protein misfolding cyclic amplification (PMCA) and other related in vitro PrP^{Sc} replication and detection techniques, will lead to a broader and deeper understanding on all aspects of prion diseases and prion agents, including the development of effective prevention and therapeutics for prions.

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Chapter 12
Alternative Models of Prion Diseases

Pedro Fernandez-Funez, Edward Málaga-Trillo, and Diego E. Rincon-Limas

Abstract Prion diseases encompass a diverse group of lethal neurodegenerative disorders associated with the accumulation of misfolded conformers of the prion protein (PrP) in brain neurons. Modeling these diseases in mice and hamsters has led to major advances in our understanding of prion transmission and pathogenesis. However, laboratory rodents are also expensive, time-consuming, and limiting for systematic studies. Genetically tractable animal models, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the zebrafish *Danio rerio*, have recently made significant contributions to PrP pathogenesis. Here, we discuss recent applications of these three nonmammalian models to various relevant areas, including PrP processing, trafficking, misfolding, neurotoxicity, as well as unraveling its elusive endogenous function. Now that these alternative models have staked a claim on PrP biology, we anticipate that they will expand their range of applications and contributions in the next few years, including the generation of nonmammalian models of prion transmissibility.

Keywords Animal modeling • Brain neurons • Prion protein • Prion diseases • PrP pathogenesis

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Abbreviations

CFP	Cyan fluorescent protein
GFP	Green fluorescent protein
GSS	Gerstmann–Sträussler–Scheinker
Hsp70	Heat shock protein 70
PrP	Prion protein
PrP^{C}	Cellular PrP
PrP^{Sc}	Scrapie PrP
SCA	Spinocerebellar ataxia
WT	Wild type

12.1 The Prion Protein in Disease

Prion diseases are incurable neurodegenerative conditions affecting humans and other mammals that lead to motor dysfunction, dementia, and death (Aguzzi et al. 2008; Colby and Prusiner 2011). Genetic and biochemical evidence point to the prion protein (PrP) as the causative agent in the pathogenesis of sporadic, inherited, and transmitted prion diseases. PrP is a membrane-anchored glycoprotein of poorly understood function that is highly enriched in the brain. The conformational change from the normal "cellular" prion protein (PrP^{C}) into the "scrapie" (PrP^{Sc}) isoform has been proposed as the main molecular event leading to the formation of transmissible prions (Prusiner 1998). This is a well-documented process associated with dramatic changes in the biochemical properties of PrP as well as in its tertiary and quaternary structures (Soto and Satani 2010). Unfortunately, the precise molecular and cellular mechanisms mediating PrP conformational conversion remain largely unknown.

Similarly enigmatic is our current understanding of the mechanisms responsible for neurodegeneration in prion diseases. Several lines of evidence argue that the transmissible and neurotoxic agents are distinct PrP entities, most likely different conformational or aggregation states (Sandberg et al. 2011; Lasmezas et al. 1997; Brandner et al. 1996). A number of atypical forms of PrP have been proposed as neurotoxic species, including transmembrane (Ctm) PrP, cytosolic (cyt) PrP, and misfolded intermediaries of PrP conversion such as PrP* and PrP^{L} (Harris and True 2006; Hill and Collinge 2003). However, the nature of the neurotoxic species and its mechanisms of action are still unknown. Alternative model organisms can play an important role in answering some of the most fundamental questions in PrP biology, including PrP function, processing, trafficking, and folding, due to their fast and flexible manipulation.

12.2 Investigating Biological Processes in Nonmammalian Models

Rodents account for a large majority of the laboratory animals used in biomedical research owing to their genetic, physiological, and anatomical similarities to humans. Chicks, frogs, fish, and a number of invertebrates also play essential roles in laboratory research. The unique characteristics of each animal provide singular advantages for specific areas of research. For instance, chick embryos are ideal for grafting experiments, while frog and zebrafish embryos are amenable to gene expression manipulation by direct injection. Zebrafish and nematodes are uniquely suited for direct observation of developmental events in their translucent embryos. Finally, nematodes and fruit flies are ideal models for genetic studies because of their short life cycles and their expansive and dynamic toolbox.

Mice and hamsters have played pivotal roles in elucidating the proteinaceous nature of the infectious particle, isolating multiple strains, and describing the so-called species barrier (Groschup and Buschmann 2008). However, small, nonmammalian models like worms, flies, and zebrafish are starting to gain ground in the study of PrP biology thanks to their genetic amenability, low cost, and compatibility with large-scale screens. Additionally, worms and flies are particularly well suited to study PrP pathobiology because they lack endogenous PrP and its close homologues Doppel and Shadoo, providing a "clean" background to study the consequence of expressing PrP. This chapter discusses the prowess and versatility of these organisms toward unraveling the enigmatic function of PrP and uncovering the molecular bases of PrP-related pathologies.

12.3 Modeling Prion Diseases in C. elegans

12.3.1 Big Genetics in a Small Package

The roundworm *Caenorhabditis elegans*, the brainchild of Sidney Brenner, is one of the best-suited organisms for genetic studies and has been a laboratory workhorse for almost 40 years (Brenner 1974; Brenner 2009). *C. elegans* has a life cycle of 3.5 days and two sexes, hermaphrodites and males, but a single hermaphrodite can produce ~300 offspring by self-fertilization, a terrific advantage for genetics research. Its body is transparent, so it is easy to track cells and follow biological processes in a living animal (Sulston et al. 1983). This fantastic feature led to the tracing of the cell lineage of its 959 somatic cells and the wiring of the 302 neurons that constitute

its nervous system. The function of many of these neurons has been defined by laser ablation, which allows behavioral studies in the context of altered neuronal networks (Bargmann et al. 1993). Finally, *C. elegans* is amenable to chemical screens, unbiased forward and reverse genetic screens, and is particularly susceptible to gene inactivation by RNAi (Fire et al. 1998).

Genes and molecular pathways involved in development, apoptosis, aging, and neuronal function are highly conserved between *C. elegans* and humans (Kaletta and Hengartner 2006). Accordingly, several human neurodegenerative diseases have been modeled in worms, including Huntington's, Alzheimer's, and Parkinson's disease, to name a few (Dimitriadi and Hart 2010). Expression of proteins involved in human proteinopathies leads to phenotypes consistent with neurodegeneration in the worm nervous system, including protein aggregation, neuronal loss, and behavioral changes. These phenotypes were later exploited to identify novel genes involved in neurodegeneration and to screen for potential therapeutic compounds (Markaki and Tavernarakis 2010).

12.3.2 PrP Toxicity in Worms

The first model of PrP-dependent pathology in *C. elegans* expressed cytosolic mouse PrP fused to cyan fluorescent protein (CFP) (Park and Li 2008). The authors were investigating whether PrP lacking the N-terminal signal sequence and the GPI anchor site (PrP[23–231]-CFP) would be toxic in the body wall muscle cells (Table 12.1). As expected, PrP[23–231]-CFP accumulated in the cytoplasm and induced muscle pathology evidenced by disruption of filamentous (F)-actin, as well as developmental delay, severe mobility defects, and shortened "dumpy" body (Fig. 12.1a–c). Interestingly, worms expressing PrP[23–231]-CFP with a protective mutation (Q167R) did not display the aberrant phenotypes of wild-type (WT) PrP, whereas the P102L mutation linked to Gerstmann–Sträussler–Scheinker syndrome (GSS, an inherited form of prion disease) showed enhanced phenotypes. Notably, this toxicity correlated with the formation of conformers that were insoluble in sarkosyl, but not protease resistant (Park and Li 2008).

A second model of prionopathy in worms used full-length human PrP targeted to mechanosensory neurons (Bizat et al. 2010). While the expression of WT human PrP had no effect on touch response assays, the expression of a mutant with eight extra copies of the octarepeat (PG13) induced progressive loss of response to touch. This phenotype presented with abnormal PrP distribution in cytoplasmic and axonal bodies, and changes in the biochemical properties of PrP, including sarkosyl insolubility and partial resistance to protease digestion. From here, the authors fully exploited the versatility of the worm: they rescued the PrP-PG13 phenotypes with a known protective drug (quinacrine), demonstrated the role of a known pathway involved in neurotoxicity (Fyn kinase), and tested another potential modifier with neuroprotective activity in other diseases (Sirtuin). This approach confirmed the conservation of known pathogenic mechanisms and introduced a new

Table 12.1 Alternative models in prion protein biology

Organism	References	PrP transgene or morpholino	Reported phenotype
Worm	Park and Li (2008)	Mouse PrP[23–231]-CFP	Cyt PrP accumulation and insolubility, mobility defects, and muscle pathology
		Mouse PrP[23–231]-Q167R-CFP	Q167R no phenotypes
		Mouse PrP[23–231]-P102L-CFP	P102L enhances PrP phenotypes
	Bizat et al. (2010)	Human PrP	No phenotypes
		Human PrP (PG13)	Neuronal dysfunction, abnormal distribution in axons, insolubility, and limited PK resistance
	Park et al. (2011)	Mouse PrP (3F4)	Dose-dependent mobility defects, short lifespan, and limited PK resistance
Fly	Raeber et al. (1995)	Heat shock hamster PrP	No phenotypes
	Deleault et al. (2003)	UAS-mouse PrP and UAS-mouse PrP (PG14)	No phenotypes
	Gavin et al. (2006)	UAS-mouse PrP (P101L)	Locomotor dysfunction, brain degeneration, and 15B3+
	Choi et al. (2010)	UAS-mouse PrP (3F4) and UAS-mouse PrP(3F4, P101L)	P101L: Locomotor dysfunction, vacuolar degeneration, and abnormal synaptic architecture
	Fernandez-Funez et al. (2009)	UAS-hamster PrP	Locomotor dysfunction, vacuolar degeneration, insolubility, guanidinium resistance, and 15B3+
	Fernandez-Funez et al. (2010)	UAS-mouse PrP	Locomotor dysfunction, no brain degeneration, insolubility, and 15B3+
		UAS-rabbit PrP	No phenotypes
Zebrafish	Malaga-Trillo et al. (2009)	PrP-1 morpholino	Early embryonic lethality, gastrulation defects, and loss of cellular adhesion
	Malaga-Trillo et al. (2011)	PrP-2 morpholino	Larval lethality, eye and brain defects
	Nourizadeh-Lillabadi et al. (2010)	PrP-2 morpholino	Embryonic lethality and brain defects
	Fleisch et al. (2011)	PrP-2 KO (zinc finger nucleases)	No phenotypes during embryogenesis

protective pathway through mutations in *Sir-2* and pharmacological inhibition of Sirtuins with resveratrol. To demonstrate the relevance of these findings, the authors then showed that resveratrol rescued PrP toxicity in mouse primary neurons (Bizat et al. 2010).

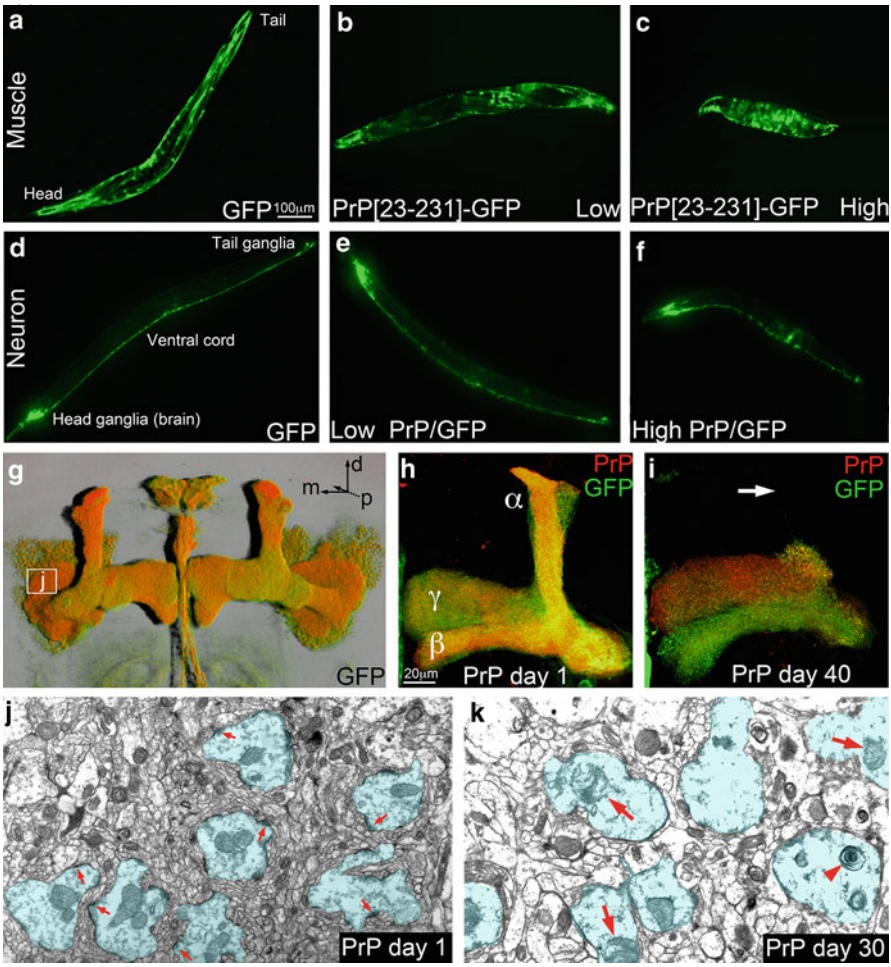

Fig. 12.1 *Deleterious consequences of PrP expression in invertebrates* **a**–**c**, *C. elegans* expressing GFP constructs in the muscle under the control of the *unc54* promoter. (**a**) Expression of GFP alone or (**b**) low levels of mouse PrP[23–231]-GFP results in normal animals. (**c**) High levels of PrP[23–231]-GFP results in "dumpy" animals with abnormal mobility. **d–f**, GFP expression in the nervous system of *C. elegans* under the control of the pan-neural promoter *ric-19*. (**d**) Expression of GFP alone or (**e**) in combination with low levels of full-length mouse PrP produces normal animals. (**f**)High levels of PrP in all the neurons results in "dumpy" animals with shorter lifespan. Images **a**–**f** were kindly provided by Dr. L. Li (Northwestern University, IL). (**g–i)** Neurotoxicity of hamster PrP in the *Drosophila* mushroom bodies. (**g**) Three-dimensional reconstruction of the mushroom bodies, the memory, and learning centers of the fly. The orthogonal axis indicates the dorsal (d), medial (m), and posterior (p) axes of the fly brain. The *box* indicates the region magnified in panel (**j**). (**h**) Flies expressing PrP develop normal mushroom body lobes, but (**i**) flies aged for 40 days exhibit degeneration of α-lobes (*arrow*). (**j** and **k)** Transmission electron micrographs of the dendritic fields of the mushroom bodies (calix, *box* in **g**). (**j**) Young flies expressing PrP develop normal synapses. The synaptic boutons of projections neurons (*colored in light blue*) are organized in glomeruli that form abundant synapses (*arrows*) with surrounding dendrites. The synaptic boutons also contain healthy mitochondria. (**k**) In contrast, flies aged for 30 days show vacuolized glomeruli that lack synapses and accumulate aberrant mitochondria (*arrows*) and multilaminate bodies (*arrowhead*)

More recently, Park and Li generated transgenic worms expressing full-length mouse PrP pan-neuronally (Park et al. 2011). The authors found that expression of WT PrP at low levels caused no detectable harm (Fig. 12.1d, e). However, high levels of PrP were associated with abnormal body shape, defective mobility, shortened lifespan, and increased protease resistance (Fig. 12.1f). The authors also confirmed that low levels of PrP inhibited proapoptotic BAX (Bcl2-associated protein X) and rescued BAX-induced dopaminergic cell death. This is the first time that PrP is shown to have anti-BAX activity in living animals, supporting other reports in mammalian cell culture systems (Roucou et al. 2004).

Taken together, these studies demonstrate that *C. elegans* can be exploited as an alternative model to study the pathobiology of PrP, as it mimics some of the biochemical and pathological features of sporadic and genetic prionopathies. Now that the door has been opened for modeling PrP neurotoxicity in this tiny worm, more applications and discoveries should be expected in the near future.

12.4 Modeling Prion Diseases in Drosophila

12.4.1 Modeling Neurodegeneration in Flies

The biology and genetics of *Drosophila melanogaster* have been studied for over a century. This little fruit fly provides an all-inclusive experimental system for investigating a wide range of biological problems, from genome architecture to complex behaviors, including aggression, addiction, and circadian rhythms (Bellen et al. 2010). The evolutionary conservation of the basic neuronal function from flies to humans, together with a small but well-organized brain, has established *Drosophila* as a remarkable model to study neurodegenerative diseases (Pandey and Nichols 2011; Rincon-Limas et al. 2012). The fruit fly genetic toolbox includes highly curated genome and transcriptome, easy mutagenesis and transgenesis, and vast collections of mutants (Graveley et al. 2011; Matthews et al. 2005; Venken and Bellen 2007; Pfeiffer et al. 2010). Among these tools, the UAS/GAL4 bipartite system is ideal for studying neurotoxic proteins because transgenes are silent until they are combined with GAL4, producing expression in highly specific spatiotemporal patterns.

Between 1998 and 2000, four independent laboratories generated suitable models of human neurodegenerative diseases characterized by protein aggregation and neuronal loss. Among these, three modeled diseases associated with expansions of polyglutamine tracks, Huntington's disease and Spinocerebellar ataxia (SCA) types 1 and 3 (Jackson et al. 1998; Warrick et al. 1998; Fernandez-Funez et al. 2000). The fourth laboratory reported a model of mutant Tau-dependent neuropathology linked to frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Feany and Bender 2000). These early models demonstrated that *Drosophila* was a superb system to model proteinopathies, which led to the

subsequent development of models of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and many more (Rincon-Limas et al. 2012). These models have produced major advances toward the identification of new molecular mechanisms mediating neurodegenerative disorders. Some of the most significant findings include the regulation of amyloid aggregation by molecular chaperones, the role of protein phosphorylation in amyloidosis and neurotoxicity, 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). *Drosophila* has also played a key role in the identification of novel pharmacological suppressors of neurodegeneration (Steffan et al. 2001; Auluck and Bonini 2002), placing *Drosophila* at the forefront of research on neurodegenerative diseases.

12.4.2 If at First You Don't Succeed…

In 1995, S. Prusiner and cols. generated the first transgenic flies expressing WT PrP from Syrian golden hamster under the control of the heat shock promoter (Table 12.1). Surprisingly, these flies displayed no PrP-associated pathology (Raeber et al. 1995). Recognizing that the promotor may have precluded the sustained accumulation of PrP, a few years later S. Supattapone and cols. created new transgenic flies expressing WT and mutant (PG14) mouse PrP under the control of UAS (Deleault et al. 2003). However, pan-neural expression of PrP-PG14 led to protein accumulation in the eyes, but not in the brain. This result suggested that *Drosophila* brain neurons possessed mechanisms that prevented accumulation of mutant PrP, making the fruit fly inadequate for studying prionopathies.

12.4.3 Try, Try Again: Neurotoxicity of Mutant PrP

More recently, S. Supattapone's group created the first transgenic flies displaying PrP-related pathology by expressing the GSS-linked mutation P102L (P101L in mouse PrP) (Gavin et al. 2006). Expression of mouse PrP-P101L in flies induced brain degeneration, shortened lifespan, progressive locomotor deficits, and accumulation of misfolded PrP species recognized by the 15B3 conformational antibody. However, aged flies expressing PrP-P101L did not accumulate detergent-insoluble nor protease-resistant PrP conformers. More recently, another group expressing PrP-P101L in flies described similar observations (Choi et al. 2010). These results indicate that flies can reproduce relevant features of PrP-P101L-associated neuropathology, including PrP misfolding and progressive neuronal dysfunction and neurodegeneration.

12.4.4 Modeling Sporadic Prion Diseases: Neurotoxicity of WT PrP

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 WT PrP to transition from its native tertiary structure into pathogenic conformers. This 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). Thus, we hypothesized that it should be possible to model sporadic PrP neurotoxicity in flies by choosing the appropriate species of PrP. We decided against using human PrP to avoid the risk of creating airborne prions potentially transmissible to humans. Instead, we selected hamster PrP (also Prusiner's choice) because these animals exhibit an aggressive disease course and high PrP^{Sc} titer, suggesting that hamster PrP is highly prone to misfold. As predicted, flies expressing WT PrP from hamster in brain neurons displayed progressive locomotor dysfunction and spongiform vacuolar degeneration (Fernandez-Funez et al. 2009) (Fig. 12.1g–k). We also found that the biochemical and structural properties of WT PrP changed over time, including sarkosyl-insolubility, resistance to denaturing agents, and immunoreactivity to 15B3. However, flies did not accumulate the 19-kDa proteinase K-resistant fragment of PrP^{Sc}, indicating that WT PrP can induce progressive neuronal dysfunction and spongiform degeneration in the absence of prototypical PrP^{Sc}. This finding is consistent with the neurotoxic isoforms PrP^{L} or PrP* described in mammalian models (Harris and True 2006; Hill and Collinge 2003).

A recent paper revealed other deleterious effects of WT PrP (Park et al. 2011). Whereas PrP from mouse had no effect in the *Drosophila* eye, it enhanced the eye degeneration induced by mutant Ataxin-3, which is responsible for SCA3. Furthermore, PrP increased the susceptibility to exogenous toxins, including the reducing agent DTT (dithiothreitol) and the oxidative stressors H_2O_2 and paraquat. Together with the observations form *C. elegans*, these results suggest that, whereas low levels of PrP may be neuroprotective, high levels perturb cellular homeostasis and predispose to cell death.

We next investigated the ability of the molecular chaperone Hsp70 (Heat shock protein 70) to regulate PrP misfolding and neurotoxicity. Hsp70 had previously demonstrated a potent protective activity in *Drosophila* models of intracellular amyloids (α-Synuclein and Ataxin-3) (Warrick et al. 1999; Auluck et al. 2002). Interestingly, flies coexpressing human Hsp70 and PrP accumulated lower levels of misfolded PrP and displayed improved locomotor activity (Fernandez-Funez et al. 2009; Auluck et al. 2002). Moreover, Hsp70 physically interacted with PrP in the lipid rafts, a highly specialized domain of the plasma membrane. 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). These results uncovered the potential therapeutic role of Hsp70 and other molecular chaperones in prionopathies.

12.4.5 PrP Conformational Dynamics: Cues from Resistant Species

To further understand how the primary amino acid sequence of PrP determines its structural dynamics, we compared flies expressing WT PrP from mammals susceptible (hamster and mouse) and resistant (rabbit) to prion diseases (Fernandez-Funez et al. 2010). Our studies confirmed that rabbit PrP did not convert into pathogenic conformations nor induced neurotoxicity. Mouse PrP produced mixed results, inducing early locomotor dysfunction, but neither spongiform degeneration nor PrP aggregation, two characteristics of hamster PrP. Thus, three highly conserved PrP sequences exhibited prominent differences in their conformational dynamics in transgenic flies. A recent study of the crystal structure of rabbit PrP identified a hydrophobic staple that links loop 3 to helix 2 and increases the stability of its globular domain (Khan et al. 2010). Interestingly, the residue responsible for this feature in rabbit PrP (S174) is not conserved in dogs and horses (N174), two other mammals resistant to prions (Fernandez-Funez et al. 2011). Thus, other mechanisms must contribute to the stability of the globular domain of dog and horse PrP. We anticipate that further function/structure analyses in flies will help better understand the rules governing PrP misfolding and pathogenesis.

12.5 Researching PrP Function in Zebrafish

12.5.1 Zebrafish as a Manipulable Vertebrate Model

The low cost, small size, and rapid ex utero development makes the zebrafish *Danio rerio* an excellent model for studying vertebrate development. Techniques for transgenesis, large-scale genome mutagenesis, protein overexpression or knockdown, and in vivo cell imaging allow to easily model human disease in zebrafish (Ingham 2009). In addition, zebrafish larvae display learning, memory, sleep, and behavioral phenotypes that are easily quantifiable (Guo 2004). Remarkably, zebrafish can grow in 96-well plate format, is DMSO tolerant, and can easily absorb chemicals through the skin, creating an excellent platform to assess compound action in a whole organism (Rinkwitz et al. 2011).

12.5.2 PrP-1 Is Required for Cell Adhesion During Gastrulation

The genome of zebrafish contains two orthologues of mammalian PrP, denoted as PrP-1 and PrP-2, which are twice as long as PrP, but encode the same domains as their mammalian counterpart (Rivera-Milla et al. 2006). Both PrP-1 and PrP-2

produce mature proteins that are glycosylated and GPI anchored. Two additional short PrP-like genes have been also reported, possibly Doppel homologues (Rivera-Milla et al. 2006; Cotto et al. 2005; Suzuki et al. 2002). PrP-1 is highly and ubiquitously expressed in the blastula and gastrula and continues to be expressed in later stages of development, although it accumulates at lower levels than PrP-2 in the CNS. PrP-1 morpholino knockdown resulted in developmental arrest with prominent defects in gastrulation (Malaga-Trillo et al. 2009) (Table 12.1). Interestingly, coinjection of zebrafish PrP-2 or mouse PrP partially rescued this phenotype, supporting the functional conservation between piscine and mammalian PrP. Upon further analysis, the PrP-1 morpholino embryos exhibited abnormal cellular integrity. This observation led to the discovery that PrP-1 modulates the stability of E-cadherin/β-catenin adhesion complexes at the plasma membrane, possibly by affecting the levels and localization of β-catenin as well as the levels and phosphorylation state of Src-related kinases (Malaga-Trillo et al. 2010).

12.5.3 PrP-2 Is Required for Brain and Eye Development

PrP-2 accumulates specifically in embryonic neuronal clusters in the zebrafish embryo and is highly expressed in the adult brain. This expression pattern resembles more closely the distribution of mammalian PrP. PrP-2 morpholino knockdown resulted in lethality and induced severe impairment of eye and brain development (Malaga-Trillo et al. 2009), including abnormalities in cranial motor neurons (Fig. 12.2a, b) (Malaga-Trillo et al. 2011). An independent PrP-2 morpholino approach also reported lethality associated with apoptosis in the trigeminal ganglion, eye, olfactory placode, and in the midbrain–hindbrain area, supporting the critical function of PrP-2 in the development of the zebrafish nervous system (Nourizadeh-Lillabadi et al. 2010). On the other hand, T. Allison and coworkers reported at the Prion 2011 meeting that inheritable mutations in PrP-2 induced by zinc-finger nucleases (4 bp deletion) caused minor behavioral abnormalities, but no obvious effects on viability or embryonic development (Fleisch et al. 2011). While this result hints at differences between PrP-2 inactivation methodologies, a more careful assessment of these results will be possible once the full data are published.

12.5.4 Insight into Mammalian PrP Function

The zebrafish studies suggest that PrP-1 exerts a complex regulatory function over pathways that control cell–cell communication (Malaga-Trillo and Sempou 2009). This is in agreement with previously reported roles of mammalian PrP in cell adhesion (Mange et al. 2002), intracellular signaling (Mouillet-Richard

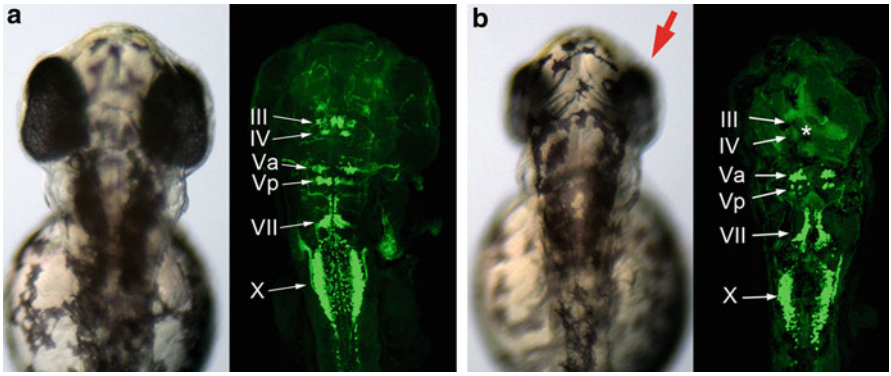

Fig. 12.2 *Neurodevelopmental roles of zebrafish PrP-2* **a** and **b**, Dorsal views of 2-day-old transgenic zebrafish larvae expressing GFP in cranial motor neurons. (**a**) Control embryos develop normal clusters of neurons (*white arrows*). *Roman numbers* identify individual cranial nerve ganglia; Va and Vp denote anterior and posterior clusters of the V cranial neurons, respectively. (**b**) Embryos depleted for *PrP-2* display a reduction in the number and size of clusters, poor axonal outgrowth, and smaller eyes

et al. 2000), and NCAM-mediated Fyn signaling leading to neurite outgrowth (Santuccione et al. 2005). However, these results should be taken with some caution because these studies were performed in cell culture and *Prnp*$^{-/-}$ mice present no obvious neurodevelopmental problems. Since some of the molecules affected by PrP-1 knockdown (E-cadherin, β-catenin, and Scr-kinases) also play important roles in neuronal function, further analyses may help clarify whether PrP-2 or mammalian PrP contribute to neuronal function and survival.

Intriguingly, while the phenotypes of PrP-1 and PrP-2 knockdown zebrafish are reminiscent of some of the neurodevelopmental and cellular defects observed for mammalian PrP, the severity of the phenotypes differs markedly between fish and mice. The absence of strong developmental phenotypes in *Prnp*$^{-/-}$ mice could be explained by selection of mutant embryonic stem cells, functional redundancy of Shadoo and Doppel, genetic compensation, or developmental plasticity (Malaga-Trillo et al. 2010; Steele et al. 2007). However, it is also conceivable that fish and mammalian PrP may have acquired novel, divergent functions during evolution. These questions underscore the importance of using a wide range of model organisms to study PrP biology.

12.6 Future Contributions

12.6.1 Novel Discoveries in PrP Pathobiology

The main advantage of worms, flies, and zebrafish is their ability to efficiently identify new genes and pathways involved in specific biological processes through modifier

screens. Since PrP misexpression induces easy to score phenotypes in *C. elegans*, it should be easy to screen for genes that either enhance or suppress those phenotypes. This could be an ideal system for identifying new cellular mechanisms involved in PrP neurotoxicity. In contrast, the highly specific phenotypes induced by PrP in flies may be inadequate for large-scale genetic screens. However, the use of automated, computerized equipment to measure quantitative phenotypes such as locomotor dysfunction may overcome this limitation. In addition, the genetic versatility of zebrafish may allow exploring the interaction of PrP with signaling pathways and the deleterious consequences of misexpressing mammalian PrP (neurotoxicity and aggregation). It will be also interesting to remove the two PrP genes in zebrafish. If they are partially redundant, the double mutant should result in stronger phenotypes, revealing new functions for PrP in vertebrate development. Finally the generation of a conditional PrP-1 mutant can contribute to determine its role in postembryonic development. Once these assays are ready, multiple strategies for testing genetic interactions will be available, including gene misexpression and genome-wide collections of RNAi constructs. Although less efficient than in vitro assays, drug screens in worms, flies, and zebrafish have the advantage of working with the intact organism, thus providing critical information about drug bioavailability, pharmacodynamics, and effectiveness in the living brain (Rincon-Limas et al. 2012).

12.6.2 The Missing Piece: Prion Transmission

The next frontier for nonmammalian models is: can they propagate prions? A naïve interpretation of the protein-only hypothesis suggests that it should be possible to promote de novo formation of PrP^{Sc} and/or propagation of PrP^{Sc} in worms, flies, and zebrafish. However, none of these models has reported the accumulation of PrP^{Sc} so far, suggesting that something may be missing in their cellular environment, particularly in invertebrates. Some authors have argued that PrP cannot be converted in invertebrates due their short lifespan, lack of conversion factors, and/or presence of potential inhibitors (Deleault et al. 2003; Abid et al. 2010). Several simple cofactors have been recently identified as specific accelerators of PrP conversion, including polyanions, RNA, and lipids (Abid et al. 2010; Supattapone 2004). These cofactors should allow the formation of PrP^{Sc} in invertebrates under the "right" conditions. Unfortunately, we do not know what these ideal conditions for in vivo PrP conversion are, although they may include a combination of physical (long incubation time and high temperature) and physiological (toxins, cellular stressors, and inflammation) factors. These are complex but testable conditions in model organisms and their identification would be a significant step forward in understanding prion formation and transmission. Generating worm, fly, or zebrafish models of prion transmission will allow the genetic dissection of the mechanisms involved in the formation of the PrP^{Sc}. This will create avenues for new discoveries in prion research and provide new opportunities for therapeutic interventions.

12.7 Concluding Remarks

Worms, flies, and zebrafish bring to the field of prion protein biology the ability to rapidly test hypotheses, carry out high-throughput genetic screens, perform sophisticated experiments, image living animals in real time, and much more. The studies described here lay the foundation for further understanding PrP function and its role in neurotoxicity. We anticipate that in the next decade worms, flies, and zebrafish will play key roles in elucidating PrP function, processing, misfolding, and pathogenesis only through this detailed knowledge we will be able to identify new molecular targets for the treatment and prevention of prion diseases.

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Chapter 13
Diagnosis of Prion Disease: Conventional Approaches

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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 in the cerebrospinal fluid (CSF) and hyperintense signal changes in the basal ganglia, thalamus and cortical areas on magnetic resonance imaging (MRI). These three tests became part of the clinical diagnostic criteria for probable CJD. 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 have to be considered in younger patients.

Keywords 14-3-3 proteins • Cerebrospinal fluid • Diagnosis • Diagnostic criteria • EEG • Molecular disease subtype • MRI • PSWCs

13.1 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 addition, sporadic

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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 diagnosis.

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 30 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, there is a substantial progress in developing other specialized investigations, including useful surrogate biomarkers in the cerebrospinal fluid and brain imaging, and clinical diagnostic criteria have been amended (Collins et al. 2006; Zerr et al. 2000, 2009).

13.2 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 patters in human fluids, the CSF analysis in dementia disorders became 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.

13.2.1 Routine

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

Table 13.1 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 oligoclonal IgG (%)
Sporadic CJD	3 (1.0)	44 (10.0)	5 (1.1)	8 (4.4)
Genetic CJD	3 (13.0)	1 (3.1)	0 (0)	0 (0)
FFI	2 (66.7)	1 (12.5)	0 (0)	1 (20.0)

IgG bands or raised cell count is an extremely rare finding (see Table 13.1). 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/μl were found in three out of 298 patients with sporadic CJD, in two with cell counts of 7 cells/μl and in one of 20 cells/μl. Total protein concentrations of >0.9 g/l were found in five of 438 patients with sporadic CJD, but none had a concentration of >1 g/l. CSF oligoclonal IgG was detected in eight out of 182 sporadic CJD patients. Among patients with other TSEs, six had elevated cell counts ranging from 6 to 14 cells/μl, 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.

As a rule, inflammatory CSF findings exclude the diagnosis of a human prion disease.

13.2.2 14-3-3

14-3-3 proteins were initially described as abundant, acidic brain proteins and their name is derived from the combination of its fraction number on DEAE-cellulose chromatography and migration position in the subsequent starch gel electrophoresis. 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. The large number of studies proved that in the appropriate clinical circumstances a positive 14-3-3 is highly sensitive and specific for sCJD diagnosis. 14-3-3 detection correlated with clinical diagnosis in 85–94% in sporadic CJD (Table 13.2). The sensitivity of 14-3-3 varies among TSE subtypes (Table 13.3). Patients with iatrogenic and gCJD have elevated 14-3-3 levels in 75% and 78% of the cases, respectively. In variant CJD (vCJD), only 40% patients were positive, while GSS and FFI patients were almost always negative in this test.

Table 13.2 Reported sensitivities and specificities of 14-3-3 in the diagnosis of sporadic CJD

Study	Method	Sensitivity (%)	Specificity in sCJD subtypes (%)						Specificity (%)
			MM1	MM2	MV1	MV2	VV1	VV2	
Pennington et al. (2009)	WB	96							67
Gmitterová et al. (2009)	ELISA (cut-off 290 pg/ml)	94	100	75	89	89	100	100	
Sanchez-Juan et al. (2007)	WB	88–91							
Green et al. (2007)	WB	89							97
Sanchez-Juan et al. (2006)	WB	85	92	78	91	65	100	90	85
Collins et al. (2006)	WB	88	91	61	86	71	90	95	
Castellani et al. (2004)	WB	87	94	70	100	57	100	84	
Van Everbroeck et al. (2003)	WB	100 94							92 96
Green et al. (2002)	Capture assay	82							94
Aksamit et al. (2001)	sICMA (cut-off 8 ng/ml)	61							100
	(cut-off 4 ng/ml)	94							49
Lemstra et al. (2000)	WB	97							97
Zerr et al. (2000)	WB	94							84
Beaudry et al. (1999)	WB	60							100
Zerr et al. (1998a)	WB	94							93
Hsich et al. (1996)	WB	96							96

Biological parameters significantly influence the sensitivity of 14-3-3 test in patients with sCJD, i.e., disease duration, codon 129 genotype, age at onset and time of the lumbar puncture. 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 vs. nonclassical CJD types (see molecular disease subtypes). In classical CJD (which basically fulfill the criteria of having the tendency to be older, homozygous

Table 13.3 Sensitivity of 14-3-3 in different forms of human transmissible spongiform encephalopathies

Disease	Total number of cases	14-3-3 positive	Sensitivity (%)
Definite or probable sCJD	413	376	91
Possible sCJD	127	79	62
vCJD	11	5	45
iCJD	10	6	60
gCJD			
E200K	13	13	100
V210I	15	15	100
GSS	5	2	40
FFI	15	0	0
Controls*	392	34	–

*other neurological diseases

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.

13.2.3 Tau/p-tau

Tau concentration in CSF of CJD patients is highly increased and its quantitative analysis is a good diagnostic tool for CJD. Several studies revealed that the optimum cut-off point for CJD is at 1.300 pg/ml. This cut-off is three times higher than levels reported for Alzheimer's dementia, and in the latter, only rare cases display such extreme tau levels as CJD patients. Determination of tau has shown to yield specificity and sensitivity comparable to those for 14-3-3 testing. Concerning the phosphorylated tau isoforms in CSF of CJD, tau phosphorylated at threonine 181 (p-tau) was significantly raised in sCJD as well 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.

13.2.4 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 13.4).

Table 13.4 Other reported cerebrospinal fluid candidates for markers of human prion diseases

Study	Proposed CJD marker	Level in CSF
Manaka et al. (1992)	Ubiquitin	Elevated
Choe et al. (2002)	ApoE	Elevated
Minghetti et al. (2002)	Prostaglandin E(2)	Elevated
Guillaume et al. (2003)	H-FABP	Elevated
Kettlun et al. (2003)	Matrix metalloproteinase	Elevated
Schmidt et al. (2004)	LDH-1	Elevated
Cartier et al. (2004)	Fibronectin, thrombospondin, and heparan sulfate proteoglycan	Elevated
Zerr et al. (2004)	Plasminogen	
Sanchez et al. (2004), Piubelli et al. (2006)	Cystatin C	Elevated
Stoeck et al. (2005)	IL-4, IL-8, and IL-10	Elevated
Silveyra et al. (2006)	Acetylcholinesterase	Altered glycosylation pattern
Holsinger et al. (2006)	BACE1	Increased activity
Stoeck et al. (2006)	TGF-β	Reduced
Albrecht et al. (2006)	Beta-nerve growth factor	Elevated
Jesse et al. (2009)	GFAP	Elevated
Alberti et al. (2009)	Neurofilament heavy subunit	Elevated
Gawinecka et al. (2012)	Desmoplakin	Elevated

13.3 Magnetic Resonance Imaging

13.3.1 General Introduction

MRI has played an important role in the diagnosis of CJD (Tschampa et al. 2005; Urbach et al. 1998). 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 mainly rely on DWI and FLAIR MRI (Matoba et al. 2001; Young et al. 2005).

With the emergence of more sensitive MRI techniques, such as FLAIR (fluid-attenuated inversion recovery) and DWI (diffusion-weighted image), cortical signal increase was additionally observed in sCJD patients and hyperintense basal ganglia were detected more frequently (Fig. 13.1). Using FLAIR- and diffusion-weighted imaging, signal increase of 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

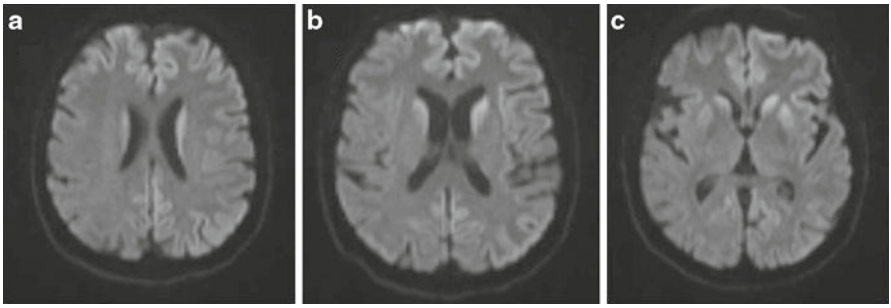


Fig. 13.1 DWI from patient with CJD representing. (**a**) hyperintensities cortical, (**b**) symmetric both caudate nucleus plus cortical, and (**c**) striatum right > left plus cortical

and for the mesencephalon. In general, the most sensitive technique to date seems to be DWI, followed by FLAIR and T2 imaging.

13.3.2 Test Sensitivity

In the diagnosis of sCJD, the hyperintense signal abnormalities in the striatum have a sensitivity of 58–78% (Choi et al. 2009; Cohen et al. 2011; Fulbright et al. 2006) and among non-CJD dementia patients, the specificity is higher (82–93%) (Choi et al. 2009; Fulbright et al. 2006). With the introduction of diffusion-weighted imaging, MR changes are detected earlier at the disease (Collins et al. 2006; Demaerel et al. 1999; Heinemann et al. 2007b; Holsinger et al. 2006; Hsich et al. 1996; Jesse et al. 2009; Josephs et al. 2009; Kelley et al. 2008) and serial imaging studies allowed to follow the disease progression by MR imaging (Kelley et al. 2008; Kettlun et al. 2003; Korczyn 1991). DW imaging improves interobserver reliability (Demaerel et al. 1999).

13.3.3 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). In single case reports, basal ganglia lesions were only developed during the disease course (Demaerel et al. 1999; Parazzini et al. 2003; Tomita et al. 2004; Tribl et al. 2002; Matoba et al. 2001). According to Ukisu and colleagues, on DW MRI cortical 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). In the late stage of the disease, the diffusion changes may disappear (Arruda et al. 2004; Matoba et al. 2001; Shyu et al. 1996; Tribl et al. 2002).

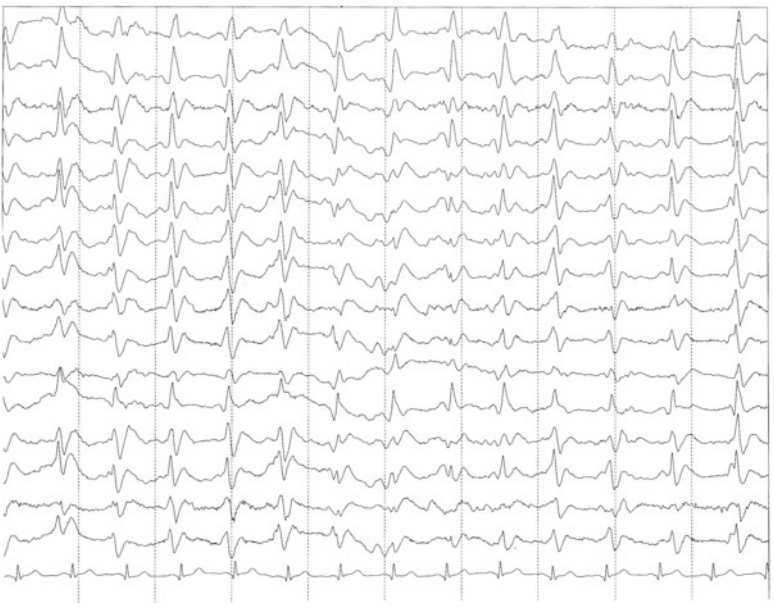


Fig. 13.2 Typical periodic sharp wave complexes

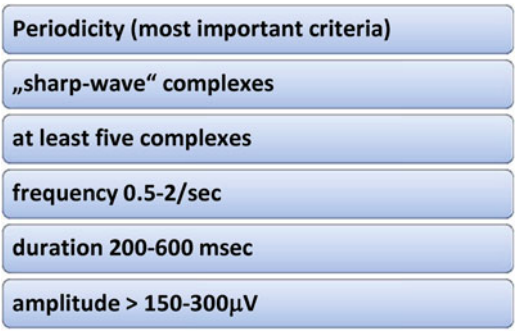


Fig. 13.3 EEG criteria (according to Steinhoff et al.)

13.4 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 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. 13.2) are observed in 60–70% of all cases after about 12 weeks (median) from disease onset but might occur as early as 3 weeks after onset. They may disappear at late 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. 13.3). According to these criteria, PSWCs are detectable in two-thirds of CJD patients at mid and late disease stages (sensitivity 64%). The sensitivity increases when several EEG recordings are performed. Specificity is comparable high (89%), and a good interrater agreement was achieved (kappa 0.95).

13.5 Molecular Disease Subtype Specific Diagnosis

Recently, a molecular basis has been defined, which might explain the clinical and pathological disease heterogeneity. 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. 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. 13.4).

The discovery of several distinct molecular CJD subtypes explains many features observed in sporadic CJD patients. The clinical presentation at early disease stage is 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 13.5 gives an overview of the diagnostic investigations in distinct molecular CJD subtypes.

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 of a long time CJD diagnosis was based on the triad: dementia, myoclonus, and PSWC in EEG, we might speculate that the frequency of so-called classical myoclonic CJD type was overestimated in earlier studies because of 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 and has the lowest sensitivity in MV2 and MM2 patients. This can easily be explained by quantitative analysis of this protein in CSF. Figure 13.5 displays results of the 14-3-3 test ELISA in various CJD subtypes, demonstrating low levels in MM2 and MV2 patients. Similar results were obtained for CSF tau protein (Sanchez-Juan et al. 2006; Heinemann et al. 2007a).

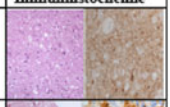
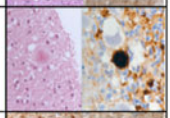
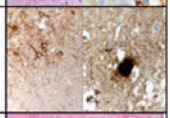
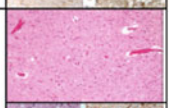
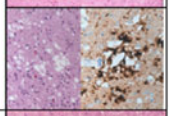
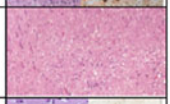
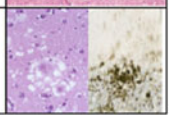
	molecular subtype	clinical signs	neuropathological findings	PrP-Immunhistochemie
frequent	MM1/MV1	dementia, cortical anopia, myoclonus, short disease duration (average 4 months)	severe damage of the occipital cortex (spongiosis, neuronal loss, astrogliosis), synaptic PrP–deposition	
	MV2	ataxia, dementia, extrapyramidal symptoms, long disease duration (average 18 months)	focal cortical damage, amyloid-(„Kuru"-) plaques, focal plaque-like PrP-deposition	
	VV2	ataxia at onset, late dementia (average disease duration 7 months)	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 (months)	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, giosis and neuronal loss, PrP deposition (florid plaques)	

Fig. 13.4 Molecular CJD subtypes

Table 13.5 Different values of the technical investigations EEG, CSF, and MRI stratified by CJD subtype

			MM1/MV1	VV1	MM2	MV2	VV2	CJD
EEG	PSWCs		+					
CSF	14-3-3		+	+	(+)	(+)	+	
MRI	Cortex		+	+	+	+	?	
	Basal ganglia		+		(+)	+	+	(+)
	Thalamus	Hyperintensity			(+)	+	+	+
		Pulvinar sign				(+)		+

A recent analysis of a multicentre international study aimed to describe the brain MRI findings associated with each of the sCJD molecular subtypes. MRI scans were evaluated in 211 CJD patients with various disease subtypes. 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.

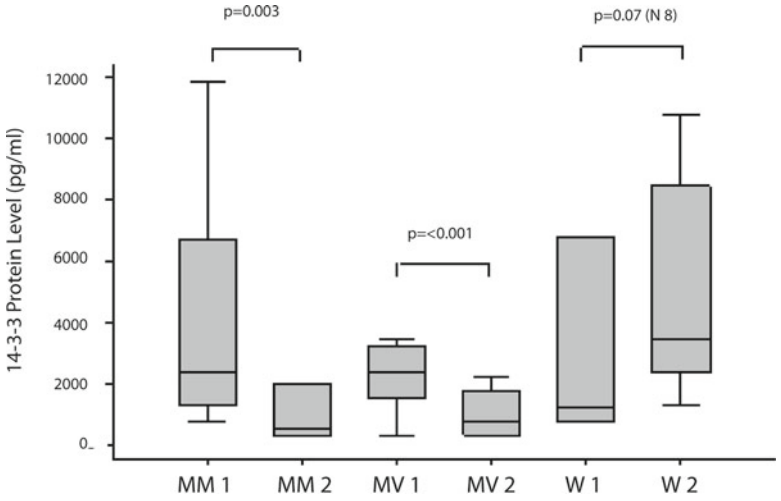


Fig. 13.5 14-3-3 test in various CJD subtypes

Table 13.6 MRI findings and subtypes

Subtype	More than three 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% $p=0.057$

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 13.6 and Fig. 13.6).

The most characteristic MRI lesion patterns were 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.

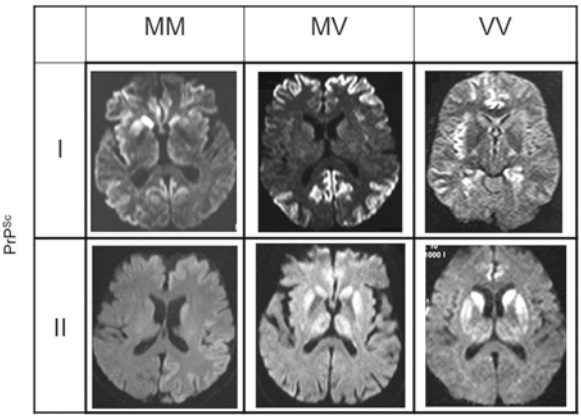


Fig. 13.6 Characteristic MRI findings in sporadic CJD subtype

13.6 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.

Only limited data are available for genetic TSE. Data from the literature are restricted to single case reports on patients with rare mutations. The most comprehensive studies have been carried out in genetic CJD with E200K and V210I mutations and in fatal familial insomnia (FFI).

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 the codon 200 and 210 (Korczyn 1991; Ladogana et al. 2005). In these patients, the sensitivity of the EEG is almost the same as in sCJD (Zerr et al. 1998a).

So far there has been only limited information available about biochemical markers in genetic transmissible spongiform encephalopathies (gTSE), although they represent 10–15% of human TSEs. However, there is a special interest in studying biochemical 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).

Concerning CSF 14-3-3 testing, sensitivity varies across the spectrum of genetic mutations. Apparently, the types of mutation significantly influence the biomarker concentration in the CSF and, thus, test sensitivity. According to current information, changes in the CSF of patients with familial genetic forms of CJD (gCJD) are comparable to those found in sCJD samples. Table 13.7 gives an overview. 14-3-3 proteins are detectable in patients with an E200K and V210I mutation (Rosenmann

Table 13.7 CSF marker in genetic TSE*

	14-3-3	tau	s100b	NSE
gCJD $n=117$	83	86	87	64
Insert $n=16$	68	80	78	50
GSS $n=10$	10	40	50	0
FFI $n=23$	13	7	20	0

*increased levels, %

et al. 1997; Zerr et al. 1998a) but only in rare cases in FFI and GSS (Zerr et al. 1998a, b).

In a multicenter EC-funded study on biomarkers in CJD, 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. However, numbers were too low to draw any definite conclusions (Ladogana et al. 2009).

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 patients group 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 (Ladogana et al. 2009).

Some data are available concerning MRI changes in gCJD cases, based on few larger studies and case reports. Of special importance, thalamostriatal diffusion reductions have been shown to precede disease onset in E200K prion mutation carriers and might therefore serve as an early diagnostic marker (Lee et al. 2009).

A series of case reports and a small number of larger imaging studies have described MRI changes in different gCJD point mutations (20–22). They illustrated a variety of distinctive features found mainly in the basal ganglia, thalamus, and cortical areas. In patients with E200K mutation, 50% were reported to display high signal intensities. Using FLAIR or DWI, the sensitivity is apparently even higher (Fulbright et al. 2008). Most common changes are reported in gyrus cinguli and are less notable in other cortical areas. An overview of reports from the literature is given in Table 13.8.

Table 13.8 Review of the literature, MRI in patients with E200K mutation

	References (author/date)	Sample size	BG	Cortex
E200K	Choi et al. (2009)	1	+	+
	Cohen et al. (2011)	31	+	+
	Farbu et al. (2007)	2	+	−
	Fulbright et al. (2006)	4	+	
	Fulbright et al. (2008)	15 patients 22 controls	+	+
	Ghoshal et al. (2009)	4	+	−
			+	−
			+	+
			+	+
	Heinemann et al. (2007b)	21	+	
	Kovacs et al. (2005)	175; 66 MRI	+	
	Lee et al. (2009)	14 E200K patients 20 E200K carriers thalamostriatal diffusion reductions before disease onset 20 controls	+	
	Masullo et al. (2010)	1	+	+
	Mancuso et al. (2009)	1	+	
	Nozaki et al. (2010)	37	+	
	Seror et al. (2010)	12 E200K 20 controls	+	
	Tsuboi et al. (2005)	1	+	+
V210I	Furukawa et al. (1996)	1	−	−
	Heinemann et al. (2007b)	15		
	Kovacs et al. (2005)	67; 46 MRI		
	Mastrianni et al. (2001)	4	−	−
	Nitrini et al. (2001)	2	+	+
	Shyu et al. (1996)	1	+	

13.7 Differential Diagnosis

The differential diagnosis of sCJD includes a large number of neurological and psychiatric diseases. 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. Because of the typical clinical presentation in CJD might be a rapid evolving neurological disorder, the spectrum of differential diagnosis also comprises some potentially treatable conditions. Figure 13.7 and Table 13.9 give an overview on differential diagnoses in CJD.

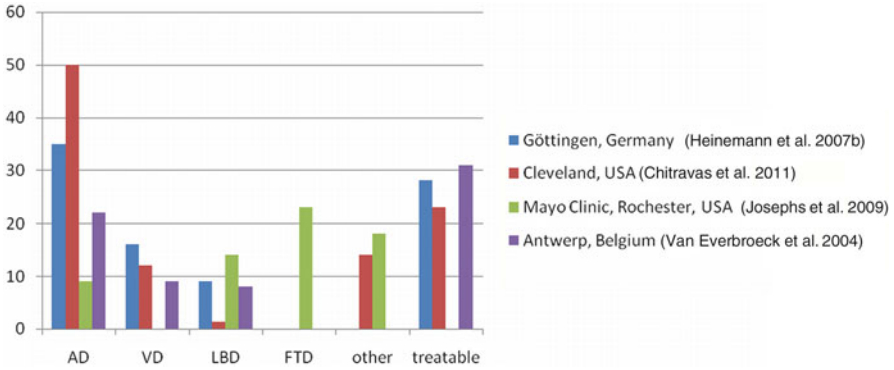


Fig. 13.7 Diagnosis of rapid progressive dementia in % in various retrospective analysis of surveillance/neurology units

With respect to the clinical syndrome, we could demonstrate that ataxia (77%) and dysmetria (48%) was significantly more frequent in CJD than in AD or DLB patients (Edler et al. 2009). Comparable results for the occurrence of ataxia in CJD were seen by Parchi et al. (1996), Tschampa et al. (2001), and Van Everbroeck et al. (2004) as shown in Table 13.10.

On contrary, hypokinesia was found four to five times more often in AD and DLB than in CJD patients. Investigating constellations of movement disturbances using the principal component analysis, prominent statistical effects are seen, when comparing CJD with AD patients (Table 13.10). In patients presenting ataxia without hypokinesia, the diagnosis of CJD was the most likely diagnosis. In AD, hypokinesia without ataxia is more typical.

13.8 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). Criteria for sporadic CJD have been amended by 14-3-3 CSF test and more recently, MRI. They are displayed in Fig. 13.8.

Table 13.9 Overview of differential diagnoses in CJD

Publication (title, author, journal)	Patients (*n*) center	Rapid progressive dementia—final/pathology diagnosis
Rapidly progressive diffuse Lewy body disease Gaig et al. (2011); Mov Disord	6 Neurology Service, Hospital Clinic of Barcelona, Barcelona, Spain	*All LBD*
Treatable neurological disorders misdiagnosed as Creutzfeldt–Jakob disease. Chitravas et al. (2011) Ann Neurol	71 (year: 2006–2009) US National Prion Disease Pathology Surveillance Center	1. *Alzheimer disease* (50%) 2. *Vascular dementia* (12%) 3. *Treatable diseases* (23%): immune-mediated disorders, neoplasia (often lymphoma), infections, and metabolic disorders 4. *Other* (14,1%): e.g.: tauopathy, LBD, FTLD, and Huntington's diseas3e
Clinical features of rapidly progressive Alzheimer's disease. Schmidt et al. (2010); Dement Geriatr Cogn Disord	32 (year: 1993–2004) retrospective analysis German National Prion Disease Surveillance Center	*All AD* 32 neuropathologically confirmed cases differentially diagnosed as AD out of a group with rapidly progressive dementia
Rapidly progressive neurodegenerative dementias. Josephs et al. (2009); Arch Neurol	22 (year: 2000–2007) Department of Neurology, Mayo Clinic, Rochester, MN	*CJD* (36%), *FTD plus MND* (23%), *tauopathy* (18%), *LBD* (14%), and *Alzheimer disease* (9%)
Rapidly progressive young-onset dementia. Kelley et al. (2008); Cogn Behav Neurol	22 (year: 1996–2006) Department of Neurology, Mayo Clinic, Rochester, MN	*CJD* ($n=3$; probable CJD: $n=4$), *FTLD-MND* ($n=1$), *unknown neurodegenerative disorder* ($n=4$), *adult onset leukodystrophy* ($n=1$), *PML* ($n=1$), *MELAS* ($n=1$) *microangiopathy/microvascular ischemia* ($n=1$), and *other* ($n=2$) *metabolic etiologies* (14%)
Creutzfeldt–Jakob disease in Germany: a prospective 12-year surveillance. Heinemann et al. (2007b); Brain	2,170 (year: 1993–2005) German National Prion Disease Surveillance Center	*CJD* (66%): definite CJD (35%), probable CJD (26%), possible CJD (5%), and *other* (34%): *Alzheimer's disease* (35%), *vascular dementia* (16%), *LBD* (9%), *MSA* (3%) potentially treatable (28%): *malignancies/paraneoplastic* (6%), *metabolic dysfunction* (8%), and *psychiatric disease* (9%)
Differential diagnosis of 201 possible Creutzfeldt–Jakob disease patients. Van Everbroeck et al. (2004)	201 (year: 1998–2004) Laboratory of Neurobiology, University of Antwerp, Wilrijk, Belgium	*Final diagnosis:* *CJD (30%), AD (22%), VD (9%), DLB (8%), and other (31%)*: most frequent were viral encephalopathy, paraneoplastic syndromes, metabolic encephalopathy ($n=3$), and Hashimoto encephalitis

Table 13.10 Significance levels comparing the movement disturbances of CJD patients with differential diagnoses

Symptoms	CJD vs. AD	CJD vs. DLB	CJD vs. non-CJD
Oppenheim reflex	$p=0.0542$	$p=0.2126$	$p=0.0115$
Gordon reflex	$p=0.0542$	$p=0.2126$	$p=0.0323$
Babinski reflex	$p=0.2901$	$p=0.7851$	$p=0.0315$
Athetosis	$p=0.0939$	$p=0.4069$	$p=0.0421$
Dystonia	$p=0.0088$	$p=0.1895$	$p=0.0016$
Hypokinesis	$p=0.0018$	$p=0.0148$	$p=0.0033$
Dysmetria	$p=0.0222$	$p=0.0136$	$p=0.0016$
Ataxia	$p=0.0001$	$p=0.0003$	$p=0.0001$

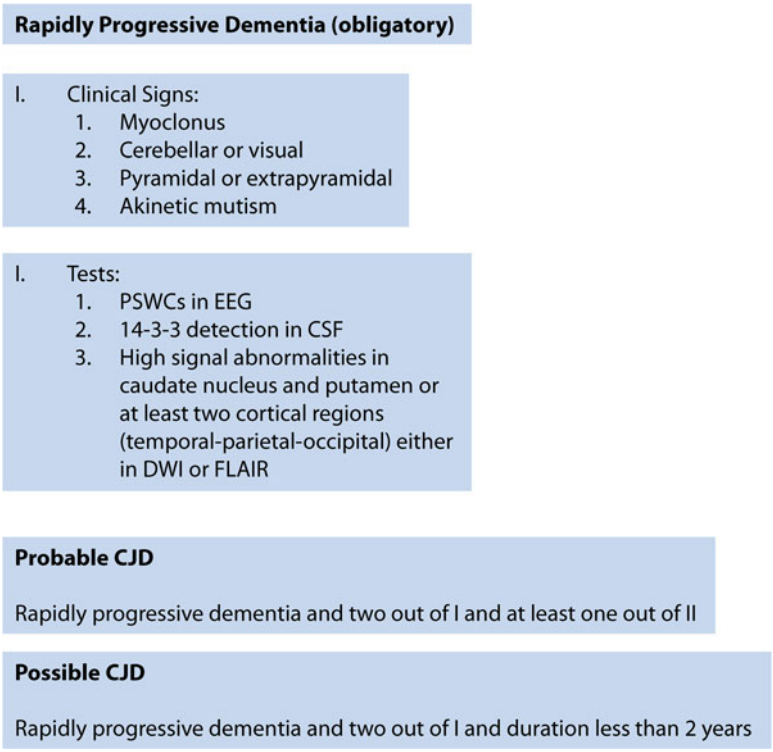


Fig. 13.8 Criteria for sporadic CJD

13.9 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. In the differential diagnosis of neurodegenerative disorders, elevated levels of these proteins support the diagnosis of sCJD with a sensitivity of 85–95% and specificity of 80%. Recently, it has been demonstrated that advanced brain imaging techniques significantly contribute to the clinical diagnosis on one hand, but might also help in the early differentiation in molecular disease subtypes in sporadic CJD on the other hand. Clinical diagnostic criteria are amended and are based on detailed algorithm.

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Chapter 14
Quaking-Induced Conversion Assays for the Detection and Diagnosis of Prion Diseases

Jason M. Wilham, Christina D. Orrù, Sarah Vascellari, Andrew G. Hughson, and Byron Caughey

Abstract The seeding activity of prions has been exploited for the development of a number of ultrasensitive assays for transmissible spongiform encephalopathies (TSEs). Among the more practical assays are those that use recombinant PrP^{Sen} ($rPrP^{Sen}$) as a substrate for prion-seeded conversion into amyloid fibrils, shaking rather than sonication, and fluorescence detection in multiwell plates. These include the amyloid seeding assay (ASA), real-time QuIC (RT-QuIC), and enhanced QuIC (eQuIC). Recent applications of RT-QuIC to the antemortem diagnosis of sporadic Creutzfeldt–Jakob disease (sCJD) using cerebrospinal fluid (CSF) showed improved specificity (nearly 100%) relative to assays for other CSF markers. Moreover, the RT-QuIC can be quantitative and as sensitive as animal bioassays, allowing measurements of prion seeding activity in CSF and nasal fluids. In hamster CSF, the time course of seeding activity accumulation can vary markedly with route of scrapie inoculation. To enhance sensitivity and to cope with inhibitor-laden sample types such as blood plasma, an immune capture step was integrated with RT-QuIC, giving rise to the eQuIC assay. eQuIC can detect up to 10^{14}-fold dilutions of human CJD brain homogenate in blood plasma and discriminate plasma samples from scrapie-infected and uninfected rodents. Although further work is required to fully validate various applications of RT-QuIC, eQuIC, and related tests, these assays improve prospects for practical prion detection in humans, animals, biomaterials, and the environment.

Keywords Amyloid seeding assay • Prion protein • Real-time QulC • sCJD • Transmissible spongiform encephalopathies • TSE

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14.1 Introduction

Among the greatest practical challenges facing the TSE/prion field is the need for better, faster, and more specific tests for prions that are sensitive enough to detect the smallest traces of TSE infectivity. Such tests will not only help prevent TSE infections by allowing detection of infectivity sources, but also will facilitate early diagnosis and therapeutic intervention in infected humans and animals.

It has long been known that TSE-associated forms of prion protein, e.g., PrP^{Sc}, PrP^{Res}, or PrP^{TSE}, can induce or seed the conversion of their normal counterpart, PrP^{C} or PrP^{Sen}, to forms that, like PrP^{Sc} itself, are higher in beta sheet content, polymeric, and more protease-resistant (Caughey et al. 2009; Kocisko et al. 1994). The development of a continuous in vitro conversion reaction by Soto and colleagues allowed extraordinarily sensitive detection of prion seeding activity in an assay called protein misfolding cyclic amplification (PMCA) (Castilla et al. 2006; Chen et al. 2010; Saa et al. 2006b; Saborio et al. 2001). In this reaction, test samples are mixed with brain homogenates from uninfected animals, and subjected to cycles of intermittent sonication. Prion seeding activity within the test sample induces the conversion of PrP^{Sen} in the brain homogenate to PrP^{Res}, which may ultimately be detected by proteinase K treatment (to eliminate unconverted PrP^{Sen}) and immunoblotting. With serial PMCA (sPMCA) reactions, as little as ~1 ag (10^{-18} g) of initial PrP^{Res} seed could be detected within ~3 weeks (Saa et al. 2006b), amounting to PrP^{Res} 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 detection of prions in numerous tissues and sample types (Castilla et al. 2006; Jones et al. 2011). Although 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^{Sen} 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, and (4) the overall time required for optimal sensitivity is prohibitive for many routine surveillance, screening, or diagnostic applications.

Various 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, multiwell plate formats, fluorescence readouts, shaking rather than sonication, and markedly reduced overall assay times (for recent reviews, see (Orru and Caughey 2011; Orru et al. 2012b)). In this chapter, we will review the more recent developments and applications of these assays.

14.2 $rPrP^{Sen}$ as a Substrate in Prion-Seeded Reactions

Initial studies showed that $rPrP^{Sen}$, like PrP^{Sen} derived from mammalian cells (Kocisko et al. 1994), could be induced to convert to specific partially protease-resistant forms in PrP^{Sc}-seeded cell-free conversion reactions (Kirby et al. 2003). However, the

reactions gave limited yields that did not allow for substantially amplified PrP^{Sc} detection. A subsequent study described a highly sensitive assay in which fluorescently labeled $rPrP^{Sen}$ was used to coaggregate with BSE-associated seeds that were then detected by fluorescence activated cell sorting (Trieschmann et al. 2005). This report described the discrimination of sera from BSE-infected vs uninfected cattle. Atarashi and colleagues then developed a $rPrP^{Sen}$-based PMCA assay (rPrP-PMCA) which, like previous PMCA reactions, was a sonicated, microtube-based reaction. Prion-infected tissues seeded the polymerization of $rPrP^{Sen}$, yielding partially proteinase K-resistant amyloid fibrils by immunoblotting (Atarashi et al. 2007; Peden et al. 2012). Further studies showed that the sonication, which is known to be fickle and difficult to standardize, can be replaced by aggressive shaking of the reaction tubes, giving rise to what we now call the standard *qu*aking-*i*nduced *c*onversion (S-QuIC) assay. Meanwhile, Colby et al. (2007) developed the amyloid seeding assay (ASA), which monitored the formation of prion-seeded $rPrP^{Sen}$ amyloid fibrils more conveniently using the amyloid-sensitive dye, thioflavin T (ThT). Other major advantages of the ASA were its higher throughput multiwell plate-based format and shaking mode of agitation. A disadvantage of the ASA reaction conditions was the relatively rapid spontaneous (prion-independent) fibrilization by the $rPrP^{Sen}$ substrate, often within twice the lag phase of the prion-seeded reactions. Thus, the assessment of ASA results depends on careful comparisons of fibrillization lag times, making interpretation increasingly difficult with low titer samples.

14.3 RT-QuIC

To address shortcomings of the previous assays, Atarashi, Wilham, and colleagues then combined positive attributes of the S-QuIC and ASA assays in developing the *r*eal-*t*ime QuIC (RT-QuIC) assay; namely, the greatly reduced spontaneous fibrillization (i.e., background) of S-QuIC was combined with the multiwell plate format and ThT detection of the ASA. In RT-QuIC assays, a small aliquot (usually 2–15 μl) of test sample is combined with a reaction mix containing an appropriate $rPrP^{Sen}$ substrate, ThT, and little if any detergent or chaotropic agent. The 96-well 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 either the average or total fluorescence from replicate wells is plotted as a function of reaction time (Fig. 14.1). Often, when plotted in this way, a stepwise increase in fluorescence is observed which reflects the fact that individual replicates can have somewhat different "lag phases" prior to sudden rapid growth of fibrils until the available substrate is consumed. We have applied RT-QuIC to the detection of sheep and rodent-adapted scrapie and chronic wasting disease (CWD) of cervids (Wilham et al. 2010). In the case of the hamster-adapted scrapie strain 263 K, RT-QuIC sensitivity for detection of brain homogenate dilutions was comparable to bioassay

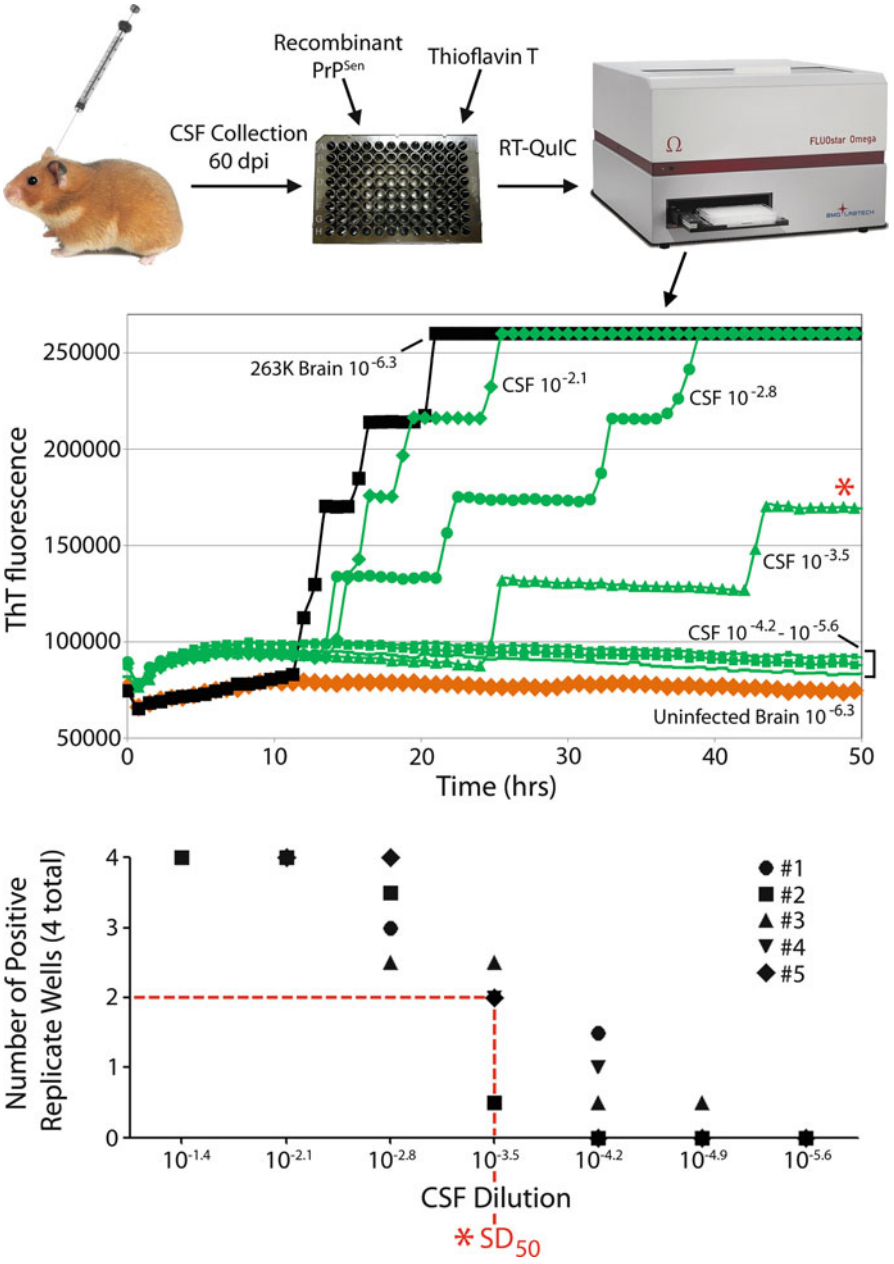

Fig. 14.1 RT-QuIC end-point dilution analysis of prion-infected hamster CSF. Upper figure is a representative end-point dilution RT-QuIC assay of prion seeding activity in CSF from an intracerebrally inoculated hamster at the early stage of clinical disease (60 dpi). Serial 1:5 dilutions of 2 μl of CSF were subjected to RT-QuIC analysis (*green traces*) as previously described (Wilham et al. 2010). The vertical axis shows the average ThT fluorescence from four replicate reactions seeded with each CSF dilution. The stepwise increases in fluorescence represent individual positive reactions with rapid fibril assembly to maximum fluorescence as is typical of many seeded polymerization reactions. The lower figure shows the number of positive replicate wells out of four at each CSF dilution for five separate hamsters. * SD_{50} value: the CSF dilution giving positive reactions in 50% of the replicates as seen here with the $10^{-3.5}$ dilution

by intracerebral inoculation of hamsters. Prion seeding activity was also detected in cerebral spinal fluid and nasal lavages of Hyper (HY) transmissible mink encephalopathy-infected hamsters (Bessen et al. 2010).

14.4 End-Point Dilution Quantitation by RT-QuIC

Two potential approaches to measuring prion seeding activity by RT-QuIC include end-point dilution titrations and comparisons of lag phases. Although lag phases tend to be inversely proportional to seed concentration within individual experiments as discussed previously (Peden et al. 2012; Wilham et al. 2010), they can be variable between experiments and influenced by factors other than seed concentration. Consequently, we have favored the end-point dilution approach which determines the sample dilution giving positive reactions in 50% of replicate reactions, i.e., the 50% seeding dose or SD_{50}. Back calculations then establish the SD_{50} per unit of the original specimen. Multiple applications of end-point dilution RT-QuIC to four independent scrapie-infected hamster brain tissue homogenates demonstrated consistent SD_{50} per gram brain determinations of 10^{11}–10^{12}, which were ~1 log higher than the 50% lethal dose (LD_{50}) per gram measured by end-point dilution bioassay in corresponding hamster experiments.

End-point dilution RT-QuIC analyses also allowed the first in vitro measurements of seed activities in the CSF (~10^5 SD_{50}/ml) and nasal lavages (10^3–10^6 SD_{50}/ml) from prion-infected hamsters (Wilham et al. 2010). Such substantial levels of seeding activity, as well as infectivity (Bessen et al. 2010), in nasal fluids is consistent with the detection of PrP^{Res} in the olfactory epithelia of hamsters (Bessen et al. 2010; Wilham et al. 2010) and humans (Zanusso et al. 2003). These results suggest that the nasal cavities may be a significant source of prion shedding from infected individuals and that nasal fluids may be valuable, and easily accessible, TSE diagnostic specimens. Adding to the potential seriousness of nasal prion shedding are observations that irritation of the nasal cavity can increase the release of prion seeding activity into nasal fluids by another 100–1,000-fold (Bessen et al. 2011).

CSF is also a revealing diagnostic specimen (see below), and 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. 2012a). 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 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 to 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.

14.5 RT-QuIC for Human TSEs: Improved Diagnostic Specificity

Human CJD detection and diagnosis using RT-QuIC is particularly promising. RT-QuIC can detect up to 10^{10}-fold dilutions of sporadic CJD (sCJD) brain homogenates containing ~1 fg of PrP^{Res} while maintaining specificity with respect to a variety of non-CJD brain tissue controls (Atarashi et al. 2011b; Peden et al. 2012). Interestingly, for reasons that remain unknown, the lag phases in RT-QuIC reactions seeded with variant CJD (vCJD) tend to be much longer than those for reactions seeded with comparable dilutions of sCJD brain homogenate (Peden et al. 2012).

These initial findings encouraged further evaluations of RT-QuIC as a diagnostic test for sCJD using cerebrospinal fluid (CSF) samples (Atarashi et al. 2011b). Antemortem CJD diagnoses can be complicated and problematic, relying on a battery of tests including clinical signs, EEG, MRI, and assays for CSF markers such as the 14-3-3 and tau proteins that are not specific for TSE. The likelihood that the RT-QuIC detects a TSE-specific marker led to expectations of greater CJD specificity than is possible with currently available diagnostic tests based on nonspecific CSF markers. Indeed, Atarashi and colleagues demonstrated in analyses of CSF samples from sCJD and non-CJD patients from Japan and Australia that the RT-QuIC had >80% sensitivity and 100% specificity in identifying CSF specimens from CJD patients (Atarashi et al. 2011b). Moreover, they obtained positive reactions from all of the different sCJD subtypes. Comparisons with the 14-3-3 test revealed that the RT-QuIC was substantially more specific for CJD overall (Table 14.1).

Importantly, the diagnostic utilility of RT-QuIC CSF testing has been confirmed by blinded analyses of other large panels of CSF specimens from sCJD and non-CJD patient controls by McGuire et al. (2012) in the UK. Their exploratory study of 55 CSF samples from neuropathologically confirmed sCJD patients and 53 controls yielded 91% sensitivity and 98% specificity, while a confirmatory test of 67 sCJD samples and 51 controls gave 87% sensitivity and 100% specificity (Table 14.1). Overall, the results from both the Atarashi and McGuire reports (Atarashi et al. 2011a, b; McGuire et al. 2012) indicate that RT-QuIC has the potential to provide greater specificity for sCJD than currently available CSF tests. Further efforts are under way to implement, evaluate, and standardize RT-QuIC as a sCJD diagnostic test in multiple centers around the world.

14.6 eQuIC

Our initial failures using RT-QuIC to detect prion seeding activity in blood and blood plasma suggested that it would be necessary to first isolate the PrP^{Sc} seeds from blood-borne inhibitors of the reaction. Moreover, blood tends to have very low prion concentrations (Brown et al. 1998, 1999; Cervenakova et al. 2003; Gregori et al. 2004), suggesting that it would be helpful to concentrate PrP^{Sc} from blood to

Table 14.1 Sensitivities and specificities for CSF RT-QuIC and 14-3-3 tests in neuropathologically confirmed sCJD cases

	Japan/Australia		UK	
	Atarashi et al. (2011b)		McGuire et al. (2012)	
	RT-QuIC	14-3-3	RT-QuIC	14-3-3
sCJD	29/34	27/34	109/123	116/123
Negative controls	0/49	9/49	1/103	36/103
Sensitivity (All)	85%	79%	89%	94%
Sensitivity (MM)	84%	76%	90%	95%
Sensitivity (MV)	100%	50%	88%	95%
Sensitivity (VV)	75%	100%	95%	100%
Specificity	100%	82%	99%	65%

The fractions give the composite published numbers of positive/total assay results from sCJD or negative control patients compiled from multiple tests by the referenced groups. Overall sensitivity percentages are shown for analyses of sCJD patients with the designated *Prnp* codon 129 genotypes (*in parentheses*). Overall specificity percentages indicate the overall proportion of negative control samples that gave negative reactions

improve the sensitivity of detection. Toward both of these goals, we incorporated a front-end PrP^{Sc}-selective immunoprecipitation step to the RT-QuIC reaction. Concurrently we found that assay sensitivity could often be improved by the addition of a $rPrP^{Sen}$ substrate replacement step during the reaction as has been shown to be helpful in PMCA reactions (Rubenstein et al. 2010). The combination of these two assay modifications is what we call the enhanced QuIC or eQuIC (Orru et al. 2011). The antibody that we used (monoclonal IgM antibody 15B3) is selective for PrP^{Sc} and other abnormal PrP oligomers over monomeric PrP^{Sen} (Biasini et al. 2009; Korth et al. 1997). 15B3-coated magnetic beads are incubated with the test sample (e.g., 0.5 ml blood plasma), washed, and added directly to the RT-QuIC reaction well. After 24 h of shake/rest cycles, 90% of the liquid is removed, leaving most of the beads and nascent seeded amyloid in the well, and fresh RT-QuIC buffer including rPrP substrate is added to continue the reaction.

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. 2006b). 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). Thus, current work is aimed at finding methods to better cope with variations in human plasma. 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).

eQuIC also has readily detected prion seeding activity naturally present in the blood of scrapie-infected hamsters (Orru et al. 2011), mice, and sheep (unpublished data), as indicated by the discrimination of plasma samples from infected and uninfected controls. Collectively, the blood plasma data provide evidence that eQuIC can serve as the basis for a blood test for prion infections. Additional work will be required to determine the extent to which eQuIC sensitivity is affected by matrix variations in different plasma samples. Other in vitro assays have also discriminated blood samples from infected and uninfected individuals (Edgeworth et al. 2011; Rubenstein et al. 2010; Saa et al. 2006a; Trieschmann et al. 2005). Hopefully, assays including the eQuIC will also be applicable to the detection of minute concentrations of prions in other biological and environmental sample types, including those containing substances that are otherwise incompatible with prion-seeded amplification reactions such as RT-QuIC and PMCA.

14.7 Further Technical Considerations

Finally, from a practical perspective we would like to address some issues that often arise when RT-QuIC assays are initiated in new laboratories. First 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 resists spontaneous fibrillization in the presence of comparable negative controls. The more the sensitivity is pushed by increasing overall reaction times, the more the spontaneous fibrillization becomes an issue. We assume that there may be multiple methods for preparing appropriate $rPrP^{Sen}$ substrates, but some types of preparations do not work well, often because they are prone to prion-independent fibrillization. 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 other successful groups have published should minimize such problems. Spontaneous fibrillization can also be greatly reduced by optimizing reaction temperature and salt concentrations for given 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 can be reused, albeit with reduced yields. A second cost-cutting measure that appears to be possible 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 of RT-QuIC. In fact, in many cases a single reading at the end of the overall reaction should suffice for discriminating positive and negative samples. A challenge that we have encountered is the lack of plate shakers with optimal control of temperature, shaking-rest cycles, and shaking motion (i.e., double orbital vs orbital). Eventually, there should be no technical reason why such an approach cannot dramatically improve assay throughput per unit cost.

14.8 Conclusions

Considerable progress has been made recently in the development of more practical assays for TSE prions. Clearly, the RT-QuIC has strong potential to improve the antemortem diagnosis of sCJD in humans. Further work will be required to validate the applicability of the robust RT-QuIC and the highly sensitive eQuIC to the diagnosis of other human and animal TSEs. Assays such as RT-QuIC and eQuIC are becoming sufficiently sensitive, rapid, and high throughput to address routine needs in medicine, agriculture, wildlife management, and research that have not been met previously. Ultimately, the availability of multiple ultrasensitive tests for prion detection and TSE diagnostics will be crucial. TSE diagnoses have dire implications for both the individual and society, emphasizing the need for accurate primary tests and the ability to confirm positive reactions with both repeats of the same test and applications of confirmatory tests. Given the apparent rarity of actual TSE infections in most human and animal populations, it is likely that even tests with extremely low false-positive rates will yield more false positives than real positives in any surveillance of general, rather than clinically targeted, populations. The eventual availability of independent and highly specific primary and confirmatory tests will likely reduce false-positive rates to acceptable levels.

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Chapter 15
The Tyr-Tyr-Arg Prion-Specific Epitope: Update and Context

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Abstract The production of PrP^{Sc}-specific antibodies has proven challenging, but much has been learned since the production of the first PrP^{Sc}-specific antibody in 1997 by Korth and colleagues. Some of the challenges that have been overcome, and the novel approaches that have been applied over the past 15 years will be reviewed and presented in this summary of prion immunology.

Keywords Antemortem diagnostic testing • Prion protein • PrP^{Sc}-specific antibodies

15.1 Introduction

Prion diseases are transmissible neurodegenerative disorders for which no effective therapeutic agent, prophylactic regimen, nor definitive antemortem diagnostic test exists. Given the impact of prion diseases on human public health, and the economic consequences of a major outbreak in livestock, addressing these longstanding goals of prion research have become a major priority. There is a building body of evidence and opinion that the development of PrP^{Sc}-specific Abs and PrP^{Sc}-targeting immunotherapies may be able to address all three of these elusive goals.

15.2 Current Antemortem Diagnostic Testing

Current antemortem diagnostic analysis for Creutzfeldt–Jakob disease (CJD) consists of the combined use of clinical observations, imaging systems, genetic testing, and the detection of various biomarkers of disease. Current antemortem tests include MRI,

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periodic sharp wave complexes in EEG, and the detection of biomarkers such as 14-3-3, α-synuclein, S-100β, total tau, and NSE, which offer varying degrees of sensitivities and specificities (Zerr et al. 2009; Chitravas et al. 2011; Perry and Geschwind 2011). The practice of combining antemortem test results can improve the sensitivity and specificity of a putative or probable diagnosis. Despite the valuable role that this combinational approach plays in patient care and infection control, all current methods of testing are insufficient for a definitive diagnosis in life, with the exception of the clinical syndrome occurring in a known PRNP mutation carrier.

15.3 Current Definitive Diagnostic Testing

The definitive diagnosis of prion diseases is currently limited to the postmortem analysis of brain material at autopsy. Immunohistochemistry (IHC) is performed to determine the presence or absence of amyloid plaques and spongiform degeneration in brain sections. The presence or absence of PrP^{Sc} in brain tissue homogenates (BH) can be determined on immunoblots, utilizing the differential PK resistance exhibited by the infectious and non-infectious forms of PrP. Prion disease subtype can also be done through the analysis of immunoblot banding profiles that are characteristic of different forms of CJD (Parchi et al. 1997, 1998, 1999). While current methods of definitive postmortem diagnoses are informative, they are unsatisfactory for two main reasons: (1) the delay of a definitive diagnosis until after autopsy, and (2) current immunoblot detection of infectious PrP relies upon the central assumption that all infectious PrP is resistant to proteinase K (PK) digestion for specificity. This assumption has been shown to be untrue (Tzaban et al. 2002; Pastrana et al. 2006; Thackray et al. 2007; Kim et al. 2011). PK digestion of RML BH has also been shown to reduce the detectable signal in immunoblots by up to 58% (Tattum et al. 2010). Thus, the reliance upon PK digestion for the specificity of immunoblot detection of infectious PrP may lead to false negative results.

The sensitive and specific detection of PrP^{Sc}, the only known component of the disease-causing agent, is the only biomarker sufficient for a definitive antemortem diagnosis for prion disease. This major goal of prion research can be achieved through the development and application of PrP^{Sc}-specific Abs.

15.4 Important Benefits of PrP^{Sc}-Specific Antibodies

PrP^{Sc}-specific Abs are highly desired for many reasons: (1) They offer hope for a definitive antemortem test, (2) they offer the potential for improvements in patient care, infection control, food safety, and the screening of donors and donated tissues, (3) PrP^{Sc} Abs are powerful research tools for the study of prion diseases, (4) the development of PrP^{Sc}-specific Abs lends itself to the exploration and development of immunotherapies for the treatment and prevention of prion disease.

15.4.1 Definitive Antemortem Diagnosis of Prion Disease

PrP^{Sc}-specific Abs offer hope for a definitive antemortem test that is minimally invasive without relying on differential proteinase K susceptibility for the specific detection of infectious PrP. A definitive antemortem diagnosis, as early in the disease process as possible, has many wide ranging and important benefits for the patient and for society in general.

A definitive antemortem diagnosis would provide important answers to family and physicians that could lead to genetic testing, family counseling, and preparation for subsequent stages of the disease. A prompt diagnosis would also assist in the administration and development of treatments and improve inclusion in clinical studies. Even a definitive negative result can be of great value to enable the proper channeling of health care provision, as many patients with suspected sCJD are misdiagnosed and actually have treatable neurological disorders (Chitravas et al. 2011).

15.4.2 Surveillance and Risk Management

Answers are needed early on for risk management, infection control, handling of tissue, and decontamination of instruments. A definitive noninvasive antemortem test would improve surveillance and participation in prion disease surveillance systems, and thus, would improve patient care, research, and epidemiology.

Importantly, a sensitive and definitive antemortem test would allow for earlier exclusion from donor eligibility lists, the rapid recall of contaminated material before recipient exposure, and the rapid follow-up of patients exposed to contaminated material or medical instruments. The application of PrP^{Sc}-specific Abs may also be useful for risk mitigation through the clearing of infectious PrP proteins from donated blood and blood products. This is especially true in cases where the destruction of large pools of donated material may be impractical or have highly unacceptable consequences, such as when patients require life-sustaining products despite the potential risk of iatrogenic infection.

15.4.3 Research Application

PrP^{Sc}-specific Abs would be instrumental in structural studies such as mapping PrP^{Sc}-specific conformational epitopes and PrP^{C}-PrP^{Sc}-binding sites (Ushiki-Kaku et al. 2010). Such antibodies may provide probes for dissecting the mechanics of prion protein conversion in disease. They would be invaluable in tracing routes of infection and localization of infectious PrP in the body. PrP^{Sc}-specific Abs would also be very useful in definitively testing the efficacy of other potential treatments for the clearance of PrP^{Sc}.

15.4.4 Potential Treatment of Disease and Prevention of Infection

Experiments involving passive immunization against normal prion protein in vitro have shown that the propagation of PrP^{Sc} can be delayed or even cleared by treating infected cells in tissue culture with anti-PrP^{C} mAbs (Enari et al. 2001; Peretz et al. 2001; Pankiewicz et al. 2006), or with Fab fragments prepared from anti-PrP^{C} mAbs (Alexandrenne et al. 2009). Experiments in vivo where PrP^{C}-specific mAbs have been injected I.P. into mice have also had some success regarding peripheral infection (Mallucci et al. 2007; Song et al. 2008). However, major concerns have been raised about serious side effects, and the lack of clearing of PrP^{Sc} from the central nervous system in vivo. Even greater concerns have been raised regarding potentially devastating side effects and the possible induction of autoimmune disease when actively immunizing with the purpose of breaking self-tolerance to normal host PrP. Some of the areas of particular concern include the inappropriate activation of signaling cascades (Mouillet-Richard et al. 2000), immunosuppression (Cashman et al. 1990), widespread complement-mediated cell lysis (Bendheim et al. 1992); the induction of apoptosis, and interference with the natural functions of the highly conserved PrP^{C} proteins such as metal binding, cognition, and immunity (Solforosi et al. 2004); strong neuronal loss, astrogliosis, microglial activation (Lefebvre-Roque et al. 2007); and cell lysis, hematopoietic disruption, and neuronal apoptosis (Li et al. 2010).

The application of passive immunity in vivo and any successful immunization scheme against prion disease must be done in a host-friendly manner. The ability to induce the production of PrP^{Sc}-specific Abs lends itself to the development of passive and active immunization protocols with less concern for the induction of autoimmunity.

15.5 Development of a Definitive Antemortem Test for Prion Disease

The development of a minimally invasive and definitive antemortem test for prion disease requires the sensitive and specific detection of disease-causing PrP^{Sc}. An antemortem brain tissue biopsy can provide high concentrations of PrP^{Sc} for testing if prion disease is present. However, an antemortem brain tissue biopsy is not an ideal source of material due to its highly invasive nature, risks to medical personnel, and the fact that it is of very limited benefit to the patient. An ideal antemortem test would be performed on samples that are readily obtained from minimally invasive techniques. Samples such as blood, CSF, and urine are ideal candidates that are easily and routinely acquired. However, sensitivity becomes an issue when trying to detect the low concentrations of PrP^{Sc} that exist in such dilute and highly complex samples of bodily fluids. The combination of sensitive PrP^{Sc}-specific Abs with concentration methods, amplification methods, and sensitive detection systems has the potential to provide a definitive antemortem test for prion infection.

15.5.1 Concentration of PrP Species from Dilute and Complex Biological Samples

Some common nonspecific methods of concentrating and partially purifying PrP^{Sc} include bead chromatography, column chromatography, phosphotungstate (PTA) precipitation (Safar et al. 2005), and sarkosyl precipitation (Chen et al. 2010; Morales et al. 2008). These methods exploit the diverse properties of various proteins in order to separate and concentrate proteins of interest from dilute and highly complex samples.

The specific immunoconcentration of PrP^{C} and PrP^{Sc} can be done by using Ab bound to paramagnetic beads or columns. PrP^{C} and PrP^{Sc} can be brought down together using an anti-PrP Ab, or PrP^{Sc} can be specifically concentrated from dilute biological fluids using PrP^{Sc}-specific Abs (Paramithiotis et al. 2003; Miller and Supattapone 2011; Orru et al. 2011).

15.5.2 Amplification of PrP^{Sc} Signal

Achieving the sensitivity required to detect minute amounts of PrP^{Sc} in very dilute and complex biological fluids is one of the many challenges inherent in developing an antemortem test for PrP^{Sc}. To address this challenge, a few main methods of amplifying PrP^{Sc} in dilute samples have been developed. Protein Misfolding Cyclic Amplification (PMCA) (Saborio et al. 2001), Serial Protein Misfolding Cyclic Amplification (sPMCA) (Chen et al. 2010; Rubenstein et al. 2010, 2011), and Real-Time Quaking Induced Conversion (RT-QUIC) (Atarashi et al. 2011a, b; Orru et al. 2011) have been very successful in amplifying PrP^{Sc} in biological fluids. However, great care must be taken to avoid false positives that may result from the amplification of minute cross contamination or through PrP^{Sc}-independent fibril formation (Rubenstein et al. 2010; Cosseddu et al. 2011).

15.5.3 Ultrasensitive Detection Methods for a Definitive Antemortem Test for PrP^{Sc}

In recent years, a number of ultrasensitive methods for the detection of PrP^{Sc} in biological fluids have been reported in the literature. Two of these methods, PMCA-coupled SOFIA, and rtQuIC-coupled PrP^{Sc}-specific IP (IP-RT-QuIC) with ThT fluorescence detection, involve the massive amplification of PrP^{Sc} with concurrent massive dilution of PrP^{C} to discern PrP^{Sc} vs. PrP^{C}. Another recent ultrasensitive detection method utilizes a solid state capture matrix consisting of stainless steel particles coupled with direct immunodetection (Edgeworth et al. 2011).

Regardless of the method(s) used to increase test sensitivity for antemortem PrP^{Sc} detection, PrP^{Sc}-specific Abs are very highly desired to avoid the sensitivity pitfall associated with the reliance on PK digestion, and the specificity issue associated with relying upon the massive amplification of PrP^{Sc} to discern PrP^{Sc} vs. PrP^{C}.

15.6 Production of PrP^{Sc}-Specific Antibodies: Overcoming Self-Tolerance

The unique character of prion diseases, in which the structure of a ubiquitous normal host protein undergoes template-mediated conformational conversion to a pathological form, leads to unique challenges and opportunities. Overcoming these challenges requires a combinational approach with some insightful innovation.

The challenge of overcoming self-tolerance for the production of PrP^{Sc}-specific mAbs can be avoided by using $Prnp^{0/0}$ null animals that do not recognize PrP proteins as self. However, this approach normally results in the production of IgM Abs (Tayebi et al. 2004, 2009, 2011; Nikles et al. 2005; Buchholz et al. 2006). IgM Abs is not as useful as IgG Abs in diagnostic applications due to low affinity, and they are not as useful in therapeutic applications for the following reasons: IgM cannot cross the blood–brain barrier, they diffuse through the tissues poorly, they provide only short-lived immunity, and they can cause the inappropriate activation of 3Cb complement.

B-cell tolerance to self proteins can be overcome by using one or a combination of strategies such as virus-like particles, carrier proteins, carrier micro-beads, harsh adjuvants, intensive inoculation schemes, and epitope expansion.

In addition to overcoming self-tolerance by technical means, one can also use a hypothesis-based approach to target an epitope that is uniquely exposed in, or attached to, the infectious form of PrP.

15.6.1 Overcoming B-Cell Tolerance to Self Proteins

Unfortunately, the exposure of PrP null mice to PrP^{Sc} proteins without the use of harsh adjuvants, carriers, or Virus Like Particles (VLPs), etc., regularly induces the production of IgM Ab in mice (Tayebi et al. 2004, 2009, 2011; Nikles et al. 2005; Buchholz et al. 2006). This seemingly default tendency of the murine immune system to produce IgM upon exposure to PrP^{Sc} is manifested at the T helper (Th) cell level. Completely overcoming self-tolerance and the induction of IgG vs. IgM anti-self mAbs depends upon how Ag is presented to the immune system.

The use of Virus-Like Particles (VLPs) engineered to display self peptides tends to induce VLP specific T helper type 1 (Th-1) cell assistance that is required for the

induction of anti-self IgG production by B cells. Examples of this are RNA phage Qβ VLPs (Kozlovska et al. 1996), Papilloma VLPs (Chackerian et al. 2001), C-type retroviral display of PrP on retrovirus-like particles such as Murine Leukemia Virus (MLV) (Nikles et al. 2005), and PrP displayed on Bovine Papilloma Virus type 1 (BPV-1)-like particles (Handisurya et al. 2007).

Carrier proteins such as Keyhole Limpet Haemocyanin (KLH) (Paramithiotis et al. 2003), Blue Carrier Protein (BCP) (Pilon et al. 2007), and Leukotoxin Carrier Protein (LKT) (Hedlin et al. 2010) can be used in a similar fashion to induce anti-self IgG production by B cells.

Solid phase immobilization/concentration of PrP^{C} and PrP^{Sc} using inert material, such as magnetic beads, can also be used as a carrier to properly present Ag to the immune system (Tayebi et al. 2004).

Several successful examples of the use of strong adjuvants and intensive immunization protocols to enhance the immune response to PrP^{Sc} immunization have been published. Some of the adjuvants that have been used include Complete Freund's adjuvant (Korth et al. 1997), CpG oligodeoxynucleotide (ODN 1826) TLR9 agonist (Spinner et al. 2007), and Emulsigen-D (Hedlin et al. 2010). Intensive immunization protocols can include large amounts of immunogen and many injections over long periods of time. Such treatments have been useful for the production of PrP^{Sc}-specific Ab, but may be impractical or too harsh for use in human beings.

15.6.2 Epitope Expansion

The principles of epitope expansion can be used to produce highly immunogenic recombinant peptides for immunization and the induction of auto-antibodies. Epitope expansion exploits the tendency of the mammalian immune system to strongly respond to antigenic epitopes that are regularly arranged at a spacing of 5-10 nm and are repeated in close proximity to each other (Chackerian et al. 2001; Bachmann et al. 1993; Bachmann and Zinkernagel 1996; Fehr et al. 1997). For example, the YYR epitope published by Paramithiotis et al. (2003) can be serially repeated, either forward and/or backwards to produce a much more immunogenic peptide. In this publication, Paramithiotis et al. designed and used an innoculum consisting of a peptide with the Amino Acid (AA) sequence CYYRRYYRYY conjugated with KLH and formulated in Freund's complete adjuvant to immunize mice and to successfully produce PrP^{Sc}-specific monoclonal Abs.

15.6.3 Hypothesis-Driven PrP^{Sc}-Selective Epitope

Complex proteins have epitopic regions which are normally sequestered from exposure to the immune system by tertiary structural elements and post-translational modifications. It stands to reason that epitopic regions of PrP which are normally

buried in PrP^{C} become exposed during the conformational conversion of PrP^{C} to PrP^{Sc}. Such putative "immunologically nonself" epitopes are unlikely to be recognized as self-antigens. These epitopes could induce a robust and specific immune response to PrP^{Sc} with less concern for the inducement of autoimmune disease than targeting self-epitopes common to PrP^{C}. The hypothesis-based approach for the selection of an epitope target and the combinational approach for overcoming self-tolerance are demonstrated in a manuscript published in Nature Medicine by Paramithiotis et al. (2003). This paper describes the development of the first PrP^{Sc}-specific IgM mAbs 1A12 and 17D10.

15.6.3.1 Approach Used by Paramithiotis and Colleagues

Paramithiotis et al. used a hypothesis-driven approach to determine a conformational PrP^{Sc}-specific epitope for immunization and a multifaceted immunization protocol to overcome self-tolerance to PrP^{Sc} in mice expressing endogenous PrP.

Immunizing with peptides-containing multiple tyrosines [Y] has been known to be favorable for the production of conformationally specific mAbs (Price 1995). It had been demonstrated that the conversion of recombinant mouse prion protein to a beta-sheet enriched form is accompanied by increased solvent exposure of tyrosine [Y] side chains (Zou and Cashman 2002). It was also observed that the majority of tyrosine residues in the structured domain of PrP appear in pairs, in a highly conserved manner, across mouse, hamster, sheep, bovine, and human PrP proteins. Two bi-tyrosine AA pairs [YY], located in helix one and beta strand two, are in conjunction with a C-terminal arginine [R] which creates a YYR motif. The mapping of the bi-tyrosine for PrP^{Sc}-specific exposure suggested that the YYR motif in β-strand 2 was the most likely candidate for the production of PrP^{Sc}-specific mAbs.

In order to produce mAbs against the putatively PrP^{Sc} specific YYR epitope, the authors engineered a peptide using the principles of epitope expansion. The YYR motif was repeated forward, backwards, and forward again with an N-terminal cysteine [C] residue to create the immunizing peptide CYYRRYYRYY. The CYYRRYYRYY peptide was then conjugated to the carrier protein KLH, and formulated in Freund's complete adjuvant. Monoclonal antibodies were developed by immunizing and thrice boosting mice expressing endogenous PrP^{C}. The initial screenings of hybridomas were done by testing antibody reactivity on 4-MAP-Tyr-Tyr-Arg-coated plates. Rabbits were also immunized in this manner to produce polyclonal PrP^{Sc} specific IgG Abs.

15.6.3.2 Outcome of the Approach Used by Paramithiotis and Colleagues

Forty PrP^{Sc} selective Abs, including ten PrP^{Sc} specific mAbs, were produced of IgG and IgM isotypes. IgM mAbs 1A12 and 17D10 displayed PrP^{Sc}-specific immunoprecipitation from several scrapie-infected species which include mouse, hamster, sheep, and bovine species. 1A12 and 17D10 also reacted to the surface of living dendritic cells from

scrapie-infected sheep lymph nodes. Moreover, these mAbs have been shown to recognize low concentrations of PrP^{Sc} in the infected mouse spleen. Rabbit polyclonal antisera and mouse monoclonal antibodies directed against YYR peptide immunoprecipitate PrP^{Sc} from prion-infected animal and human brains, but not from uninfected brains.

15.6.3.3 Review and Subsequent Developments

In subsequent experiments, YYR mAbs were able to reduce PrP^{Sc} cellular content in scrapie-infected ScN2a neuroblastoma cells in a concentration and time-dependent manner. This suggests that a prion-specific humoral immune response may be effective in treating prion infection, while sparing the normal cellular isoform of the prion protein (Cashman and Caughey 2004). Moreover, PrP^{Sc}-specific rabbit polyclonal antisera and murine mAbs do not recognize antigens at the cell surface of normal dissociated splenocytes and brain cells, despite the presence of YYR motifs in PrP^{C} and in other non-prion proteins. The apparent lack of YYR-specific antibody reactivity with PrP^{C} or other cell surface proteins suggests that prion immunoprophylaxis may be possible in animals with minimum risk of altering the expression and function of PrP^{C}.

Recently, through strategic expansion of the epitope and application of a leukotoxin-based delivery platform, the YYR epitope has been translated to a vaccine which induces robust PrP^{Sc}-specific immune responses in ruminants (Hedlin et al. 2009).

A thermodynamic algorithm has been developed to predict regions of the prion protein which are most likely to be exposed by misfolding (Guest and Plotkin 2008). These epitopes, as either univalent or multivalent vaccines of other PrP^{Sc}-epitopes, may offer the opportunity for effective and targeted prion immunotherapy.

Another hypothesis-based approach is to discover and target sites on PrP^{Sc} that are involved in the binding of PrP^{Sc} to PrP^{C} and/or in the conversion of PrP^{C} to PrP^{Sc}. Recently, it has been published that the interaction between PrP^{C} and PrP^{Sc} is mediated by specific sites, which have been mapped to six putative 'binding and conversion domains' (PrP-BCD) through peptide and antibody competition studies (Li et al. 2009). These PrP-BCDs suggest that there are PrP^{Sc}-specific BCDs that could be targeted for the production of PrP^{Sc}-specific Abs. Such Abs would not only be specific, but they would likely interfere with the binding and conversion process and be prime candidates for a putative immunotherapy.

15.7 A Brief History of the Design and Production of PrP^{Sc}-Specific Antibodies

The production of PrP^{Sc}-specific antibodies has been challenging. Only a few laboratories have demonstrated success since the first PrP^{Sc}-specific mAb was produced by Korth et al. (1997). Their successes, innovations, and novel contributions are briefly summarized in Table 15.1 below:

Table 15.1 A Brief History of the Design and Production of PrP^{Sc} specific Antibodies

PrP^{Sc}-specific Ab	References	Ig class	Production strategy	PrP^{Sc}-specificity	Epitope(s) recognized	Published comments	Additional comments
15B3	Korth et al. (1997)	IgM	PrP null mice inoculated with Rec Bov PrP in Freund's complete adjuvant	Bov, Mu, Hu	GSDYEDR, YYRPVDQYS, and CITQYQRES QAYY	15B3 also binds infectious and noninfectious oligos and aggregates Biasini et al. (2008)	Orru et al. used 15B3 for pre-QuIC concentration Orru et al. (2011)
10 mAbs including: 1A12, and 17D10; plus 30 pAbs	Paramithiotis et al. (2003)	Mu IgM mAbs, plus Goat and Rabbit pAbs	BALB/c mice inoculated with KLH conjugated to the synthetic peptide CYYRRY YRYY in Freund's complete adjuvant (FCA); Pabs were raised in goats and rabbits inoculated with KLH conjugated CYYR peptides in FCA	Bov, Mu, Hu, Ham, and Sheep BH infected with scrapie. Surface of dendritic cells from scrapie-infected sheep lymph nodes	YYR	YYR mAbs reduce PrP^{Sc} cellular content in ScN2a neuroblas toma cells Ca shman and Caughey (2004). YYR with LKT carrier vaccine induces robust PrP^{Sc}-specific immune response in ruminants Hedlin et al. (2009)	Hypothesis-driven approach to determine a conforma tional specific epitope for immunization

3 mAbs including the IgG1 mAb V5B2	Curin Serbec et al. (2004)	IgG1	BALB/c mice inoculated with KLH conju gated to the synthetic peptide CITQYER ESQAYY corresponding to AAs 214–226 of the C-terminus of HuPrP	Hu sCJD without PK digestion, specificity disrupted by denaturation	CITQYERE SQAYY	V5B2 recognizes dimers, oligomers, PrP^{Sc} and fibril-like aggregates Ulrih et al. (2006); scFv from V5B3 Skrlj et al. (2010); humanized scFv from V5B3 Skrlj et al. (2011); V5B2 binds truncated form of PrP^{Sc} ending in Y226 not full length PrP^{Sc} Kosmac et al. (2011)

(continued)

Table 15.1 (continued)

PrP^{Sc}-specific Ab	References	Ig class	Production strategy	PrP^{Sc}-specificity	Epitope(s) recognized	Published comments	Additional comments
OCD4 DNA binding mAb and DNA binding protein g5p	Zou et al. (2004), Yokoyama et al. (2001)	anti-DNA mAb and a DNA binding protein	OCD4 produced according to Yokoyama et al. (2001) against nuclear DNA; g5p produced in E. coli trans formed with a Ff gene 5 plasmid	Hu BH from sCJD type 1, sCJD type 2, vCJD, fCJD, and PK digested fragments from sCJD, fCJD, and GSS BH; BH from scrapie-infected sheep, deer, hamsters, and mice; and BH from BSE-infected cattle.	DNA bound specifically to PrP^{Sc}		

bS-36, bS-43, Sha-9, Sha-29, and Sha-52	Morel et al. (2004)	1 IgG and 4 IgM	βS series Abs were raised against a mutated form of β-folded Murine rPrP (23–231). Sha series Abs were raised against PK treated native SAF from Syrian hamster-infected brain (263K). Both series were produced in PrP null mice	βS-43, βS-36, SHA-52, and SHA-9 bind Scrapie-infected Sheep BH and hCJD BH SAFs; SHA-29 binds PrPSc from hCJD BH	SAF	Biasini et al., stated that PrP^{Sc}-specific but epitope-independent binding may be due to solubilization conditions induced by detergent (Biasini et al. (2008)	Authors also reported on 9 mAbs that were raised against proteins, peptides, or haptens that are unrelated to PrP but specifically IP'd PrP^{Sc} through binding at the Fc portion of the Ab (and not the paratope).

(continued)

Table 15.1 (continued)

PrP^{Sc}-specific Ab	References	Ig class	Production strategy	PrP^{Sc}-specificity	Epitope(s) recognized	Published comments	Additional comments
IgG 89–112, and IgG 136–158 Motif grafted Abs	Moroncini et al. (2004, 2006)	Motif grafted human IgG1 Ab β12	Genetically engineered β12 IgG Ab with its HCDR3 region replaced with AA peptide sequence of 2 putative PrP^{C}-PrP^{Sc} binding sites of murine PrP^{C} (AA 96–104 and Aas 133–158)	Human, murine, and hamster PrP^{Sc} from infectious BH, but not PrP^{C}	AAs 89–112 and 136–158 of murine PrP^{C}, respectively	Moroncini et al. (2006) IHC showed PrP^{Sc}-specific staining with motif grafted Ab 89–112 on Hu brain with sCJD, vCJD, and GSS. EM of GSS cerebellum showed IgG 89–112 was confined to amyloid plaques Solforosi et al. (2007)	Biasini et al. demonstrated that IgG 89–112 and IgG 136–158 also bound multiple types of infectious and noninfectious PrP oligomers and aggregates Biasini et al. (2008)

45 Motif grafted Abs	Solforosi et al. (2007)	Motif grafted human IgG1 Ab β12	Duplicated the strategy by Moroncini et al., and added an additional PrP^{C}-PrP^{Sc} binding site (AA 19–33)	IP BH from RML-infected mice as well as the PK digested fragments PrP27-30	AAs 89–112, 136–158, and 19–33 of murine PrP^{C}, respectively		Biasini et al. demonstrated that IgG 89–112, IgG 136–158, and IgG 19–33 also bound multiple types of infectious and noninfectious PrP oligomers and aggregates Biasini et al. (2008)
P1:1	Jones et al. (2009)	IgM	Aggregated PrP 106–126 in Freund's complete adjuvant, inoculated s.c. into $PrnP^{0/0}$ mice.	Full length and/or PrP^{Sc} in vCJD, sCJD, and GSS from Human BH	Aggregated form of PrP 106–126		

(continued)

Table 15.1 (continued)

PrP^{Sc}-specific Ab	References	Ig class	Production strategy	PrP^{Sc}-specificity	Epitope(s) recognized	Published comments	Additional comments
6H10	Horiuchi et al. (2009)	IgG2b	Non-denatured purified PrP^{Sc} from infected mice inoculated into $PrnP^{0/0}$ mice, hybridomas screened with non-denatured PrP^{Sc}	Non-denatured, PK digested, and aggregated PrP^{Sc} from infected mice, sheep, and cattle			
3B7 (IgG2a mAb); plus 2C4, 1B12, and 3H6 (IgG2b mAbs)	Ushiki-Kaku et al. (2010)	one IgG2a, and three IgG2b mAbs	Native PrP^{Sc} in TitreMax Gold inoculated into PrP null mice. Hybridomas screened by reactivity to denatured and native mouse scrapie BH	3B7, 2C4, and 1B12 IP'd PrP^{Sc} from scrapie-affected mice and hamsters, and CWD-affected white-tailed deer. 3H6 only IP'd PrP^{Sc} from scrapie mouse BH			Authors declare that 3H6 mAbs are the first probes to detect a species-specific PrP^{Sc} conformation during prion propagation

W68 and W261	Petsch et al. (2011)	W268 is IgG1	NaPTA precipitated RML6 PrP^{Sc} mixed with ABM adjuvants inoculated i.p. into $PrnP^{0/0}$ mice. Hybridomas made with P3X63Ag8.653 cells and screened for reactivity to mouse recPrP	W68 and W261 specifically IP'd PrP^{Sc} and the PK resistant core from scrapie-infected BH. W261 also IP'd PrP^{Sc} from the BH of CWD-infected white-tailed deer and all pathological isoforms of CJD in Hu BH

(continued)

Table 15.1 (continued)

PrP^{Sc}-specific Ab	References	Ig class	Production strategy	PrP^{Sc}-specificity	Epitope(s) recognized	Published comments	Additional comments
Four PRIOC IgM mAbs	Tayebi et al. (2011)	IgM	RML mouse BH pretreated with congo red, heating, or untreated before PK digestion and absorption to microbeads. FVB/N Prnp null mice inoculated s.c. with beads in Complete Freund's adjuvant. Hybridomas screened for reactivity to full length and truncated mouse PrP and recombinant PrP	PRIOC1 specificly IP'd PrP^{Sc} from RML BH, and human type 4 CJD BH after PK digestion. PRIOC mAbs 2, 3, and 4 all strongly IP'd RML BH and human type 4 CJD BH whether it was denatured or not. PRIOC mAbs could not detect PrP^{Sc} from infected Ovine or Bovine BH	PRIOC1 and -2 recognize a(n) eptitope(s) between AA 90–109 of PrP. PRIOC3 and 4 weakly bind an epitope between AA 170–189 of PrP		

Abbreviations: *mAb* monoclonal antibody, *s.c.* subcutaneous(ly), *i.p.* intraperitoneal(ly), *i.v.* intravenous(ly), *PBS* phosphate-buffered saline, *Ag* antigen, *ELISA* enzyme-linked immunosorbent assay, *ELIFA* enzyme-linked immunofluorescent assay, *BSE* bovine spongiform encephalopathy, *I.P.* immunoprecipitation, *AA(s)* amino acid(s), *NMR* nuclear magnetic resonance, *FCA* Freund's complete adjuvant

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Chapter 16
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 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 in the difficulty of early diagnosis at a time before significant neurological damage has already occurred. 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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16.1 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.

16.2 Treatment

16.2.1 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).

16.2.2 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 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 other common 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, CSF protein tests, and the cerebral MRI are helpful, but these tests are not absolutely specific, generally playing a supportive role (Chap. 13). There are, currently, no noninvasive, clinical diagnostic tests completely validated for sCJD. In vCJD, the illness progression is typically slower, with a median illness duration of around 14 months and there is a potentially useful, disease-specific (albeit somewhat invasive), test in the form of tonsil biopsy (Chap. 13). However, the presentation of vCJD is very nonspecific, typically consisting of psychiatric features without specifically 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.

The development of disease-specific tests might allow accurate diagnosis at earlier, lesser, stages of neurological impairment; recent reports of a blood test for vCJD and a CSF test for sCJD may prove useful (Edgeworth et al. 2011a, b; Atarashi et al. 2011; McGuire et al. 2012) (the current status of diagnostic test development is reviewed in Chap. 13).

16.2.3 Disease-Modifying Treatment

A systematic review has 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, Anti-oxidants, 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 neuro-protection. 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).

16.2.4 Preventative Treatment

Given the often fulminating disease course (for example 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) in *PRNP*-D178N mutation carriers is planned in Italy (reference to Chap. 7 (2)).

16.2.5 Treatment: Discovering Potential Treatments

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 well 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 favor the last of these (Weissmann and Aguzzi 2005; Zanusso and Monaco 2005).

The selection of potential treatments has been based on their potential effects on PrP^{C}, the conversion of PrP^{C} to PrP^{Sc} (the abnormal disease-related prion protein), or the aggregation and accumulation of PrP^{Sc} in tissues. Experimental work has shown that PrP^{C} is required for successful transmission of prion disease and, while the normal role of PrP^{C} is 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). As a result, one therapeutic approach is based on removal of PrP^{C} by using antibodies against PrP^{C}. Immunomodulatory approaches to treatment are reviewed in Chap. 7(3). Another set of approaches is to identify molecules that could stabilize PrP^{C}, prevent its conversion to PrP^{Sc}, destabilize PrP^{Sc}, or to break up aggregations of PrP^{Sc}. The last of these 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 were released. A useful review of therapeutic approaches to prion diseases was published in 2005 (Weissmann and Aguzzi 2005).

Three general steps can be taken to identify possible treatments: in vitro cell-free studies, cell culture studies, and in vivo animal experiments. These entirely reasonable, desirable steps have potential limitations: success in a chemical or cell-line setting 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 assays at least allow for relatively rapid, high-throughput searches for anti-prion disease compounds (Kocisko and Caughey 2006).

Quinacrine was suggested as a treatment on the basis of in vitro work (Korth et al. 2001). Subsequently, it has been used in animal experiments and humans, with no significant efficacy (Collins et al. 2002; Collinge et al. 2009). Animal experiments involving intra-cerebro-ventricular administration of Pentosan Polysulphate (PPS) showed promising results (Doh-Ura et al. 2004). Subsequent treatment of human prion disease has suggested some slowing of disease progression in some cases (most convincingly in vCJD) but without effects in most cases, without consistent effects on brain disease-related PrP and without halting progression (Tsuboi et al. 2009; Honda et al. 2012; Bone et al. 2008).

16.2.6 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 established 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, etc.) 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. 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.

16.2.7 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 was 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.

16.2.8 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, the Flupirtine trials succeeded in this (Otto et al. 2004).

16.3 Decontamination

16.3.1 The Background to Decontamination Concerns

The existence of iatrogenic CJD justifies the development of decontamination procedures for prion disease (ref to appropriate chapter section).

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. In relation to the last point, 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 2M sodium hydroxide are effective but not practical in routine practice (ACDP REF). Various autoclaving protocols involving 134–137 C reduce infectivity but cannot be relied upon for its complete removal (ACDP ref). 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 UK (Hilton et al. 2004; de Marco et al. 2010). 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. 2006).

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 (for example, 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 UK, because of estimates of vCJD subclinical infection prevalence in the population, with the potential involvement of reticulo-endothelial 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.

16.3.2 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. Updated UK ACDP guidelines can be found on the relevant website (refs below 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, and an enzyme-detergent method (Baxter et al. 2005; Rogez-Kreuz et al. 2009; Jackson et al. 2005).

16.3.3 Assessment of Decontamination Methods

As the ultimate nature of prion infectivity remains uncertain, determination of infectivity and the effectiveness of decontamination processes has been 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. However, they are expensive and time-consuming.

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).

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). Therefore, general extrapolation of any specific experimental determination of decontamination is not necessarily valid.

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Chapter 17
Immunomodulation

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Abstract The underlying pathogenesis of prion diseases (prionoses) is related to an autocatalytic conformational conversion of 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) Colby (Cold Spring Harb Perspect Biol 3:a006833, 2011). Currently, all prion diseases are without effective treatment and are universally fatal [Trevitt and Collinge (Brain 129:2241–2265, 2006); Muller-Schiffmann and Korth (BioDrugs 22:45–52, 2008); Brazier et al. (Expert Rev Anti Infect Ther 7:83–105, 2009); Li et al. (Immunotherapy 2:269–282, 2010); Wisniewski and Goni (Expert Rev Vaccines 9:1441–1452, 2010)]. The conformational change of PrP in prion diseases is associated with a negative gain of function; however, prion-like protein conformational changes are increasingly being recognized as a normal biological trait in mammals and lower species [Tuite and Serio (Nat Rev Mol Cell Biol 11:823–833, 2010); Hou et al. (Cell 146:448–461, 2011); Moresco (Cell Res 21:1643–1645); Si et al. (Cell 140:421–435, 2010); Wickner (Semin Cell Dev Biol, 22:469, 2010)]. The growing understanding of these protein conformational changes in biological processes opens the possibility of therapeutic targeting, when this phenomenon occurs in association with disease. The past experience with bovine spongiform demic of chronic wasting disease (CWD), has highlighted the need to develop prophylactic and/or therapeutic approaches. In Alzheimer's disease (AD), also a conformational neurodegenerative disorder, both passive and active immunizations have been shown to be highly effective in model animals at preventing disease and cognitive deficits, with emerging data from human trials suggesting that this approach can partially ameliorate amyloid plaque and tau pathology [Selkoe (Nat Med 17:1060–1065, 2011); Wisniewski and Boutajangout (Brain Struct Funct 214:201–218, 2010)]. Human prion diseases are most commonly sporadic; hence the therapeutic need is primarily to stop progression; however, in animals the majority of

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prionoses are infectious and the emphasis is on prevention of transmission. 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, whilst at the same time producing a modulated systemic immunity that is unlikely to be associated with toxicity. Nevertheless, 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 autoimmune toxicity. Our results using an attenuated Salmonella vaccine strain expressing the prion protein showed that mucosal vaccination can protect against prion infection from a peripheral source, suggesting the feasibility of this approach. The current epidemic of CWD, with its potential to spread to human populations, emphasizes the importance of developing such immunomodulatory approaches more fully.

Keywords Amyloid β • Chronic wasting disease • Conformational disorders • Creutzfeld–Jakob disease • Oligomers • Prion • Vaccine

17.1 Bovine Spongiform Encephalopathy, Variant Creutzfeldt–Jakob Disease, and Chronic Wasting Disease

Interest in developing potential therapeutics for prionoses has 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). BSE was first identified among cattle in the United Kingdom in 1985, with its emergence being related to the practice of feeding meat-and-bone meal from animal carcasses to cattle (Collee and Bradley 1997; Harman and Silva 2009). The original source of the BSE is unknown, but is presumed to be either a sporadic BSE case or a more infectious strain of scrapie. 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 (Harman and Silva 2009). This led to the emergence of variant CJD (vCJD) with the first case being recognized in 1996 (Mackay et al. 2011). Since the original report in 1995 a total of 225 probable or confirmed cases of vCJD have been diagnosed, 176 in the Great Britain, 25 in France, 5 cases in Spain, 4 cases in Ireland, 3 in the USA, and a few cases elsewhere (for latest numbers, see http://www.cjd.ed.ac.uk/vcjdworld.htm). It has been difficult to predict the expected future numbers of vCJD. The most recent modeling suggests about 390 further cases between 2,010 and 2,179 (Mackay et al. 2011; Garske and

Ghani 2010). Since the vCJD agent is present at high levels in lymphatic tissue, screening for PrP^{Sc} was performed in 2004 on sections from lymph nodes, tonsils, and appendices archived in the United Kingdom. Three out of 12,674 randomly selected cases showed evidence of subclinical infection, leading to a prediction that about 4,000 vCJD further cases may occur in the United Kingdom (Hilton 2006). A second larger study of anonymous tonsil biopsies performed in 2008 found no positive samples (Clewley et al. 2009), while the most recent tonsil biopsy study revealed one positive result from 9,160 patients from the 1961 to 1985 birth cohort, given a similar prevalence result to the 2004 study (de Marco et al. 2010). There is much uncertainty about future predictions of vCJD prevalence since there is a lack of knowledge regarding the time of incubation, the number of patients who could be infected from a given dosage of BSE agent, and other factors which can govern an individual's susceptibility to clinical infection. Furthermore, it is not known if all subclinical infections will progress to disease and also whether the reported screening of lymphoid tissue would capture all subclinical cases. A complicating factor for estimating future numbers of vCJD is the documentation of several transfusion-associated cases. These occurred after incubation periods of 6–8 years. One of these disease-associated donations was made more than 3 years before the donor became symptomatic, suggesting that vCJD can be transmitted from silently infected individuals (Brown et al. 2006). An additional point of concern is regarding the cases with a methionine/valine (MV) genotype at the 129 codon of the PRNP gene. So far all the clinically symptomatic cases of vCJD had the MM codon 129 genotype. However, two clinically non-symptomatic patients with the MV genotype were found to be infected (one from a blood transfusion and the other was found to be positive from a random appendix and tonsil specimen survey of the population) (Ironside 2010; Brown 2008). The finding of such carriers raises the possibility of secondary spread of infection via blood transfusion, surgical procedures, or tissue transplants from individuals who likely have much longer (or possibly lifelong) asymptomatic infection. Currently, no method exists for the screening of vCJD blood contamination (Puopolo et al. 2011; Zaman et al. 2011), although such assays are in development (Edgeworth et al. 2011). Hence, the risk of vCJD spreading via blood transfusion remains a possibility. In the USA, BSE surveillance methods are felt by many to be inadequate and there may be asymptomatic carriers of vCJD in the USA who likely acquired their infection elsewhere and are donating to the US blood supply (Bishop et al. 2006; Clarke et al. 2007). Furthermore, several atypical strains of scrapie (atypical/Nor98 scrapie) and BSE (BSE-H and BSE-L) have been documented with possibly greater transmissibility potential to humans (Tranulis et al. 2011; Kong et al. 2008). It has been suggested that atypical BSE may be responsible for a type of CJD (type MV2) previously thought to be sporadic given similarities in the Western blot pattern of the PrP^{Res} (Tranulis et al. 2011; Casalone et al. 2004). Another study noted the presence of ~14 and ~7 kDa fragments of PrP^{Res} in BSE-H which are similar to those found in some CJD cases (Biacabe et al. 2007). Hence, it is possible that a percentage of CJD cases thought to be sporadic are in fact of infectious origin. Consistent with this hypothesis, it has been shown

that BSE-L is transmissible to non-human primates (cynomolgus macaque monkeys) (Comoy et al. 2008) and to transgenic mice expressing either normal or elevated levels of human M129 PrP with a higher transmission rate than that observed with classical BSE (Kong et al. 2008; Beringue et al. 2008), suggesting that there is no significant species barrier between BSE-L in cattle and humans.

Chronic wasting disease (CWD) appears to be the most infectious prionoses to date, affecting free-ranging and farmed ungulates (white-tailed deer, mule deer, elk, and moose) (Williams 2005; Aguzzi and Sigurdson 2004; 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 (Williams 2005; Williams and Young 1980, 1982). CWD has been detected in 19 states of the USA, three Canadian provinces, and in South Korea (Gilch et al. 2011). In captive cervid populations, up to 90% of animals have been reported to be prion positive, while in wild cervid populations the prion infection prevalence has been as high as 30%. Transmission of CWD is thought to be mainly horizontal via a mucosal/oral route (Gilch et al. 2011; Beekes and McBride 2007; Safar et al. 2008). The occurrence of CJD among three young deer hunters from this same region raised the speculation of transmission of the CWD to humans (Belay et al. 2004). Autopsy of these three subjects did not show the extensive amyloidosis characteristic of the vCJD and CWD (Liberski et al. 2001). However, like BSE, CWD is transmissible to non-human primates (squirrel monkeys) (Marsh et al. 2005; Race et al. 2009a). 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 that there is a significant species barrier which is greater than 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 recently 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. Future transmission studies using non-human primates and human PrP expressing Tg mice will need to be repeated with all known CWD strains and also will need to take into account multiple PrP polymorphisms in the CWD source and human PrP (Collinge 2010; Johnson et al. 2011). 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 (Safar et al. 2008; Angers et al. 2006, 2009; Mathiason et al. 2006, 2010; Race et al. 2009b; Haley et al. 2009; Tamguney et al. 2009). Therefore, the possibility of transmission to humans needs to be closely monitored. A recent study 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 30% in some endemic areas to <1% in states in which CWD has only recently been discovered, such as West Virginia and New York (Williams 2005; Sigurdson 2008). Overall, over 6.6 million deer and 6.9 million total cervid species are harvested annually in the USA. It is therefore certain that human exposure has occurred and continues to occur, by direct contact in hunters and game processors, by consumption of venison, or by contact with products from cervids. Furthermore, the preclinical 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 (Smith et al. 2011; Johnson et al. 2007). The likely exposure of humans to CWD-infected tissue is difficult to estimate. In a 2006–2007 population survey conducted by FoodNet in the USA, it was found that of 17,373 survey responders 18.5% had hunted deer or elk and 1.2% had hunted deer or elk in CWD endemic areas (Abrams et al. 2011). Of the 11,635 responders who had consumed venison, 88.6% had obtained it from the wild (Abrams et al. 2011). Hence, it is likely that a large population is being exposed to CWD-infected food. 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 to develop better vaccination/immunomodulation approaches to prevent CWD transmission and uncontrolled spread. The development and testing of such potential approaches are discussed below.

17.2 The Immune System and Prion Infection

PrP^C is expressed in T and B lymphocytes, natural killer (NK) cells, platelets, monocytes, dendritic cells (DC), and follicular dendritic cells (FDC) (Aguzzi and Heikenwalder 2005). Because of this expression pattern and the lack of an immune response to a self-antigen, the immune system is an active player very much involved in the peripheral replication of the prion agent and its ultimate access to the CNS

(Wisniewski and Goni 2010; Aucouturier et al. 2000). Paradoxically immune suppression with, for example, splenectomy or immunosuppressive drugs increases the incubation period (Aucouturier et al. 2000), while nonspecific 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). Lymphatic organs such as the spleen, tonsil, lymph nodes, or gut-associated lymphoid tissue (GALT) contain high concentrations of PrP^{Sc} long before PrP^{Sc} 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^{Sc} 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 whilst avoiding potential autoimmune toxicity (Sigurdsson and Wisniewski 2005; Goni et al. 2008; Wisniewski et al. 2007). 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.

17.3 In Vitro Studies

A precise understanding of the molecular mechanisms and pathways involved in the PrP^{C} to PrP^{Sc} conversion remains to be elucidated; however, there is abundant 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 minor differences in the primary sequence of PrP^{C} in different species. For example, it has recently been shown that amino acid polymorphisms at positions 170 and 174, in the β2-α2 loop, are critical for transmission within and between species (Sigurdson et al. 2009, 2010, 2011). It is not surprising that antibodies that may alter or mask the critical epitopes on PrP^{C} and/or PrP^{Sc}, involved during the mutual conformational complementarity required in prion propagation, will be inhibitory for prion replication. This was initially demonstrated in 1988 when an anti-PrP^{C} polyclonal antibody was used ex vivo on a prion preparation prior to

inoculation; a two-log reduction in infectivity was noted (Gabizon et al. 1988). Using scrapie-infected cells it was later shown that an anti-PrP mAb, 6 H4 directed to residues 144–152, was able to clear infection in vitro (Enari et al. 2001). In the same year Peretz et al. (2001) used a number of different PrP-specific Fab fragments for scrapie clearance in chronically infected N2a cells. 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. We also tested a panel of anti-PrP 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 critical PrP domains. We have also shown that 6D11 is able to inhibit prion infection in vivo, with a very significant prolongation of the scrapie incubation period and a reduction of the severity of symptomatic disease; however, all treated animals eventually came down with infection (Pankiewicz et al. 2006). Interestingly, it has been reported that deleting residues 32–134 in the PrP molecule but not residues 23–134 produces symptoms of neuropathology, suggesting that residues 23–31 (KKRPKPGGW), a positively charged segment, are required for a toxic phenotype (Westergard et al. 2011). This segment is involved in binding glycosaminoglycans that may inhibit toxicity most likely by interfering with a membrane-associated target site, consistent with the hypothesis that chaperones or facilitator molecules that interact with different parts of the PrP molecule are involved in its conformational transitions. Given the fact that so many parts of the PrP molecule are involved in different stages of the PrP^{C} to PrP^{Sc} conformational change, it is likely that in the more complicated in vivo situation, an optimal strategy will be the concurrent use of two or more anti-PrP mAbs (or derivatives thereof) which target a number of the critical regions for the PrP^{C} to PrP^{Sc} conversion. This greater difficulty of obtaining in vivo therapeutic effects with passive immunization is highlighted by some subsequent studies where anti-PrP antibodies which were able to clear PrP^{Sc} infection in tissue culture only produced a minimal (but statistically significant) delay in the incubation period when used in vivo (Petsch et al. 2011).

17.4 Passive Immunization for Prion Infection

In an initial passive anti-PrP immunization study using wild-type CD1 mice, we showed, using mAbs 8B4 (to mouse PrP residues 34–52) and 8 H4 (to mouse PrP residues 175–185) given immediately after challenge with 139A scrapie by intraperitoneal (ip) injection (50 μg/week), that this 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). In another study using higher doses (4,000 μg/week ip) of either ICSM 18 (to mouse PrP residues 146–158) or ICSM 35 (to mouse residues 95–105), it was shown 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 (White et al. 2003). This type of approach could be used immediately following accidental exposure in humans to prevent future infection. However, passive immunization has not been found to be effective closer to the clinically symptomatic stages of prion infection. Also, passive immunization would be an approach that is too costly for animal prion diseases. As mentioned above, passive immunization has also been shown to be effective at inhibiting prion infection when initiated immediately or within 30 days after peripheral prion infection (Sigurdsson et al. 2003; White et al. 2003; Sadowski et al. 2009). Presumably the lack of greater therapeutic efficacy of these passive immunization approaches is related to the poor blood–brain barrier (BBB) permeability of the anti-PrP antibodies. Interestingly, Song et al. (2008) showed therapeutic efficacy with anti-PrP antibodies up to 120 days post-inoculation, using direct intraventricular infusion. However, a potential problem with such an approach is that neuronal apoptosis has been reported to occur in response to some anti-PrP antibodies being directly applied to the CNS (Solforosi et al. 2004; Lefebvre-Roque et al. 2007), suggesting that the characteristics of the antibodies to be used will be an important factor determining the probability of toxic side effects.

17.5 Active Vaccination for Prion Infection

In AD model mice it has been definitively shown that active immunization can prevent the onset of cognitive deficits and the development of amyloid lesions (Wisniewski and Sigurdsson 2010). Significantly, this method of treatment is associated with consistent cognitive benefits in the mice (Wisniewski and Boutajangout 2010a; Morgan et al. 2000; Janus et al. 2000; Sigurdsson et al. 2004; Asuni et al. 2006; Goni et al. 2010). An antibody-mediated response is critical for a therapeutic response, since similar results have been obtained with passive immunization (Bard et al. 2000). Active immunization has been tried in humans for AD by Elan, with significant toxicity resulting from the vaccine (Gilman et al. 2005; Wisniewski and Frangione 2005; Wisniewski 2005). In the human phase 2A clinical trial of the Elan AD vaccine (called AN-1792), 18 out of 372 patients worldwide developed symptoms of meningitis or meningoencephalitis with symptoms apparently responding to immunosuppression in most patients (12 patients out of the 18 responded fully) (Selkoe 2011; Gilman et al. 2005). Evidence suggests that patients who developed anti-Aβ titers benefited cognitively from vaccination, including patients among the 12 that initially had complications, although these benefits were modest (Selkoe 2011; Gilman et al. 2005; Hock et al. 2003). The lack of more

dramatic clinical benefits in these patients has been suggested to be related to the vaccination starting too late in the progression on the disease process (Selkoe 2011; Wisniewski and Boutajangout 2010b). Limited autopsy data documented that vaccination resulted in both amyloid clearance and a reduction in tau-related pathology (Selkoe 2011; Boche et al. 2010; Holmes et al. 2008). Hence, it appears that if future protocols can resolve safety issues and the timing at which treatment should begin, a vaccine approach will prove to have important therapeutic value in patients (Wisniewski and Frangione 2005; Weiner and Frenkel 2006). Numerous AD vaccination clinical trials are ongoing (Selkoe 2011; Wisniewski and Konietzko 2008; Lemere and Masliah 2010).

In part because of this success in AD models, similar experiments with anti-PrP antibodies were initiated in prion infectivity culture models as well as active and passive immunization studies in rodent models. Earlier in vivo studies had shown that infection with a slow strain of PrP^{Sc} blocked expression of a more virulent fast strain of PrP, mimicking vaccination with a live attenuated organism (Manuelidis 1998). While we first demonstrated that active immunization with recombinant PrP delayed the onset of prion disease in wild-type mice, the therapeutic effect was relatively modest and eventually all the mice succumbed to the disease (Sigurdsson et al. 2002). This limited therapeutic effect may be explained by the observation that antibodies generated against prokaryotic PrP often do not have a high affinity towards the critical portions of PrP^{C} that are involved in binding and replication and that the anti-PrP titers generated were relatively low (Polymenidou et al. 2004a); however, these anti-PrP^{C} titers correlated well with the increase in the incubation period. Other groups attempted to use active immunization in wild-type animals to prolong the incubation period, with some failing to break immunological tolerance and produce a therapeutic response (Polymenidou et al. 2004b), while in other studies active immunization was verified to have a partially therapeutic effect (Magri et al. 2005; Schwarz et al. 2003; Pilon et al. 2007). These conflicting results reflect methodological differences in terms of the immunogens, immunization methods, adjuvants, animal models, and PrP^{Sc} strains used for challenge, as well as highlighting the difficulty in breaking tolerance to a self-protein. The possibility that immunological approaches that reduce levels of PrP^{C} or limit its availability for conversion to PrP^{Sc} could be effective closer to symptomatic disease is supported by a study where it was shown that the behavioral deficits, impaired neurophysiological function, and early hippocampal spongiform pathology of prion infection could be completely rescued by genetically knocking out the expression of endogenous neuronal PrP^{C} (Mallucci et al. 2007).

The amalgamation of the above data suggested that by departing from the classical vaccination approach and using some specific properties of antibodies, it would be possible to interfere with one or more stages of the initiation or progression of prionoses. A critical issue is that the self-nature of the precursor protein has to be taken into account. Specifically, immunomodulation for prion disease has to overcome tolerance to the original PrP to raise antibodies that will interfere in a neutralizing way primarily with PrP critical binding sites that are involved with one or more of the following functions: facilitation of invasion, the PrP^{C} to PrP^{Sc} interaction, or

interactions with host factors. Concurrently, any humoral or cellular autoimmune cytotoxic effect has to be avoided or minimized. A delicate balance between quality and quantity of therapeutically active immunoglobulin molecules has to be accomplished without knowing a priori what would be the ideal nature of the required immunogen and what could be the best route by which to present it to the immune system.

A potential ideal means of using immunomodulation to prevent prion infection is by mucosal immunization. One important reason for this is that the gut is the major route of entry for many prion diseases such as CWD, BSE, TME, and vCJD. Furthermore, mucosal immunization can be designed to induce primarily a humoral immune response with a secretory IgA response in the gut, avoiding the cell-mediated toxicity that was seen in the human AD vaccine trial. Such a secretory IgA response can prevent entry of the prion agent into the body and has a greater potential to be highly effective, despite the relatively low anti-PrP antibody titers which can be generated. We have been developing prion vaccines that target gut-associated tissue, the main site of entry of the prion agent. One of our approaches is to express PrP in attenuated Salmonella strains, where one or more genes responsible for virulence have been deleted, as a live vector for oral vaccination. Live attenuated strains of *Salmonella enterica* have been used for many years as vaccines against salmonellosis and as a delivery system for the construction of multivalent vaccines with broad application in human and veterinary medicine (Mastroeni et al. 2001; Moreno et al. 2010). A main 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; Tacket et al. 2000; Kirkpatrick et al. 2006). Ruminants and other veterinary species can be effectively immunized by the oral route using live *Salmonella*, to induce humoral mucosal responses (Villarreal-Ramos et al. 1998; Chabalgoity et al. 2000). Salmonella targets M-cells, antigen sampling cells in the intestines, which may also be important for uptake of PrP^{Sc} (Mabbott and MacPherson 2006; Sigurdsson and Wisniewski 2005; Heppner et al. 2001). Hence, this approach is more targeted than prior vaccination studies, likely explaining the improved efficacy (Goni et al. 2005, 2008). The Salmonella vector can also express one or more tandem copies of PrP, producing and delivering a protein product that might simulate the three-dimensional sites critical for the PrP^{C}-to-PrP^{Sc} interaction (Goni et al. 2008). The rationale takes into account that if tolerance is broken, the bulk of the B-cell response will be devoted to producing dimeric secretory IgA in the mucosa, with a discrete, in terms of conventional vaccination experience, systemic IgG level, which will help to maintain a desired number of antibodies with a low risk of autoimmune pathology. Because the V region genes recombined would be the ones selected by the gut immune system through mesenteric lymph nodes, and typically producing neutralizing IgA molecules, most probably the genes selected and the recombinations would be different from the ones obtained by systemic immunization, with a lower possibility of autoimmune inflammatory complications. Our experience,

using 139A scrapie prions in wild-type CD-1 mice, suggests that in animals which have a good anti-PrP mucosal IgA response and a systemic anti-PrP IgG response, full protection against oral challenge with the prion agent is possible (Goni et al. 2005, 2008). Further development of mucosal immunization, which aims for high specificity rather than high antibody response, is likely to lead to an effective means of preventing prion disease in animal and human populations at risk for prion exposure. Since some of the antibodies raised in a mucosal immunization might be effective to neutralize PrP^{Sc} invasion, binding, conversion, or progression, successfully vaccinated animals could be used to produce monoclonal antibodies with the required therapeutic characteristics. Potential therapeutic monoclonal antibodies could be humanized and used to prevent a potential infection or progression. Evidence suggests that more than one monoclonal antibody would be necessary to obtain an effective protection. This approach is actively being pursued in our laboratory, as well as by other groups.

Trials using mucosal immunization to prevent CWD in white-tailed deer are ongoing. We have performed oral inoculations in white-tailed deer, using attenuated salmonella expressing deer PrP. The animals were inoculated orally three times, and three additional boosts were performed which included tonsil and rectal inoculations with the inoculum supplemented with polymerized recombinant deer PrP. Control animals were inoculated with an attenuated salmonella not carrying any foreign protein (Wisniewski et al. 2011). Both groups of animals produced IgA anti-salmonella in plasma, saliva, and feces. However, the vaccinated group had a low titer of anti-PrP IgG and IgA in plasma, as well as anti-PrP IgA in the saliva. Deer immunoglobulins were precipitated from plasma, saliva, and feces, semi-purified, and the heavy and light chains recognized in blots. The purified IgG and IgA from the plasma of a vaccinated animal reacted in Western blots strongly against polymerized PrP and salmonella antigens and to a lesser extent to monomers and dimers of mouse, sheep, and deer recombinant PrP. Control deer showed a reaction only against the salmonella antigens in similar blots. Both groups were challenged with homogenized CWD-infected brains included in food bait. These results show for the first time that a specific antibody response against the self-antigen PrP can be elicited in the biological fluids of large cervid mammals (Wisniewski et al. 2011). We are expecting quantitative results of infection progression or protection, before the end of the year.

Another active immunization approach, with encouraging preliminary results, is to induce active immunization which more specifically targets PrP^{Sc} or the specific shared β-sheet conformation of the pathological conformers found in prionoses and other neurodegenerative diseases. One such approach is based on the hypothesis that in PrP^{Sc} certain epitopes are exposed, normally buried in PrP^{C}. Prior studies have shown that the conversion of recombinant PrP to a more PrP^{Sc}-like state is associated with increased exposure of tyrosine side chains (Zou and Cashman 2002; Paramithiotis et al. 2003). Use of a peptide based on these exposed epitopes was tested in sheep and it gave rise to a PrP^{Sc} selective IgG immune response (Hedlin et al. 2010). We have developed a novel immunomodulatory method that specifically

targets pathological β-sheet-rich conformations, by immunizing with a polymerized British amyloidosis (pABri)-related peptide which has no sequence homology to PrP, amyloid β, or other human proteins. We showed that the pABri peptide through conformational mimicry induces a humoral immune response that recognizes Aβ and neurofibrillary tangles that characterize AD (Goni et al. 2010). This immunogen was able to cognitively rescue AD model mice (Goni et al. 2010) and in human PrP expressing transgenic mice able to induce an immune response to PrP (unpublished data). This type of immunomodulation has the potential to induce a humoral immune response to pathological conformers without the risk of autoimmune using a non-self immunogen, without any risk of autoimmune toxicity.

17.6 Conclusions

None of the conformational neurodegenerative disorders has 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. Recent autopsy and imaging data from clinical trials also are suggestive that this approach can reduce AD pathology. The prion diseases are much less common than AD; however, the past outbreak of vCJD originating from BSE, and the current epidemic of CWD, 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. 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. This approach may be particularly suitable to stem the current epidemic of CWD with its 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 presymptomatic diagnosis are developed). An additional 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 that are central to the pathogenesis of many neurodegenerative conditions.

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