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Bioassays and inactivation of prions

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Abstract

The experimental study of prions requires a model for their propagation. However, because prions lack nucleic acids, the simple techniques used to replicate the propagation of bacteria and viruses are not applicable. For much of the history of prion research, time-consuming bioassays in animals were the only option for measuring infectivity. Although cell models and other in vitro tools for the propagation of prions have been developed, they all suffer limitations, and animal bioassays remain the gold standard for measuring infectivity. A wealth of recent data suggests that β-amyloid (Aβ) and tau proteins, which aggregate in Alzheimer's disease, and α-synuclein, which aggregates in Parkinson's disease, also become prions. Cell and animal models that recapitulate some of the key features of cell-to-cell spreading of these additional prions have been developed.

The unusual resistance of prions to inactivation was an early indication that they represented a different class of infectious agents to bacteria or viruses. Incomplete inactivation of prions has led to medically induced, or iatrogenic, Creutzfeldt–Jakob disease (CJD). Worldwide, the number of elderly individuals who undergo surgery has increased, and due to the unusually long asymptomatic phase of CJD, there are a growing number of cases in which surgical instruments are used on an asymptomatic patient and then reused repeatedly before a CJD diagnosis is confirmed. Recent data suggesting the potential for iatrogenic transmission of Aβ amyloidosis has raised renewed interest in the field of prion inactivation.

BIOASSAY OF PRIONS

The first reports of sheep scrapie are thought to date from the 18th century, and the description of an analogous disease in humans was reported early in the 20th century. The first successful transmission of these diseases to animals, in the 1930s and 1960s, respectively, marked the start of the experimental era of prion research. Initial attempts to transmit sheep scrapie were unsuccessful, likely due to insufficient observation periods. When transmission of sheep scrapie to sheep was finally observed, it was more than one year after inoculation (Cuillé et al. 1936); transmission of sheep scrapie to goats required >2 years (Cuillé et al. 1939). Similarly, experimental transmission of kuru and Creutzfeldt–

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Jakob disease (CJD) to chimpanzees took ~2 years following intracerebral inoculation (Gajdusek et al. 1966; Gibbs et al. 1968). Early attempts to transmit other neurodegenerative diseases produced ambiguous results, and it has only been in the last decade that a wealth of data has accumulated to support the idea that most, if not all, neurodegenerative diseases are caused by proteins that change their conformation to an aggregation-prone state and become self-templating—i.e., prions (Prusiner 2012).

Although the bioassays originally performed in large animals for the prototypical prions were cumbersome, important information was obtained. Bioassays in sheep demonstrated the resistance of prions to formalin and heat (Gordon 1946; Stamp et al. 1959; Pattison et al. 1960). In addition, transmission of sheep scrapie to goats provided the first evidence of prion strains, with inoculated animals showing one of two distinct phenotypes: “drowsy” due to the lethargy manifest during the clinical phase of scrapie, or “hyper” because these animals were highly irritable and easily aroused (Pattison et al. 1961). The difficulties of performing such experiments are highlighted by the calculation of prion titer. An endpoint titration, in which serially diluted samples were inoculated into naïve animals, required an entire herd of goats to quantify the concentration of prions in a single sample (Pattison 1966).

Transmission of scrapie to rodents

The experimental transmission of scrapie to rodents marked a turning point in prion research, enabling many new experimental studies to be performed. The time to disease onset following inoculation (incubation period) for goat-passaged sheep scrapie in mice was around one year (Chandler 1961), but subsequent serial passage in mice shortened and stabilized incubation periods to ~4 months (Chandler 1962).

Another important advance came with transmission of prions to hamsters. Inoculation of the “Chandler isolate” of mouse-passaged prions into hamsters, followed by serial passage, resulted in incubation periods as short as ~60 days (Kimberlin et al. 1977). The shorter incubation periods and the finding that prion titers in terminal hamsters were ~10-fold higher than mice made this the preferred model despite the increased cost of housing hamsters over mice.

Incubation-time assay

Despite the advances of the prion-infected hamster model, endpoint titrations were still time consuming and costly. Typically, ten 10-fold dilutions of brain homogenate were each inoculated into 4–6 hamsters, and the animals were monitored for at least 6 months. Based on observations that the time interval from inoculation to onset of illness increased as the prion dose decreased (Eklund et al. 1963; Hunter et al. 1963), experiments were performed to determine if incubation time could be used to reliably measure titer. Extensive studies showed that the measurement of incubation periods predicted prion titer with a similar precision to that obtained by endpoint titration, but this new approach required less time and fewer animals (Prusiner et al. 1980; Prusiner et al. 1982).

Transgenic mice

The generation of transgenic (Tg) mice overexpressing mouse or hamster PrP genes provided animal models with even shorter incubation times than wild-type hamsters (Prusiner et al. 1990; Carlson et al. 1994). When mice were developed in which the endogenous mouse PrP gene was ablated (*Prnp*$^{0/0}$ mice), it presented new opportunities for transgenic mouse studies. *Prnp*$^{0/0}$ mice did not develop prion disease and did not support prion replication (Büeler et al. 1993). Importantly, mice expressing human PrP on a wild-type mouse PrP background were resistant to infection with CJD prions, but became susceptible when backcrossed to the *Prnp*$^{0/0}$ background (Telling et al. 1995). Transgenes encoding PrP from a range of species have subsequently been used to bioassay various natural and passaged prion strains and to study transmissibility between species (reviewed in Watts et al. 2014b).

Cultured cells

Although the gold standard for measuring prion infectivity remains animal bioassay, typically in wild-type or Tg rodents, these studies are time consuming and expensive. The finding that the amount of PrP remaining after limited proteolysis with proteinase K (PK) closely correlates with prion infectivity (McKinley et al. 1983) provided a simple tool to measure the disease-associated conformation of the prion protein, PrP^{Sc}, and helped to identify cell models capable of propagating prions. Mouse neuroblastoma cells (N2a) were shown to propagate mouse-passaged sheep scrapie derived from the Chandler isolate (Race et al. 1987; Butler et al. 1988); the resulting prion-infected cells are referred to as ScN2a. Subsequently, N2a cells were shown to propagate a subset of prion strains, including 22L and 139A but not ME7, 87V, or 22A (Bosque et al. 2000; Nishida et al. 2000). A limited number of other cell lines were identified that stably propagate prions, including the mouse hypothalamic neural cell line GT1 (Schätzl et al. 1997) and the fibroblast-derived line 3T3 (Vorberg et al. 2004). Rat pheochromocytoma cells (PC12) have been reported to propagate mouse prions (Rubenstein et al. 1984; Rubenstein et al. 1992), but the biology of this system remains unclear. A rigorous study of the susceptibility of N2a and Cath.a-differentiated (CAD) cell subclones to multiple prion strains identified the CAD5 line with the broadest strain sensitivity reported to date (Mahal et al. 2007). The differential sensitivities of cell lines were used to develop a scrapie cell panel assay to discriminate between prion strains (Mahal et al. 2007).

Despite the success of PK-based approaches in identifying cell models, subsequent studies have revealed that a portion of PrP^{Sc} can be PK-sensitive, depending on the strain (Safar et al. 1998); in CJD, up to 90% of PrP^{Sc} was found to be PK-sensitive (Safar et al. 2005). It is therefore important that any findings identified in cell models are validated by animal bioassays.

Cell-free assays

The “protein-only” hypothesis of prion propagation suggested that it should be theoretically possible to model this process in vitro. The first substantive step toward this goal was the partial denaturation of PrP^{Sc} and the incorporation of new cellular prion protein, PrP^{C}, into a protease-resistant conformation (Kocisko et al. 1994). This technique was used to study the

molecular level of transmission barriers due to differences in primary sequence (Kocisko et al. 1995). However, conversion was substoichiometric, in contrast to the exponential growth of PrP^{Sc} in vivo. It is possible that this difference reflected linear growth of PrP^{Sc}, as opposed to repeated fragmentation and growth that would be required for more rapid prion propagation. Subsequent studies to fragment growing prion aggregates using sonication led to the protein misfolding cyclic amplification (PMCA) assay (Saborio et al. 2001). Refinement of the PMCA technology showed that in addition to replicating PK-resistant PrP, infectious PrP^{Sc} could be generated (Castilla et al. 2005). Parallel studies using shaking to fragment PrP^{Sc} seeds led to the quaking-induced conversion (QuIC) assay (Atarashi et al. 2008); however, the QuIC methodology has yet to demonstrate the replication of prion infectivity.

BIOASSAYS FOR NON-PrP PRIONS

Animal assays

Alzheimer's disease (AD) is characterized by neuropathological aggregates of two proteins: Aβ and tau. Experimental models for iatrogenic Aβ amyloidosis have been developed based on intracerebral inoculation of brain homogenates from AD patients into Tg mice producing human Aβ (Kane et al. 2000; Meyer-Luehmann et al. 2006) and into nonhuman primates (Baker et al. 1994; Ridley et al. 2006).

Because Aβ amyloidosis does not lead to a lethal phenotype, these animals had to be euthanized for neuropathological analysis to assess transmission and disease progression. We had previously shown that in PrP prion diseases progression could be monitored in vivo using a luciferase reporter driven by the glial fibrillary acid protein (GFAP) promoter (Tamgüney et al. 2009). We demonstrated that GFAP upregulation correlated with Aβ load in two Tg mouse lines and that upregulation of GFAP could be monitored in vivo in these lines (Watts et al. 2011). Using this paradigm, we were able to demonstrate that synthetic Aβ alone was sufficient to induce Aβ amyloidosis in a susceptible Tg mouse line (Stöhr et al. 2012). Moreover, we showed that the Aβ isoform-specific phenotypes from familial and sporadic AD could be serially propagated in vivo, defining various Aβ prion strains (Watts et al. 2014a). Different synthetic Aβ strains were also induced by varying the refolding conditions, which resulted in different neuropathological phenotypes following intracerebral inoculation into Tg mice (Stöhr et al. 2014; reviewed in Watts et al. 2016).

Seeded aggregation of tau has also been modeled in Tg mice, most notably in one line expressing full-length human wild-type tau, termed ALZ17 (Probst et al. 2000). Inoculation of ALZ17 mice with brain homogenate from aged Tg mice expressing human tau with the disease-associated P301S mutation induced neurofibrillary tangles along with neuropil threads and coiled bodies (Clavaguera et al. 2009). Subsequent studies demonstrated that inoculating tau oligomers isolated from AD patient samples into the ALZ17 mice resulted in tau neuropathology similar to that seen in AD patients (Lasagna-Reeves et al. 2012). Following the initial transmission of human tau prions to Tg mice, inoculation experiments using brain homogenate from patient samples yielded transmission of distinct tau neuropathologies in the ALZ17 mice reminiscent of the neuropathologies associated with each disease inoculated (Clavaguera et al. 2013). Using a different Tg mouse line expressing

tau with the P301S mutation, termed PS19 (Yoshiyama et al. 2007), synthetic tau fibrils were used to induce progressive tau pathology (Iba et al. 2013; reviewed in Clavaguera et al. 2016).

Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are neuropathologically characterized by aggregates of hyperphosphorylated α-synuclein. Multiple Tg mouse models, many of which harbor mutations associated with familial PD, have been developed for what are referred to collectively as the synucleinopathies. One of these Tg lines expresses human α-synuclein with the mutation A53T driven by the PrP promoter, termed M83 (Giasson et al. 2002). Homozygous $M83^{+/+}$ mice develop spontaneous disease at around 1 year, at which point their brains contain extensive aggregated α-synuclein. Brain homogenate from aged $M83^{+/+}$ mice induced synucleinopathy and accelerated disease onset when inoculated into young $M83^{+/+}$ mice (Luk et al. 2012b; Mougenot et al. 2012). Similarly, intracerebral inoculation of fibrils formed from synthetic α-synuclein accelerated disease onset in $M83^{+/+}$ mice (Luk et al. 2012b), and even induced neuropathological changes in wild-type mice (Luk et al. 2012a). Likewise, brain homogenate from an aged Tg mouse expressing human α-synuclein with the A30P mutation induced synucleinopathy when inoculated into young mice of the same line (Schweighauser et al. 2015). Using patient tissues, Lewy body extract from PD brains induced α-synuclein pathology in wild-type mice and monkeys, but no behavioral deficits were observed (Recasens et al. 2014).

To study MSA patient tissues, we performed intracerebral inoculations of brain homogenate in hemizygous $M83^{+/-}$ mice, which do not develop spontaneous disease. Remarkably, initial studies with brain homogenates from two MSA patients induced a lethal phenotype in the mice ~4 months after inoculation, along with robust α-synuclein pathology throughout the hindbrain and portions of the mesencephalon (Watts et al. 2013). Subsequent inoculation of samples from 12 additional patients found that all 12 transmitted a lethal synucleinopathy, whereas control and PD patient samples had no effect (Prusiner et al. 2015; reviewed in Hasegawa et al. 2016 and Woerman et al. 2016).

Cell assays

Modeling the aggregation of other prions in cells has proven much simpler than identifying cell lines that replicate PrP prions. Expressing a fusion protein consisting of the repeat domain of human tau fused to yellow fluorescent protein (YFP) in human embryonic kidney (HEK) cells enabled tau aggregation to be monitored by the development of microscopic YFP puncta (Holmes et al. 2016). Of 29 samples from human tauopathies, 21 induced aggregate formation when incubated with these cells. Moreover, aggregate morphologies appeared to correlate with distinct disease states (Sanders et al. 2014).

Developing a high content assay with this cell line for the detection of tau prions, we adapted the model to a 384-well-plate format to facilitate automated imaging and analysis using the IN Cell Analyzer 6000 (Woerman et al. 2015). Cells incubated with crude brain homogenate from either control or progressive supranuclear palsy (PSP) patient samples yielded no significant differences when we tested the samples under the new assay conditions. However, after isolating aggregated protein from the patient samples by

precipitating with sodium phosphotungstate (PTA), tau prions from the PSP patient samples induced aggregates in ~61% of the cells while the control sample had no effect (Woerman et al. 2015).

Using an analogous concept, we developed a separate line of HEK cells capable of selectively detecting α-synuclein prions isolated from MSA patient samples (Woerman et al. 2015). These cells express full-length α-synuclein with the A53T mutation fused to YFP, which forms bright aggregates in the presence of recombinant α-synuclein prions. When we incubated the cells with crude brain homogenate from control and MSA patient samples, the MSA patient samples had no effect on aggregate formation after 4 days. However, PTA-precipitated α-synuclein prions from the MSA patient samples robustly infected the α-synuclein–YFP cells. This response was specific to MSA; PTA-precipitated aggregates from PD, DLB, and Parkinson's disease with dementia did not infect the cells, even though the concentration of α-synuclein in the samples was similar to that in the MSA patient samples (Woerman et al. 2016). Importantly, we also found that the rate of infection in the cell assay correlated with the incubation time in the Tg mouse transmission studies, providing a faster and less-expensive measure of MSA prion titer (Prusiner et al. 2015).

Notably, all of the cellular assays described above have demonstrated specificity for homotypic seeding. The tau–YFP cells are not infected by α-synuclein or Aβ prions. The α-synuclein–YFP cells are not infected by tau or Aβ prions (Woerman et al. 2015). This specificity confers the ability to rapidly bioassay distinct prions in vitro.

Cell-free assays

The QuIC paradigm has recently been applied to α-synuclein (Fairfoul et al. 2016; Hughson et al. 2016). One implementation of the assay demonstrated sufficient sensitivity to detect seeding from the cerebrospinal fluid of patients with PD or DLB (Fairfoul et al. 2016). Additionally, the QuIC assay has also been applied to measuring the seeding of tau prions (Hughson et al. 2016).

INACTIVATION OF PRIONS

Because prion propagation involves template-directed refolding of an endogenous protein, prions have the amino acid sequence of the host in which they were propagated. For example, human prions passaged in mice produce prions with the mouse PrP sequence. In other words, in interspecies infection, the prions that replicate in the host brain are not the same as those that initiated replication. This scenario is profoundly different from what occurs during a bacterial or viral infection, and is crucial in developing and implementing appropriate infection control procedures.

Inaccurate assumptions about the nature of the infectious prion agent, and a poor appreciation of potential strain differences, confounds much of the early literature on prion inactivation. Even the recent literature is not free of these errors, with authors referring to "human prion strains" when discussing mouse-passaged human prions, and prion disinfection guidelines based on experiments where the exact nature of the prion strain under examination is not clearly defined. Care must also be taken in the terminology used to

describe inactivation. Sterilization is a widely used term in infection control and is typically defined as the complete elimination of microorganisms. In practice, “elimination” is based on the sensitivity of the system used to replicate any residual infectivity. In prion biology, the lack of reliable ex vivo methods for the replication of infectivity means that bioassays in animals are required, and even then they may be of limited sensitivity compared with what can be grown in a bacterial or viral culture. Experiments starting with a low prion titer or using an insensitive detection method can lead to claims of prion “sterilization” that may not actually eliminate the potential for infection.

The unusual resistance to inactivation of the infectious agent responsible for scrapie in sheep was first observed eight decades ago. A large-scale vaccination program was implemented in the United Kingdom against the louping-ill virus starting in 1935. Sheep were immunized with a 10% homogenate, prepared from the brain, spinal cord, and spleen from sheep infected with louping-ill, which was treated with 0.35% formalin. Intracerebral inoculation into mice and sheep demonstrated that this procedure had inactivated the virus. Although the immunization campaign proved successful in reducing the incidence of louping-ill, two and a half years into the program, scrapie started to appear in sheep immunized with a single batch of the vaccine. These cases were ultimately attributed to tissue from asymptomatic scrapie-infected sheep included in that particular batch. More importantly, these cases demonstrated that scrapie was resistant to the formalin treatment that inactivated the louping-ill virus (Gordon 1946). Analogously, incomplete prion inactivation has led to iatrogenic CJD transmission. For example, standard hot-air sterilization at 180 °C for 2 hours was insufficient to inactivate CJD prions, leading to subsequent iatrogenic transmission via brain surgery (Poisson et al. 1980; Taylor 1999). Incomplete prion inactivation also led to the bovine spongiform encephalopathy (BSE) epidemic in Europe. The emergence and spread of BSE arose following changes in the rendering process that led to exposure of the resultant meat and bone meal to lower temperatures for shorter periods, recycling prions back into cattle and, ultimately, human food supplies (Wilesmith et al. 1988).

Experimental transmission studies using sheep scrapie demonstrated that drying brain and spinal cord before storing at 0 °C did not eliminate infectivity (Wilson et al. 1950). Subsequent studies reported that infectivity remained following storage at –40 °C for several years or after heating samples to 100 °C for up to 8 hours (Stamp et al. 1959). Moreover, incubation with acetylethyleneimine for a period 50% longer than that known to completely inactivate 12 different viruses, followed by lyophylization and reconstitution, did not entirely inactivate scrapie prions (Stamp et al. 1959).

Mouse-passaged sheep scrapie was shown to be highly resistant to ultraviolet irradiation, leading to the proposition that it might not contain a nucleic acid (Alper et al. 1966; Alper et al. 1967). Further experiments showed resistance to inactivation by various chemicals including glutaraldehyde, peracetic acid, or ethanol, and to extended heating such as at 160 °C for 24 hours (Dickinson et al. 1978). Additional independent transmissions of sheep scrapie to mice and serial passaging experiments led to the identification of multiple strains of mouse-passaged scrapie, each of which differed in its biological properties including its resistance to inactivation (Dickinson et al. 1978).

Due to its high titer and short incubation periods, the hamster-passaged prion strain Sc237, also known as 263K, became widely used in prion inactivation studies. Treatment of the Sc237 strain with a variety of nonionic and nondenaturing ionic detergents did not alter its infectivity. In contrast, the denaturing detergent sodium dodecyl sulfate (SDS) inactivated Sc237 prions in a concentration-dependent manner (Prusiner et al. 1980). Chaotropic ions, including thiocyanate, guanadinium, and trichloroacetate, were also effective at reducing the titer of Sc237 prions (Prusiner et al. 1981a). Partial inactivation of the Sc237 strain was also achieved by chemical modification with diethylpyrocarbonate (McKinley et al. 1981) and by digestion with PK (Prusiner et al. 1981b).

Autoclaving at 121 °C for 90 minutes was not sufficient to completely inactivate the Sc237 strain (Prusiner et al. 1984). Paradoxically, autoclaving at higher temperatures occasionally appeared to be less effective. For the 263K strain, similar levels of inactivation were seen at temperatures up to 138 °C. But with the mouse-passaged sheep scrapie and BSE strains 22A and 301V, more infectivity was observed after autoclaving at 138 °C than at 134 °C (Taylor 1999).

World Health Organization guidelines

Under the Communicable Disease Surveillance and Control Program, the World Health Organization convened a group of experts to establish guidelines for the care of patients with prion diseases, including a list of prion inactivation procedures (World Health Organization 1999). These procedures, which include immersion of surgical instruments in 1*N* sodium hydroxide (NaOH) and autoclaving at 121 °C for 30 minutes, and soaking in 20,000 ppm sodium hypochlorite (NaOCl) or NaOH for 1 hour then transferring to water and autoclaving (World Health Organization 1999), have been recommended by the U.S. Centers for Disease Control and Prevention and incorporated into hospital infection control protocols. However, these methods not only raise serious health and safety concerns, such as the handling of hot sodium hydroxide, but also can lead to pitting and corrosion of sensitive surgical instruments (Brown et al. 2005).

Noncorrosive prion inactivation

We identified the ability of branched polyamine dendrimers to render PrP^{Sc} in ScN2a cells sensitive to protease degradation (Supattapone et al. 1999), and we also showed that their efficacy in prion-infected brain homogenate was enhanced at pH ≤ 4 (Supattapone et al. 2001). Based on these observations, we explored the susceptibility of prions to a variety of denaturants under mildly acidic conditions. Although SDS at neutral pH had modest ability to inactivate prions, it became highly effective when combined with acetic acid (AcOH) (Peretz et al. 2006). To quantify the changes in infectivity upon treatment, Cox models were derived from incubation periods of serially diluted brain homogenates and used to generate the equivalent $\log_{10}$ reduction in titer. At room temperature, shaking a 1% brain homogenate containing Sc237 prions with 1% SDS and 0.5% AcOH reduced the titer by >7 $\log_{10}$ units. Nonetheless, infectivity was still present as evidenced by all mice succumbing to disease (Peretz et al. 2006). Increasing the concentration of the reagents and temperature improved prion inactivation. At 65 °C, using a 2% SDS–1% AcOH solution required 2 hours to remove all detectable infectivity from the Sc237 brain homogenate; however, 4% SDS–1%

AcOH at 65 °C or 2% SDS–1% AcOH at 121 °C inactivated all detectable prions within 30 minutes (Peretz et al. 2006).

To directly compare prion inactivation between the BSE strain and its mouse-passaged analog 301V, we derived Cox models for each strain. Brain homogenates from mice infected with BSE or 301V prions were incubated with a range of treatments including “acidic SDS” for various times and temperatures. Treatments that showed low levels of inactivation produced similar reductions in prion titer of 301V and BSE prions; however, with more stringent procedures, there were significant differences. BSE prions were >1,000-fold more resistant to elimination by autoclaving at 134 °C for 15 minutes than 301V prions, suggesting that any extrapolation from rodent-passaged strains to their parent strain must be interpreted cautiously (Giles et al. 2008).

Inactivating prions bound to surfaces

All early prion inactivation studies were performed on solutions or suspensions of homogenized prion-infected tissue. To generate a more translational model for surgical instruments and machinery used in the processing of animal carcasses, researchers in the laboratory of Charles Weissmann used short sections of stainless steel suture wire as a surface for prion contamination. These wires can be incubated in prion-infected brain homogenate, subjected to various inactivation procedures, and directly bioassayed by implantation into the brains of naïve mice (Zobeley et al. 1999). Incubation periods in mice implanted with prion-contaminated wires are longer than with standard intracerebral inoculation of brain homogenate, effectively producing a lower dynamic range in the bioassay. It is unclear whether this delay is due to the quantity of prions bound to the surface of a ~5 mm section of stainless steel wire compared with 30 μL of 1% brain homogenate, the limited desorption of prions from a wire, or the ability of prions to initiate infection from the bound state.

Stainless steel wires incubated with the 263K strain were subjected to various inactivation protocols. Caustic chemical treatments including NaOCl, NaOH, and a phenolic disinfectant were all effective at eliminating infectivity (Fichet et al. 2004). Autoclaving at 134 °C for 18 min was only effective at removing detectable infectivity when the wires were immersed in water, but not when the wires were simply placed on a support (Fichet et al. 2004). Enzymatic cleaners alone were only partially effective (Fichet et al. 2004; Yan et al. 2004), and enzymatic cleaner in combination with vaporized hydrogen peroxide (Fichet et al. 2004) or alkaline detergent followed by hydrogen peroxide gas plasma sterilizer (Yan et al. 2004) were required to remove all detectible infectivity from the wires.

Although 2% SDS–1% AcOH at 65 °C for 2 hours was sufficient to eliminate all detectable Sc237 prion infectivity in brain homogenate, the majority of mice implanted with Sc237-contaminated wires that were subject to the same treatment succumbed to prion disease, suggesting that prions on stainless steel surfaces are more difficult to inactivate than those in solution (Peretz et al. 2006). Similarly, procedures effective at inactivating BSE prions in solution, such as 4% SDS–1% AcOH at 65 °C for 18 hours, were not completely effective at reducing all infectivity on BSE-contaminated wires, which required autoclaving in the presence of acidic SDS (Giles et al. 2008).

Refinement of the steel wire model

The steel wire model has become widely adopted, and contamination is typically achieved by incubating the wires overnight in prion-infected brain homogenate. To model surgical procedures more closely, steel wires were transiently inserted into a brain collected from an asymptomatic prion-infected mouse. Wires that contacted infectious brain tissue for as little as 5 minutes retained considerable infectivity when bioassayed by permanent insertion into reporter mice (Flechsig et al. 2001). Transient insertion of prion-contaminated wires into brains of naïve mice for as little as 30 minutes was sufficient to transmit infection without significantly reducing the remaining infectivity on the wire (Flechsig et al. 2001). Subsequently, transient insertion of a wire contaminated with 263K prions for as little as 5 minutes was shown to efficiently transmit prion disease (Yan et al. 2004).

To determine how readily prions were able to adhere to steel wires, we tested contamination by transient insertion into brains, as well as the duration of incubation in brain homogenate, from terminally ill mice infected with the mouse-passaged sheep scrapie strain RML. Surprisingly, the shortest contact times tested—30-second insertion and 15-minute incubation with 10% brain homogenate—showed that wire surfaces adsorbed a similar level of infectivity as the longer contact times (Table 1). This suggests that wires, and by inference stainless steel surgical instruments and meat-processing equipment, can be rapidly saturated with prion infectivity.

Inactivation of human prions

Early inactivation studies on CJD prions were performed under the assumption that CJD represented a single disease. However, it is now understood that there are multiple well-defined human prion strains. Moreover, multiple prion strains are not uncommon within a single brain (Parchi et al. 2009). A polymorphism at residue 129 of human (Hu) PrP, encoding methionine (M) or valine (V), has an allele frequency in the general population of ~0.6/0.4. Although MV heterozygotes represent around half of the population, they account for only 10% of sporadic CJD (sCJD) cases (Parchi et al. 1999). Additionally, biochemical analysis of sCJD prions can be broadly classified into two groups (termed type 1 and type 2), depending on the size of the PK-resistant fragment of PrP. These strain types also correlate strongly with genotype, and of the 6 possible genotype/strain-type combinations, MM individuals with type 1 prions, termed MM1, account for ~70% of sCJD cases (Parchi et al. 1999). The 6 sporadic strain types represent at least 4 biologically distinct strains (Bishop et al. 2010). The genetic human prion diseases, including fatal familial insomnia (FFI) and Gerstmann–Sträussler–Scheinker (GSS), represent additional unique strains.

The first CJD transmission studies were performed in primates (Gibbs et al. 1968), which proved impractical for multiple prion inactivation experiments. Using a guinea-pig passaged CJD isolate, infectivity was reduced >1,000-fold with NaOCl at concentrations of 0.5% or above within 15 minutes, whereas potassium permanganate and a range of detergents had lesser effects even up to 4 hours (Brown et al. 1982). Treatment with 1*N* NaOH at room temperature for 1 hour was also effective (Brown et al. 1984), as was steam autoclaving at 121 °C or 132 °C for 1 hour (Brown et al. 1986). However, the results of these experiments must be interpreted with caution, as all of these experiments were not only performed on

guinea pig prions, but also the exact strain of the original CJD prion strain used is not known.

Transmission of an unusual human prion disease isolate, likely to represent GSS, to mice led to the Fukuoka-1 (FU-1) strain (Tateishi et al. 1979). The American Neurological Association published precautions for handling CJD tissues, recommending steam autoclaving for 1 hour at 132 °C or immersion in 1*N* NaOH for 1 hour at room temperature as "fully effective" (Rosenberg et al. 1986). However, treatment of the FU-1 strain with *2N* NaOH for 2 hours reduced infectivity but failed to fully inactivate it (Tateishi et al. 1988). Serial passage of the FU-1 strain in mice led to the M1000 strain, which was used in combination with the steel wire model to test various prion inactivation strategies (Lawson et al. 2007). Soaking in NaOH or autoclaving had limited impact against M1000 prions bound to stainless steel. Different enzymatic cleaner formulations were slightly more effective, but only at elevated temperatures, and enzymatic cleaner in combination with autoclaving was required to remove all detectible infectivity (Lawson et al. 2007).

To determine whether the FU-1/M1000 strain was representative of human prions, we performed transmission studies in a series of Tg lines either overexpressing mouse PrP, Tg(MoPrP)4053 (Carlson et al. 1994), or lacking endogenous mouse PrP but expressing a chimeric mouse/human PrP, Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ (Giles et al. 2010), or human PrP, Tg(HuPrP)2669/*Prnp*$^{0/0}$ (Berry et al. 2013). Tg(MoPrP)4053 and Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice were susceptible to RML prions, whereas the Tg(Mo/HuPrP)1014 and Tg(HuPrP)2669/*Prnp*$^{0/0}$ lines were susceptible to human sCJD(MM1) prions. For RML prions, Tg(MoPrP)4053 mice had incubation periods around half those of Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice, likely due to a higher expression level and exact sequence match with the Tg(MoPrP)4053 line. Tg(MoPrP)4053 and Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice inoculated with RML prions from ScN2a cells showed longer incubation periods than those inoculated with RML brain homogenate (Table 2), reflecting the lower titer inoculated for the former. Interestingly, the FU-1 strain in N2a cells had similar incubation periods in Tg(MoPrP)4053 and Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice, which were between the values for each line with RML-infected N2a cells. However, FU-1 prions failed to infect Tg(HuPrP)2669/*Prnp*$^{0/0}$ mice (Table 2). FU-1 prions serially passaged in Tg(MoPrP)4053 mice resulted in shorter subsequent incubation periods in Tg(MoPrP)4053 and Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice, reflecting the higher titer of the brain homogenate inoculum compared with FU-1 infected N2a cells. However, following serial passage in Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice, FU-1 prions had shorter incubation periods in Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ mice, but longer incubation periods in Tg(MoPrP)4053 mice than the original FU-1 transmission, suggesting further adaptation of the strain in the Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$ host. Neither of the serially passaged samples were able to infect Tg(HuPrP)2669/*Prnp*$^{0/0}$ mice (Table 2). Although the FU-1 strain of mouse-passaged prions exhibits unusual transmission properties, it does not have the infectivity characteristics of common CJD strains, and its utility as a general model system for human prions is therefore questionable.

Direct assay of human prions

To measure infectivity of CJD prions following various prion inactivation procedures, we used an alternate chimeric human/mouse PrP Tg line, termed Tg(Mo/HuPrP)22372/*Prnp*$^{0/0}$ (Korth et al. 2003). Inoculation of serially diluted sCJD(MM1) prions in Tg(Mo/HuPrP)22372/*Prnp*$^{0/0}$ mice showed that infectivity could still be detected in a 10^{-8} dilution of 10% brain homogenate (Peretz et al. 2006). From these data, we derived a Cox model to quantify inactivation procedures. We determined that although exposure of Sc237 prions to 2% SDS–1% AcOH at 65 °C for 30 minutes produced a 9.0 $\log_{10}$ reduction in titer, the same treatment on sCJD(MM1) prions yielded only a 3.8 $\log_{10}$ reduction (Peretz et al. 2006). Thus, the sCJD prions were >100,000-fold more difficult to inactivate than Sc237 prions, an alarming conclusion for inactivation procedures touted as effective for health care settings but validated against the Sc237/263K prion strain. Extended incubation (18 hours) with 4% SDS–1% AcOH at 65 °C did remove all detectable CJD infectivity, as did 2% or 4% SDS with 1% AcOH in combination with autoclaving (Peretz et al. 2006).

As with the other prion strains, human sCJD prions bound to a stainless steel surface were more difficult to inactivate than those in solution. The majority of Tg(Mo/HuPrP)22372/*Prnp*$^{0/0}$ mice implanted with sCJD-contaminated steel wires that had been subjected to 4% SDS–1% AcOH at 65 °C for 18 hours still developed clinical signs of prion disease. Only autoclaving in acidic SDS removed all detectable infectivity (Peretz et al. 2006). However, it should be noted that the sensitivity of the Tg(Mo/HuPrP)22372/*Prnp*$^{0/0}$ model for sCJD prions is at least 100-fold less than that used for Sc237 prions; therefore, low levels of infectivity could still be present.

Alternatives to animal bioassays

The complete removal of protein would by definition mean the complete removal of infectivity. Using an advanced light microscopy technique—episcopic differential interference contrast microscopy—and fluorescent amyloid-binding reagents, levels below 100 fg of protein were detected on the surface of surgical instruments (Lipscomb et al. 2007). This technique was also able to demonstrate the limitations of the “wire” model for prion infection, showing that prion-contaminated wires could be cleaned more easily than flat metal surfaces, which are more representative of surgical instruments (Lipscomb et al. 2006).

The QuIC assay has been used to measure residual PrP seeding ability following treatments with NaOH, and the commercial disinfectants Environ LpH and BrioHOCl on various prion strains (Hughson et al. 2016). Although this represents an advantage in terms of throughput, it was noted that elimination of prion infectivity is not always accompanied by loss of QuIC seeding activity (Hughson et al. 2016). In addition, the QuIC assay was modified to measure residual seeding from prion-contaminated wires (Hughson et al. 2016; Mori et al. 2016). Reduction of seeding ability from wires was compared to infectivity by bioassay for hamster prions (Hughson et al. 2016), although as noted above, the utility of this strain for prion inactivation studies relevant to CJD prions is limited (Peretz et al. 2006). The application of the QuIC methodology to wires contaminated with human CJD prions may prove promising;

however, as the authors note, the relationship between QuIC seeding and infectivity of human prions remains to be determined (Mori et al. 2016).

Inactivation of non-PrP prions

To date, >95% of cases of iatrogenic CJD resulted from treatment of persons of short stature with growth hormone produced from pools of cadaver-derived pituitary glands, or use of cadaver-derived dura mater grafts during neurosurgery. With the growing understanding that most, if not all, neurodegenerative diseases are caused by different proteins adopting self-propagating, or prion, conformations (Prusiner 2012), questions have arisen whether these too could be spread iatrogenically.

Reanalysis of the brains from patients who received growth hormone and developed iatrogenic CJD showed that a number of them also contained Aβ pathology that was not present in age-matched controls (Jaunmuktane et al. 2015). This suggests that Aβ seeds from the original pituitary glands had also induced an iatrogenic Aβ amyloidosis. A similar observation was made in iatrogenic CJD patients who received dura mater grafts (Frontzek et al. 2016; Kovacs et al. 2016). Interestingly, tau pathology was not observed in any of these cases.

In the Tg mouse model, Aβ seeding activity was completely inactivated by 70% formic acid for 1 hour and reduced after incubating at 95 °C for 5 minutes (Meyer-Luehmann et al. 2006). In an analogous experiment to PrP prions, stainless steel wires were incubated in brain homogenate from aged TgAPP23 mice. Wires contaminated with Aβ prions were able to seed Aβ-amyloidosis in the brains of young TgAPP23 mice (Eisele et al. 2009). Heating Aβ-contaminated wires in PBS to 95 °C for 10 minutes did not diminish the amyloid-seeding ability. However, after exposing Aβ-contaminated wires to hydrogen peroxide plasma sterilization (Sterrad S100, long cycle), they did not seed Aβ-amyloidosis (Eisele et al. 2009). Interestingly, the same plasma sterilization procedure had minimal impact on stainless steel wires contaminated with the 263K PrP prion strain (Rogez-Kreuz et al. 2009).

Extracts of brains from AD patients that had been fixed in formaldehyde for 2 years induced a robust Aβ amyloidosis when inoculated intracerebrally into TgAPP23 mice (Fritschi et al. 2014). Brains from two TgAPP mouse models were either fixed in formaldehyde or flash frozen and were shown to seed both in vitro and in vivo, with the fixation process only moderately lowering the Aβ prion titer (Fritschi et al. 2014). The resistance of Aβ aggregates to degradation in vivo was demonstrated by inoculation of mice lacking endogenous APP with brain homogenate from aged TgAPP23 mice. Brains taken from these mice up to 6 months after inoculation were still able to seed Aβ amyloidosis in reporter mice (Ye et al. 2015).

As with PrP and Aβ prions, seeding efficiency of α-synuclein prions is only slightly reduced by formalin fixation. In the A30P Tg model, inoculation of formalin fixed tissue from an aged mouse greatly accelerated disease onset in young mice of the same strain (Schweighauser et al. 2015). α-Synuclein prion inactivation procedures published to date have only been reported to reduce protein level and have not been validated in vivo. Measuring residual protein removed from α-synuclein prion-contaminated stainless steel

grids demonstrated that harsh alkaline treatments, including 1*M* NaOH for 1 hour or 0.2% SDS–0.3% NaOH, reduced recoverable protein loads by >100-fold (Thomzig et al. 2014). A more comprehensive study using plastic, glass, aluminum, and stainless steel plates spotted with synthetic α-synuclein aggregates quantified the remaining aggregates by binding of a fluorescent dye. Interestingly, NaOH and NaOCl were among the least effective in reducing α-synuclein, especially on plastic and glass surfaces, potentially exacerbating the inactivation problem by denaturing protein on the surface rather than solubilizing it (Bousset et al. 2016).

Immunoblotting and QuIC were used to test the effect of BioHOCl on aggregated recombinant α-synuclein and Lewy body isolates from DLB patients. Longer treatments and more concentrated BioHOCl were required for the brain extracts (Hughson et al. 2016). Reduced seeding was also observed after BioHOCl treatment of aggregated tau peptide, increasing the lag phase by the equivalent of a 1,000-fold dilution of seed (Hughson et al. 2016).

CONCLUSIONS

Prions are proteins that transmit infectivity by templating their conformation onto copies of the same protein. It is therefore not surprising that conditions required to inactivate them differ from the disinfection of nucleic acid-based replicators such as bacteria and viruses. Differences in protein sequence between species also impact the efficacy of prion inactivation, such that rodent-passaged PrP prions do not necessarily predict efficacy against the natural strain from which they are derived.

With the growing incidence of neurodegenerative diseases among an aging population, it will be increasingly important to closely monitor procedures that could result in iatrogenic transmission of disease and to develop prion inactivation methods that are rigorously validated against all relevant prion strains.

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Table 1

Incubation periods in Tg(MoPrP)4053 mice following permanent implantation of wires contaminated with RML prions for different durations.

Contaminating procedure	Time	Incubation period (days)[a]	n/n_0[b]
Transient insertion of wire in terminal RML-infected brain	30 s	78 ± 2	5/5
	90 s	83 ± 1	4/4
	5 min	84 ± 4	4/4
Incubating wire in 10% RML brain homogenate	15 min	88 ± 3	3/3
	1 h	89 ± 2	4/4
	4 h	86 ± 4	4/4
	16 h	82 ± 4	4/4

[a] Data reported as mean ± standard error of the mean

[b] n, number of mice showing clinical signs of disease; n_0, number of mice inoculated

Table 2

Incubation periods of the Fukuoka-1 (FU-1) prion strain in transgenic mice.

Inoculum	Host	Incubation period (days) [a]	n/n_0 [b]
RML brain homogenate	Tg(MoPrP)4053	51 ± 3 [c]	8/8
	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	107 ± 5 [c]	4/4
CJD brain homogenate	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	78 ± 1 [c]	7/7
	Tg(HuPrP)2669/*Prnp*$^{0/0}$	143 ± 2 [c]	9/9
RML/N2a cell lysate	Tg(MoPrP)4053	77 ± 3	8/8
	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	154 ±1	8/8
FU-1/N2a cell lysate	Tg(MoPrP)4053	103 ± 2	8/8
	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	103 ± 4	8/8
	Tg(HuPrP)2669/*Prnp*$^{0/0}$	> 500	0/8
FU-1/N2a cell lysate -> Tg(MoPrP)4053	Tg(MoPrP)4053	76 ± 0	8/8
	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	82 ± 2	7/7
	Tg(HuPrP)2669/*Prnp*$^{0/0}$	> 500	0/6
FU-1/N2a cell lysate -> Tg(Mo/HuPrP)1014	Tg(MoPrP)4053	120 ± 10	8/8
	Tg(Mo/HuPrP)1014/*Prnp*$^{0/0}$	95 ± 2	8/8
	Tg(HuPrP)2669/*Prnp*$^{0/0}$	> 500	0/6

[a] Data reported as mean ± standard error of the mean

[b] n, number of mice showing clinical signs of disease; n_0, number of mice inoculated

[c] Data previously reported in (Berry et al. 2013)