Spontaneous generation of mammalian prions

Julie A. Edgeworth, Nathalie Gros, Jack Alden, Susan Joiner, Jonathan D. F. Wadsworth, Jackie Linehan, Sebastian Brandner, Graham S. Jackson, Charles Weissmann[1,2], and John Collinge[1]

Medical Research Council Prion Unit, Department of Neurodegenerative Disease, University College London Institute of Neurology, London WC1N 3BG, United Kingdom

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Prions are transmissible agents that cause lethal neurodegeneration in humans and other mammals. Prions bind avidly to metal surfaces such as steel wires and, when surface-bound, can initiate infection of brain or cultured cells with remarkable efficiency. While investigating the properties of metal-bound prions by using the scrapie cell assay to measure infectivity, we observed, at low frequency, positive assay results in control groups in which metal wires had been coated with uninfected mouse brain homogenate. This phenomenon proved to be reproducible in rigorous and exhaustive control experiments designed to exclude prion contamination. The infectivity generated in cell culture could be readily transferred to mice and had strain characteristics distinct from the mouse-adapted prion strains used in the laboratory. The apparent "spontaneous generation" of prions from normal brain tissue could result if the metal surface, possibly with bound cofactors, catalyzed de novo formation of prions from normal cellular prion protein. Alternatively, if prions were naturally present in the brain at levels not detectable by conventional methods, metal surfaces might concentrate them to the extent that they become quantifiable by the scrapie cell assay.

cell culture | scrapie | metal | Creutzfeldt–Jakob disease

Prions, the transmissible agents that cause fatal neurodegenerative diseases such as Creutzfeldt-Jakob disease in humans, or bovine spongiform encephalopathy and scrapie in animals, occur in the form of various strains and are composed principally or entirely of aggregates of misfolded cellular prion protein (PrP^C), generally referred to as PrP^{Sc}. Replication comes about by autocatalytic conversion of PrP^C to the pathogenic isoform (1).

In vitro and in vivo studies using model systems have suggested host-encoded cofactors are required to facilitate the propagation of prions (2–5). A variety of polyanionic compounds, lipids, proteoglycans from brain homogenate or purified protein have been shown to stimulate the conversion of PrP^C to PrP^{Sc} in vitro (6–10).

Steel wire exposed to scrapie prion–infected brain or brain homogenate acquires an extraordinary infectious potential (11, 12). Although the amount of protein bound was undetectable by conventional methods, the wires were as effective in eliciting disease as injection of approximately 10^6 LD_{50} units in the form of brain homogenate. We have isolated an N2a neuroblastoma-derived cell line, N2aPK1 (i.e., "PK1") (13), that is highly susceptible to infection by a wide spectrum of murine strains, including RML and 22L, but not ME7 and 301C (13, 14). When PK1 cells were grown on RML prion–coated wires, adherent cells became PrP^{Sc}-positive, in contrast to neighboring cells, which remained negative, implying that adherence to the prion-coated surface is a prerequisite for infectivity transfer (15). We exploited these observations to detect prions in RML prion–infected brain homogenates diluted as much as 10^{-10}, by adsorbing infectivity to steel wire surfaces and subjecting PK1 cells grown on these surfaces to the scrapie cell assay (SCA) (16). In the course of experiments investigating the properties of metal-bound prions, by using the SCA to measure infectivity, we observed at very low frequency several instances of positive assay results in control groups in which metal wires had been exposed only to uninfected normal mouse brain homogenate. This phenomenon was investigated in rigorous and exhaustive control experiments designed to exclude prion contamination.

Results

Apparent Generation of Spontaneous Prions on Metal Surfaces in Cell Culture. In a typical experiment, wire segments were incubated with dilute homogenate of uninfected or RML prion–infected brain, washed, and covered with susceptible PK1 cells. After 3 d, cells adhering to the wires were collected and the proportion of infected cells was determined by the SCA in endpoint dilution format (SCEPA) (13): 24 replicate 1,000-cell samples were seeded into 96-well plates, grown to confluence, and propagated for 18 d with periodic splitting. Finally, the proportion of PrP^{Sc}-positive cells in each well was determined. Because infectivity spreads efficiently from infected to noninfected cells during passaging (13, 17, 18), wells initially containing one or more infected cells end up with a high proportion of infected cells (i.e., "positive wells"). Remarkably, in nine of 16 experiments control wells scored positive, at an overall level of approximately 2% (40 of 2,268 wells).

We repeated the study on a larger scale in an off-site laboratory without previous prion exposure. All equipment and consumables were purchased new and delivered directly to the designated site. PK1 cells were cultured for 3 d in antibiotic supplemented Opti-MEM (Invitrogen) with10% FCS with (*i*) no additions, (*ii*) 10^{-4} normal mouse brain homogenate, (*iii*) noncoated steel wires, or (*iv*) wires coated with 10^{-4} normal mouse brain homogenate. The cells were harvested, and for each condition 960 one-thousand-cell replicates were placed in the wells of ten 96-well plates and subjected to SCEPA as described earlier (Fig. 1*A*) (13). Only group *iv* yielded positive wells, i.e., wells in which "spots per well" exceeded the average of control samples plus 10 SDs ($n = 66$). The histogram in Fig. 1*B* shows the distribution of spots per well: for samples *i* through *iii*, none of 2,880 wells had 81 or more spots. In contrast, in the case of cells exposed to homogenate-coated wires (i.e., group *iv*), nine wells had 81 to 160 spots and three had more than 160 spots, among 960 wells. The 12 group *iv* samples that had more than 81 spots per well, as well as 12 random samples from each control set, were expanded into eight replicate wells of a 96-well plate, grown to confluence, and assayed for PrP^{Sc}-positive cells. Four of the 12 initially positive samples still scored positive, whereas wells from all other conditions were negative.

Titration and Mouse Bioassay of Spontaneous Prion Infectivity. Prion-infected PK1 cells release infectivity into the supernatant, which can be readily quantified by the SCA (13). Conditioned

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[1]To whom correspondence may be addressed. E-mail: j.collinge@prion.ucl.ac.uk or charlesw@scripps.edu.

[2]Present address: Department of Infectology, Scripps Florida, Jupiter, FL 33458.

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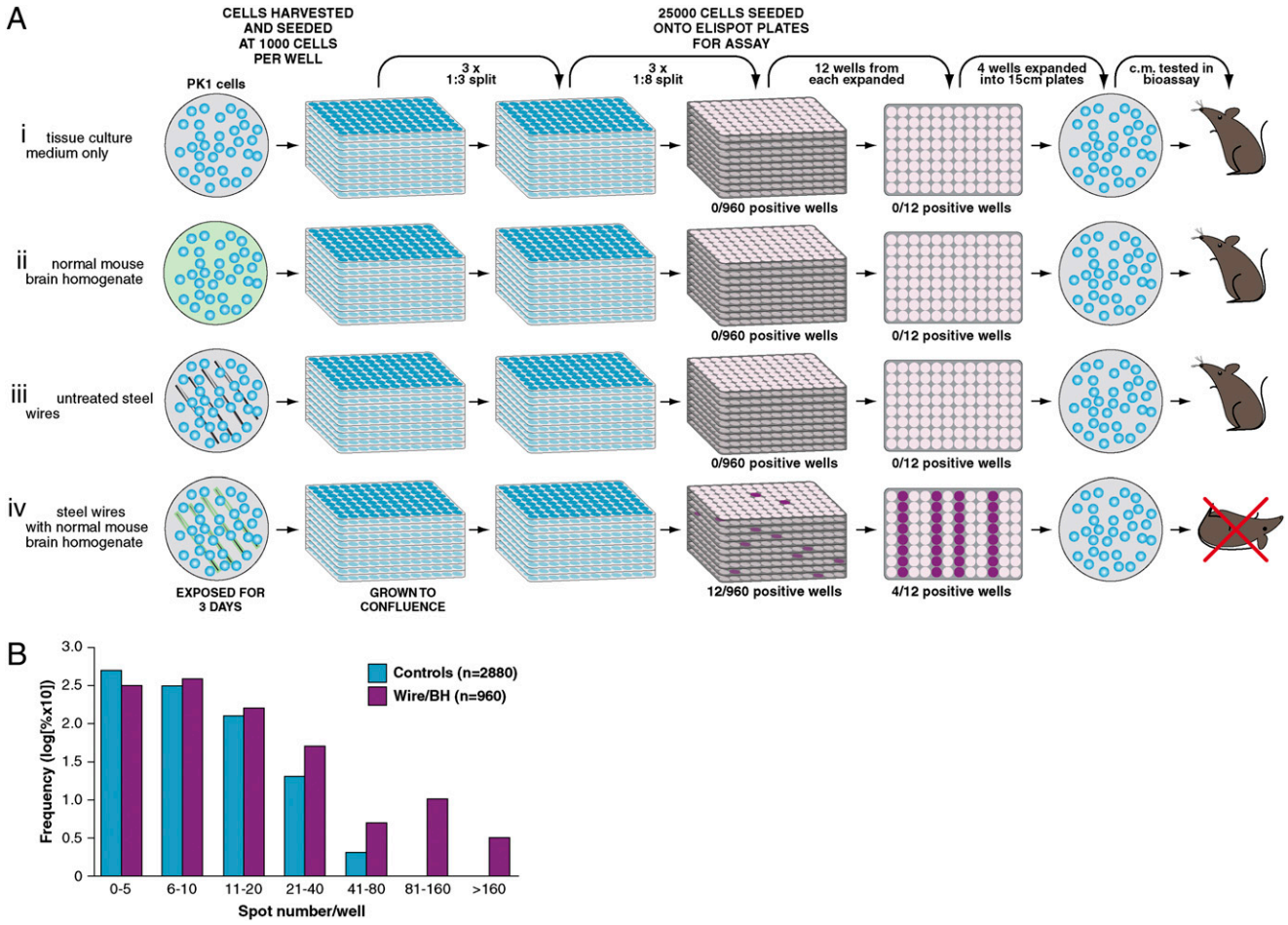

Fig. 1. Generation of "spontaneous infectivity." (*A*) Scheme of experiment: PK1 cells were exposed to (*i*) tissue culture medium only; (*ii*) 10^{-4} normal mouse brain homogenate; (*iii*) untreated steel wires; or (*iv*) steel wires coated with 10^{-4} normal mouse brain homogenate. Cells were propagated for six splits and assayed by the SCA (16). Twelve of 960 wells from group *iv* scored positive whereas all the other 2,880 wells scored negative. Cells from the positive wells and 12 wells chosen at random for the other three conditions were expanded and tested for PrP^{Sc}-positive cells. Four of the original 12 positive wells remained positive and were expanded into 15-cm dishes, along with four wells chosen at random from each of the other conditions. Conditioned medium (c.m.) from the 16 samples was concentrated ×100 and injected i.c. into groups of 10 tga20 mice each. (*B*) Histogram of the SCA spot count. Spots represent immunoreactive particles counted, i.e., PrP^{Sc}-positive cells and "background particles." Blue: Control groups (*i–iii*) all show fewer than 81 spots per well (n = 2,880). Purple: Histogram of cells exposed to homogenate-coated wires (group *iv*) is skewed to the right, with a second peak at 81 to 160 spots per well and values extending to 160, 188, 190, and 250 spots per well.

medium from the four positive wells was concentrated at 100× and assayed on PK1 cells by the SCA. In all cases, titers were approximately 10^4 LD_{50} units/mL 100× concentrated medium. Prion infectivity was confirmed by inoculating groups of 10 tga20 mice (19) intracerebrally (i.c.) with 100× concentrated conditioned medium from each of the four samples of groups (*i–iv*). Clinical scrapie at high attack rates and with mean incubation times of 82 d developed in mice inoculated with material from the four positive samples of group *iv* (Table 1), which equates to approximately $10^{4.5}$ LD_{50}/mL inoculum based on standard curves of incubation period of RML prions in tga20 mice. No transmissions were seen for samples from groups *i* through *iii*. Histopathological and immunochemical examination of brains of mice inoculated with concentrates from the negative wells, culled after 200 d, showed no evidence of disease. In contrast, the mice that developed clinical scrapie showed striking neuropathological features (Fig. 2), and the presence of PrP^{Sc} was confirmed by Western blot analysis (Fig. 3).

Strain Typing of Spontaneous Prions. Prion strains can be distinguished by distinct histopathological patterns in inoculated animals (20) or by distinctive fragment sizes and ratios of protease-resistant PrP glycoforms (21). Western blot analyses (Fig. 3) of protease-resistant PrP from brains infected with RML prions, with conditioned medium from chronically infected PK1 cells (iPK1), or from the four isolates of "spontaneous infectivity," did not reveal any differences. However, it should be noted that some biologically distinct mouse-adapted scrapie strains such as RML and 22L show indistinguishable PrP^{Sc} deposition patterns (22).

Interestingly, the histopathologic findings of brains (n = 25) inoculated with "spontaneously generated" prions was distinct from that of brains inoculated with "authentic" RML prions: the former showed intense abnormal PrP deposition predominantly in the thalamus, midbrain, and brainstem and weaker, patchy deposition in the frontal cerebral cortex (Fig. 2) whereas, notably, the hippocampus was free of abnormal PrP; in contrast, RML-inoculated tga20 brains (n = 13) showed abnormal PrP deposition over the entire cortex, basal ganglia, and very prominently in thalamus and hippocampus. Indeed, the histopathology associated with "spontaneous prions" was unlike any seen previously in our laboratory. The distinctive histopathological pattern elicited by the spontaneous prions excludes contamination with RML or

Table 1. Mouse bioassay of spontaneous infectivity

Inoculum	Source of inoculum	Animals affected*/ total	Mean incubation period, d
Concentrated conditioned medium	PK1 cells, standard culture conditions	0/10	>200
		0/10	—
		0/10	—
		0/10	—
Concentrated conditioned medium	PK1 cells, standard culture conditions plus 10^{-4} normal mouse brain homogenate	0/10	>200
		0/10	—
		0/9	—
		0/10	—
Concentrated conditioned medium	PK1 cells, standard culture conditions plus untreated steel wires	0/10	>200
		0/10	—
		0/10	—
		0/10	—
Concentrated conditioned medium	PK1 cells, standard culture conditions plus steel wires coated with 10^{-4} normal mouse brain homogenate (negatives wells)	0/10	—
		0/10	>200
		0/10	—
		0/9	—
Concentrated conditioned medium	PK1 cells in standard culture conditions plus steel wires coated with 10^{-4} normal mouse brain homogenate (positive wells)	7/7†	80 ± 0
		8/9†	86 ± 1
		7/8†	79 ± 1
		7/8†	81 ± 2
Brain homogenate (1%) used for wire coating	Normal mouse brain	0/99	>200

Groups of tga20 transgenic mice were intracerebrally inoculated with 30 μL 100× concentrated conditioned medium from cells exposed to the various experimental conditions or with 30 μL of the uninfected brain homogenate used to coat wires. Disease was elicited by only the conditioned medium from PrP^{Sc}-positive wells containing cells exposed to homogenate-coated steel wires. Values are presented as mean ± SEM where appropriate.

†A few animals were found dead in these groups at a similar period to affected mice and may also have been affected, but no biological samples were recoverable and so were disregarded.

*Affected mice were confirmed positive by histological and/or Western blot analyses.

other mouse-adapted scrapie strains used by us as a cause for these mouse transmissions.

Given the prion strain selectivity of PK1 cells (14), the spontaneously generated prions must be of a strain type permissive for propagation in these cells. We considered therefore whether in fact iPK1 cells, although originally infected with mouse-passaged RML prions, were in fact propagating a distinct strain that might have arisen by adaptation to this cell line, with strain selection or mutation to a preferred conformer (23, 24). We therefore also compared transmissions of both spontaneously derived prions and mouse RML with those from infectivity derived from the supernatant of iPK1 cells. We inoculated concentrated, conditioned medium from iPK1 into tga20 mice and harvested the brains at the preterminal stage. Surprisingly, the histopathology was distinct from that of RML prion-infected brain, but very similar to that of mice inoculated with the spontaneous prions.

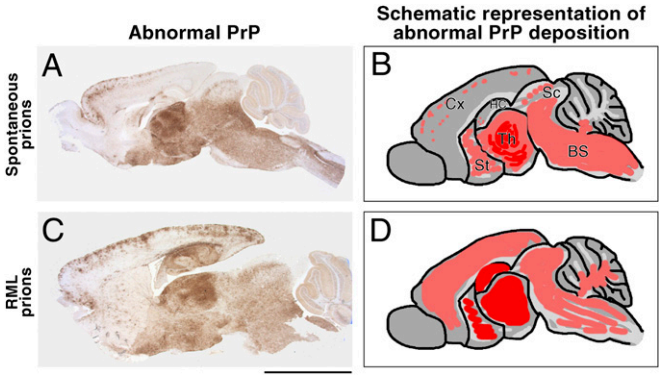

Fig. 2. Histopathologic findings in brains of tga20 mice inoculated with "spontaneous" or brain-derived RML prions. Prions derived from cell cultures exposed to metal wire coated with normal mouse brain homogenate produce a distinctive pattern of PrP deposition in the brain of inoculated tga20 mice. (*A*) Spontaneously generated prions cause deposition of abnormal PrP in thalamus, brainstem, and, to a lesser extent, superficial layers of the frontal cortex, whereas the hippocampus is strikingly spared. (*C*) Mouse-passaged RML prions cause a more intense deposition in the superficial layers of the cortex and of the thalamus, and most prominently includes the hippocampus, a feature not seen in any mice inoculated with spontaneous cell-passaged prions. (*B* and *D*) Schematic representations of abnormal PrP immunoreactivity. [Scale bar (*A* and *C*): 3.5 mm.]

Discussion

Human prion diseases may be acquired, inherited (with pathogenic germline mutation in *PRNP*) or sporadic, probably as a consequence of rare, stochastic de novo formation of prions (25).

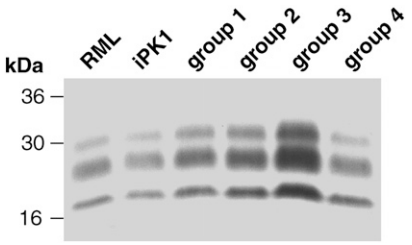

Fig. 3. Western blot analysis of protease-resistant PrP from infected mouse brains. tga20 mice were inoculated with RML-infected brain homogenate, concentrated conditioned medium from iPK1, or from the four groups of cells that became PrP^{Sc}-positive after contact with homogenate-coated wires (Groups 1-4). Brains from preterminal animals were homogenized, PK-digested, and subjected to Western blot analysis (31).

Deleault et al. (8) reported de novo generation of prions by the protein misfolding cyclic amplification (PMCA) procedure, using PrP^C purified from normal brain as substrate. However, the resulting strain could not be distinguished from RML. Generation of novel prion strains by PMCA was also reported by Barria et al. (26) and by Wang et al. in a novel PMCA system based on purified recombinant PrP, a synthetic anionic lipid and liver RNA (10).

Prion strains, even those subjected to biological cloning, may be heterogeneous at a molecular level and consist of an ensemble or quasispecies which may be selected by, and adapt in, different hosts (23, 24). According to such a model, this novel strain may have been selected from an ensemble of spontaneous prions as the preferred molecular species for stable propagation in PK1 cells, essentially adapting to PK1 cells in a similar way in which RML prions appear to have done.

PrP^{Sc} could occur at very low levels in healthy brains, almost never reaching levels that lead to disease (27). If so, "uninfected" brain homogenates might contain undetected PrP^{Sc} seeds that are concentrated on steel wires (16) and infect PK1 cells in our experiments. However, if normal brain is devoid of prions, our results mean that infectivity is generated de novo, perhaps by a mechanism in which seed formation is catalyzed by the steel surface (Fig. S1). It is also possible that brain lipids and/or RNA, known to be able to act as cofactors in some experimental systems (8, 10), could have bound to wires and played a role in triggering de novo prion formation in PK1 cells.

This raises the question as to whether "spontaneous prions" are indeed generated de novo or whether brains from uninfected animals contain a low level of prions that are concentrated by adsorption to the wire surface and thereby rendered detectable by the SCA. Differentiating between these possibilities is a challenging one but could be achieved by kinetic experiments: propagation of preexistent seed should be proportional to brain homogenate concentration; de novo seed formation would be a higher-order function of concentration (28, 29).

Methods

Culturing and Storage of N2aPK1 Cells. N2aPK1 cells (13), susceptible to several mouse prion strains, including RML and 22L, were cultured in Opti-MEM + 10% v/v fetal calf serum (OFCS; Invitrogen) supplemented with 1% penicillin/streptavidin.

Preparation and End Point Titration of RML Prions. RML prion–infected brain homogenate (designated I6200) was prepared and titrated as previously described (30).

Pretreatment of Wires. Wires, in batches of 100, were incubated with 20 mL of OFCS only (group *iii*, "untreated wires") or 10^{-4} uninfected CD-1 mouse brain homogenate diluted in OFCS (group *iv*, "coated wires") for 3 h at room temperature, washed in PBS solution five times for 15 min and air-dried. Twenty wires were placed into individual wells of a six-well tissue culture plate (Corning).

Exposure of Wires to Na2PK1 Cells and Scrapie Cell Assay in Endpoint Format. N2aPK1 cells were seeded at 3×10^5 cells per well of six-well cluster plates (Corning) containing 5 mL OFCS together with (*i*) no further additions; (*ii*) 10^{-4} uninfected CD-1 brain homogenate; (*iii*) 20 noncoated monofilament Steelex wires; or (*iv*) 20 monofilament Steelex wires coated with 10^{-4} uninfected CD-1 mouse brain homogenate. Cells were grown to confluence for 3 d at 37 °C and 5% CO_2. Cells were harvested from wells or, in the case of wire-exposed cells, wires with adherent cells were transferred to a fresh well containing 2 mL OFCS and adherent cells were rinsed off; cells were counted and seeded at 1,000 cells per well into wells of 96-well plates (Corning). The samples were assayed by the SCEPA as described previously (16).

Preparation of Concentrated Conditioned Medium from PrP^{Sc}-Positive and -Negative PK1 Cells. Cells from a single well of a 96-well plate were expanded into eight replicate wells of a 96-well plate and grown to confluence, and 25,000 cells from each well were seeded into wells of ELISPOT plates. The proportion of PrP^{Sc}-positive cells in each well was assayed as described previously for the SCA (13). Wells were deemed infected or "positive" when the "spot number" was greater than the average value given by uninfected control cells plus 10 SDs. Cells from positive wells were expanded further into 15-cm tissue culture dishes. When cells reached 60% confluence, the medium was replaced with 20 mL OFCS. Conditioned medium was collected at 24-h intervals for two successive days, pooled, and centrifuged at $100{,}000 \times g$ for 1 h at 4 °C. The pellet was resuspended in one hundredth the original volume of PBS solution to yield the "100× conditioned medium."

Testing for Transmissibility of "Spontaneous Infectivity" to PK1 Cells. Conditioned medium, concentrated ×100, was assayed at 1:10 and 1:100 dilutions by the SCA as previously described (13). The spot numbers generated in the SCA were converted to LD_{50} units/mL: a titration curve of LD_{50} units versus spot number in the SCA was fitted to the phenomenological function $S = L^n \times M / (L^n + K)$, where S is the spot number recorded in the assay, L is the number of LD_{50} units, n is an arbitrary power function, K is a saturation constant, and M is the asymptotic maximum spot number. The calibration data are fitted to this function by using Grafit software (Erithacus Software) to derive values for n, M, and K. From these values, spot numbers were converted to LD_{50} units using the relationship $L = [S \times K / (M - S)]^{0.5}$.

Testing for Transmissibility of Spontaneous Infectivity in Mouse Bioassay. Animals were cared for per institutional and home office regulations. Animals were observed daily for clinical signs and culled on diagnosis of prion disease. For each of the test conditions, groups of 10 tga20 mice were inoculated i.c. with 30 μL of 100× conditioned medium. One hundred tga20 mice were also inoculated i.c. with 30 μL of 1% CD-1 homogenate prepared from the same pool of uninfected CD-1 mouse brain homogenate used to coat the steel wires in the off-site experiment. Brains were excised and examined by histopathology and Western blotting.

Histopathology. Mouse brains were fixed in 10% buffered formol-saline, immersed in 98% formic acid for 1 h followed by 24 h in 10% formalin, and embedded in paraffin wax. Sections were cut at a nominal thickness of 4 μm. Gliosis was detected with anti-GFAP rabbit polyclonal antiserum (1:1,000; Dako). To detect abnormal PrP deposition, mounted sections were treated with 98% formic acid for 5 min, placed on an automated staining machine, heated to 95 °C in 2.1 mM Tris-HCl, 1.3 mM EDTA, 1.1 mM sodium citrate, pH 7.8, for 30 min, digested for 16 min with a low concentration of protease (iView/Ventana Medical Systems), incubated in Superblock for 10 min, then exposed to ICSM35 (1 μg/mL; D-Gen), followed by biotinylated antimouse IgG secondary antibody (SA-HRP; iView), and color was developed with 3,3′- diaminobenzidine tetrahydrochloride (iView). Hematoxylin was used as the counterstain. Photographs were taken on an ImageView digital camera (www.soft-imaging.de) and composed with Adobe Photoshop. Sections of brains were examined by the same person, who was blinded to the identity of the animal and genotype.

Western Blot Analysis. Aliquots of 10% (wt/vol) brain homogenates, with or without earlier proteinase K digestion (50 or 100 μg/mL final protease concentration, 1 h, 37 °C) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as described (31). Blots were blocked in PBS solution containing 0.05% vol/vol Tween-20 (PBST) and 5% nonfat milk powder, exposed to anti-PrP monoclonal antibody ICSM 35 (0.2 μg/mL final concentration in PBS solution with Tween-20) for 90 min, followed by antimouse IgG-alkaline phosphatase-conjugated secondary antibody for 45 min, all at room temperature. After incubation with the chemiluminescent substrate CDP-Star (Tropix), signals were visualized on Biomax MR film (Kodak), all as described (31).

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