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The authors note that the legends for Fig. 1F and Fig. 4C do not explain the error bars. For Fig. 1F, data represent mean ± SD of four determinations. For Fig. 4C, data represent mean ± SD of three determinations for XBP-1WT animals and four determinations for XBP-1Nes−/− animals. The figures and their corrected legends appear below.

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Fig. 1. Generation of XBP-1Nes−/− mice. (A) Schematic representation of xbp-1 gene-targeting strategy. F, frontal; P, posterior. (B) Southern blot analysis of genomic DNA from different brain regions and spinal cord to address the efficiency of xbp-1 deletion. (C) In vitro differentiation of primary cortical neurons from E16.5 mouse embryos transduced with lentiviral vectors to express EGFP to visualize the projection of axons and dendrites. (D) Primary neurons were treated with 5 µg/ml Tm for the indicated time points, and the levels of XBP-1, ATF4, and CHOP were analyzed by Western blot in total protein extracts. The asterisk indicates a nonspecific band. (E) XBP-1 mRNA splicing in primary cortical neurons treated with the indicated concentrations of Tm for 4 h. (F) Quantification of mRNA levels of UPR target genes in primary cortical neurons undergoing ER stress. Primary cortical neurons were treated with 5 µg/ml Tm for 8 h, and mRNA levels of grp58, pdi, wfs-1, herp, chop, and bip were analyzed in total cDNA by real-time PCR. Data represent mean ± SD of four determinations.

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Fig. 4. Prion pathogenesis in XBP-1Nes−/− mice. (A) Total levels of PrP were analyzed by Western blot in control and XBP-1Nes−/− mice infected with prions or left uninfected. Of note, accumulation of total PrPc and oligomeric PrP species is observed in prion-infected mice. (B) PrP deposition was assessed by histological analysis (PrP Olig.). (C) PrP(Δ) was determined in brain homogenates from prion infection after treatment with indicated concentrations of PK and Western blot analysis. (Lower) Quantification of five experiments as percentage of PrPres with 125 µg/ml PK. Data represent mean ± SD of three determinations for XBP-1WT animals and four determinations for XBP-1Nes−/− animals. (D) Animal survival was followed after 139A prion infection of control (n = 28) and XBP-1Nes−/− (n = 23) mice.

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Unfolded protein response transcription factor XBP-1 does not influence prion replication or pathogenesis

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Contributed by Laurie H. Glimcher, November 27, 2007 (sent for review October 3, 2007)

The unfolded protein response (UPR) is a conserved adaptive reaction that increases cell survival under endoplasmic reticulum (ER) stress conditions. X-box-binding protein-1 (XBP-1) is a key transcriptional regulator of the UPR that activates genes involved in protein folding, secretion, and degradation to restore ER function. The occurrence of chronic ER stress has been extensively described in neurodegenerative conditions linked to protein misfolding and aggregation. However, the role of the UPR in the CNS has not been addressed directly. Here we describe the generation of a brain-specific XBP-1 conditional KO strain (XBP-1Nes−/−). XBP-1Nes−/− mice are viable and do not develop any spontaneous neurological dysfunction, although ER stress signaling in XBP-1Nes−/− primary neuronal cell cultures was impaired. To assess the function of XBP-1 in pathological conditions involving protein misfolding and ER stress, we infected XBP-1Nes−/− mice with murine prions. To our surprise, the activation of stress responses triggered by prion replication was not influenced by XBP-1 deficiency. Neither prion aggregation, neuronal loss, nor animal survival was affected. Hence, this most highly conserved arm of the UPR may not contribute to the occurrence or pathology of neurodegenerative conditions associated with prion protein misfolding despite predictions that such diseases are related to ER stress and irreversible neuronal damage.

ER stress | neurodegenerative disease | prion infection | protein misfolding disorders | UPR

The endoplasmic reticulum (ER) can be thought of as a sophisticated machine for protein folding and secretion that employs an efficient system of chaperones to promote folding and to prevent abnormal aggregation or misfolding of proteins (1). The ER is responsible for executing many posttranslational modifications; it is the major calcium store and the site of cholesterol and lipid biosynthesis. A number of stress conditions can interfere with the function of this organelle, leading to abnormal protein folding in the ER lumen, resulting in a cellular condition referred to as ER stress (see reviews in refs. 1 and 2). To alleviate ER stress, cells activate an intracellular-signaling pathway known as the unfolded protein response (UPR), which aims to reestablish homeostasis by transmitting information to the nucleus about the protein-folding status in the ER lumen, triggering adaptive responses. In higher eukaryotes, ER stress stimulates three distinct UPR-signaling pathways through sensors that include inositol-requiring transmembrane kinase and endonuclease 1α (IRE1α), protein kinase-like ER kinase (PERK), and activation of transcription factor 6 (ATF6). IRE1α is a serine-threonine protein kinase and endoribonuclease that, upon activation, initiates the unconventional splicing of the mRNA encoding X-box-binding protein-1 (XBP-1) (3–5). Spliced XBP-1 is a potent transcriptional activator that increases the expression of a subset of UPR-related genes (6, 7).

The first insights about the function of XBP-1 in the nervous system came from genetic studies of human patients affected


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models (37), suggesting that the UPR has an active role in preventing neurodegeneration. Despite these strong correlations between UPR activation and protein aggregation in PrDs, the role of this pathway in the disease process has not been addressed directly and remains speculative.

XBP-1 deficiency leads to embryonic lethality due to impairment of liver function (38). To circumvent the lethal liver phenotype of XBP-1−/− mice, we targeted an XBP-1 transgene back to liver by using a liver-specific promoter (39). However, these mice died shortly after birth from severe developmental abnormalities and dysfunction of the exocrine organs, preventing the study of XBP-1 function in the CNS. To bypass this lethality, here we generated a conditional KO allele of XBP-1. XBP-1flox/flox mice were crossed with mice expressing Cre recombinase under the control of the Nestin promoter (referred to as XBP-1Nes−/−) to achieve deletion of XBP-1 in the CNS. To our surprise, XBP-1Nes−/− mice developed normally and did not show qualitative signs of spontaneous neurological impairment. To test the susceptibility of XBP-1Nes−/− mice to perturbation of ER function associated with neurodegeneration, we infected XBP-1Nes−/− mice with scrapie prions (hereafter referred to as “prion”). The extent of prion replication, neuronal loss, up-regulation of apoptosis markers, and animal survival in XBP-1Nes−/− mice was indistinguishable from that of control mice. Our results indicate that ablation of the xbp-1 gene does not drastically affect neuronal function and prion pathogenesis in vivo, suggesting that this arm of the UPR is not sufficient to confer protection against neurodegeneration.

**Results**

**Generation of a Brain-Specific XBP-1-Deficient Mouse.** Here we describe the generation of a XBP-1 conditionally deficient strain specific for the CNS (Fig. 1A). XBP-1flox/flox mice were crossed with mice expressing Cre recombinase under the control of the Nestin promoter to achieve deletion of xbp-1 in the CNS (XBP-1Nes−/−). XBP-1Nes−/− animals were viable and were born in a Mendelian ratio. To determine the efficiency of XBP-1 deletion in the CNS, we performed Southern blotting of genomic DNA samples obtained from various brain regions and the spinal cord; >95% deletion of the xbp-1 floxed allele was observed in all samples analyzed, indicating a successful targeting strategy (Fig. 1B). No spontaneous neurological dysfunctions were observed in XBP-1Nes−/− mice, as measured by the occurrence of abnormal limb clasping, tremors, wobbling gait, ruffled fur, hunched posture, reduced mobility, loss of muscle strength, changes in body weight, or signs of paralysis [supporting information (SI) Fig. 5 and data not shown]. As a positive control for all of these parameters, we followed disease progression in a mouse model of ALS, the SOD1G93A transgenic strain. Animals were followed for >12 months.

**ER Stress Responses of XBP-1-Deficient Primary Cortical Neurons.** To define the functional effects of XBP-1 deletion in neurons, we prepared primary neuronal cultures from embryonic day 16.5 (E16.5) embryos of control and XBP-1Nes−/− mice. To monitor morphological changes associated with differentiation, primary cultures were transduced with EGFP-expressing lentiviruses (Fig. 1C). Axonal outgrowth was qualitatively normal in XBP-1Nes−/− primary cultures, compared with control cultures. In addition, the levels of neuronal differentiation markers, such as Gap43, Cdkn1b, CyclinD1, Map2, and Tau, were not significantly altered in XBP-1Nes−/− primary neurons or mouse brain embryos (SI Fig. 6.4 and B). We next examined levels of XBP-1 protein in primary cortical neurons undergoing ER stress. Treatment of cells with tunicamycin (Tm) triggered XBP-1 mRNA splicing in primary cortical neurons treated with the indicated concentrations of Tm for 4 h (Fig. 1D). Quantitative real-time PCR analysis revealed that a complete deletion of the floxed allele was achieved in XBP-1Nes−/−

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**Fig. 1.** Generation of XBP-1Nes−/− mice. (A) Schematic representation of xbp-1 gene-targeting strategy. F, frontal; P, posterior. (B) Southern blot analysis of genomic DNA from different brain regions and spinal cord to address the efficiency of xbp-1 deletion. (C) In vitro differentiation of primary cortical neurons from E16.5 mouse embryos transduced with lentiviral vectors to express EGFP to visualize the projection of axons and dendrites. (D) Primary neurons were treated with 5 μg/ml Tm for the indicated time points, and the levels of XBP-1, ATF4, and CHOP were analyzed by Western blot in total protein extracts. The asterisk indicates a nonspecific band. (E) XBP-1 mRNA splicing in primary cortical neurons treated with the indicated concentrations of Tm for 4 h. (F) Quantification of mRNA levels of UPR target genes in primary cortical neurons undergoing ER stress. Primary cortical neurons were treated with 5 μg/ml Tm for 8 h, and mRNA levels of grp58, pdi, wfs-1, herp, chop, and bip were analyzed in total cDNA by real-time PCR.
of different UPR-related genes in control and XBP-1Nes type analyzed (40, 41). Thus, we compared the expression levels contains both unique and overlapping sets depending on the cell evidence suggests that the universe of XBP-1-regulated genes protein translocation into the ER, respectively. However, recent target genes in MEFs include embryonic fibroblasts (MEFs) (6) and B cells (7), and they mutually up-regulated in XBP-1-deficient neurons (Fig. 1 independent UPR genes, such as ATF4 and CHOP, was nor-
dicated the dependency of the Wolfram Syndrome gene WSF-1 in several animal models of neurodegeneration, as well as in brain damage due to ischemia, trauma, and viral infections. Thus, we decided to address the possible role of XBP-1 in neuropathological conditions triggered by protein misfolding and aggregation.

We and others previously described a strong engagement of ER stress responses in PrD mouse models (28). PrD, also known as transmissible spongiform encephalopathies, are a group of diseases that affect humans and animals characterized by neurological dysfunction, including dementia, ataxia, and psychological disturbances (29). In infectious forms of the disease, such as scrapie, the formation of PrPsc from wild-type PrPC is initiated by the exposure to exogenous infectious agents, promoting a conformational change that results in the accumulation of PrPsc. Although the nature of the infectious agent has not been completely elucidated, PrPsc seems to be the main constituent (30, 42). In agreement with previous findings, we were able to detect a significant induction of XBP-1 mRNA splicing in the brain of symptomatic animals infected with three different murine prion strains (Fig. 24). Based on this result, we analyzed the susceptibility of XBP-1Nes−/− mice to experimental PrD. Infection of mice with 139A prions triggered the up-regulation of the UPR-responsive genes, Grp58 and PDI, but not Hsp90 (Fig. 2B). Similarly, prominent phosphorylation of the stress kinases, JNK and ERK, was observed, events that have been shown to be mediated by IRE1α under ER stress conditions (43–45). To our surprise, none of these five stress markers were significantly affected in XBP-1Nes−/− prion-infected mice (Fig. 2B), suggesting that XBP-1 is dispensable for this reaction. Our results indicate that the stress responses triggered by prion replication are not enhanced in the absence of XBP-1, which could be expected if a higher basal stress or ER malfunction occurs.

We also analyzed the activation of UPR events that are IRE1α-independent. As previously described (33, 46), we found that PERK-downstream targets, such as eIF2α phosphorylation or the up-regulation of ATF4 and CHOP, were not induced by

primary neuronal cultures (SI Fig. 6C). Consistent with this result, no XBP-1 protein was detected in XBP-1Nes−/− neurons treated with Tm (Fig. 1D), but the expression of XBP-1-independent UPR genes, such as ATF4 and CHOP, was normally up-regulated in XBP-1-deficient neurons (Fig. 1E).

XBP-1 target genes have been previously defined in murine embryonic fibroblasts (MEFs) (6) and B cells (7), and they constitute a subset of UPR-related genes. Some defined XBP-1 target genes in MEFs include erdj4, edem, and sec61, which are involved in folding, ER-associated degradation (ERAD), and protein translocation into the ER, respectively. However, recent evidence suggests that the universe of XBP-1-regulated genes contains both unique and overlapping sets depending on the cell type analyzed (40, 41). Thus, we compared the expression levels of different UPR-related genes in control and XBP-1Nes−/− primary neurons. To our surprise, treatment of primary cortical neurons with Tm did not induce significant changes in the mRNA levels of Erdj4, EDEM, or Sec61, compared with XBP-1−/− MEFs (SI Fig. 7). However, the induction of other UPR genes, such as herp, pdi, and grp58, was significantly reduced in XBP-1Nes−/− neurons, demonstrating that the subset of target genes regulated by XBP-1 in neurons may differ from its targets in other cell types (Fig. 1F). In addition, we corroborated the dependency of the Wolfram Syndrome gene WSF-1 induction on XBP-1 expression (40). The expression of XBP-1-independent UPR genes, such as CHOP and BiP, was not altered in XBP-1Nes−/− neurons (Fig. 1F), suggesting that ER function and integrity were not drastically affected in this cell type by the absence of XBP-1, compared with the phenotype described in other tissues, such as liver (38), pancreas, and salivary gland (39).

**Stress Signaling in XBP-1Nes−/− Mice Infected with Prions.** As mentioned, the activation of the IRE1α–XBP-1 pathway is observed in several animal models of neurodegeneration, as well as in

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<th>XBP-1 Nes−/− + Tm</th>
<th>Control</th>
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<tr>
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Fig. 2. Stress responses in prion-infected XBP-1Nes−/− mice. (A) XBP-1 mRNA splicing was performed in cDNA obtained from the brain samples of control animals or mice infected with the murine scrapie prion strains M7, 139A, and RML. Spliced and nonspliced XBP-1 PCR fragments are indicated. As a positive control, mRNA from tunicamycin (Tm)-treated NSC34 cells is shown. (B) The expression levels of Grp58, PDI, and Hsp90 and the phosphorylation of JNK and ERK were analyzed by Western blot in brain extracts from control and XBP-1Nes−/− mice not infected or infected with 139A prions at the late stage of the disease. As a control, total JNK and ERK levels are shown. Two and three control and prion-infected animals are shown to depict experimental reproducibility. (C) The expression levels of phospho-eIF2α, total eIF2α, ATF4, and CHOP were analyzed by Western blot in brain extracts from samples described in B. As a positive control, protein extracts from NSC34 cells were treated with Tm for 6 h or left untreated (NT). A longer exposure for CHOP Western blot is shown. (D) In parallel, the levels of processed ATF6 were measured by Western blot. As a positive control, liver extracts from mice injected with Tm for 12 h (Tm) or left untreated (NT) are shown. The asterisk indicates a nonspecific band.
prion infection (Fig. 2C). Only a slight up-regulation of CHOP and ATF4 was observed in XBP-1Nes−/−-prion-infected mice (Fig. 2C), implying minor compensatory effects of the PERK pathway in the absence of XBP-1. Similarly, no ATF6 processing was observed in prion-infected brains at the late stage of the disease (Fig. 2D). Thus, this PrD model offers a unique condition to evaluate XBP-1 functions, a hallmark of PrD, and no differences were observed in the sensitivity of PrP to proteinase K (PK) treatment, even at high concentrations of PK (Fig. 4C), indicating that prion replication and misfolding were not altered in XBP-1Nes−/− mice. Finally, we determined the survival of XBP-1Nes−/− mice after 139A prion infection. In all, 23–28 animals per group were used in these experiments to provide confidence in the results of the survival analysis (control, n = 28; XBP-1Nes−/−, n = 23). As shown in Fig. 4D, the survival curve of XBP-1Nes−/− mice was virtually indistinguishable from control mice, with a median survival of 159 and 157 days after birth, respectively (the P value is nonsignificant).

**Discussion**

XBP-1 is an essential regulator of the UPR and constitutes the most conserved ER stress pathway in evolution (1). Characterization of IRE1α-XBP-1 UPR-responsive genes revealed that this pathway controls adaptive processes involving global changes of ER function, including ERAD, ER and Golgi biogenesis, protein folding, and translocation into the ER (6). For example, the ectopic expression of XBP-1 in vitro induced the expression of multiple genes related to the secretory pathway, increased cell size, expanded the ER, and elevated total protein synthesis (7). Thus, XBP-1 enforces changes in cellular structure and function consistent with the requirements of professional secretory cells.

![Image](9x9.jpg)

**Fig. 3.** Neuronal loss in prion-infected XBP-1Nes−/− mice. (A) The expression levels of BIM, PUMA, and pro-caspase-12 processing were analyzed in brain extracts from control (XBP-1WT) and XBP-1Nes−/− mice not infected or infected with 139A prions at the late stage of the disease. (B) Spongiform degeneration of the brain was analyzed in parallel after H&E staining in different brain regions.

![Image](28x824.jpg)

**Fig. 4.** Prion pathogenesis in XBP-1Nes−/− mice. (A) Total levels of PrP were analyzed by Western blot in control and XBP-1Nes−/− mice infected with prions or left uninfected. Of note, accumulation of total PrP and oligomeric PrP species is observed in prion-infected mice. (B) PrP deposition was assessed by histological analysis (PrP olig.). (C) (Upper) PrPres was determined in brain homogenates from prion infection after treatment with indicated concentrations of PK and Western blot analysis. (Lower) Quantification of five experiments as percentage of PrPres with 125 μg/ml PK. (D) Animal survival was followed after 139A prion infection of control (n = 28) and XBP-1Nes−/− (n = 23) mice.
Attempts to define the role of XBP-1 in vivo resulted in striking phenotypes in specialized secretory organs where XBP-1 was ablated. Initial studies in RAG1−/− mice whose lymphoid system was reconstituted with XBP-1−/− stem cells showed profound defects in Ig secretion, explained by the failure of B cells to differentiate to plasma cells (47, 52, 53). To circumvent the lethal liver phenotype of XBP-1−/− mice (38), we targeted an XBP-1 transgene back to liver by using a liver-specific promoter (39). This rescued mouse died shortly after birth from a severe impairment in the production of pancreatic digestive enzymes, leading to hypoglycemia and death (39). XBP-1 deficiency resulted in severe disorganization of the ER structure in pancreatic exocrine secretory cells and salivary gland acinar cells. Thus, XBP-1 is essential for the development and function of highly secretory cells.

The exact role of XBP-1 in the CNS is unknown. A genetic link is observed between an XBP-1–promoter polymorphism and the occurrence of bipolar disorders (8), schizophrenia (54), and certain personality types in the Japanese population (55). Models of behavioral stress, such as inescapable electric foot shock, trigger XBP-1 mRNA splicing in the hippocampus (19), suggesting a connection with neuronal function. Extensive studies indicate a strong association between accumulation of protein aggregates and ER stress induction in several important neurodegenerative conditions, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, ALS, PrD, and many others (28, 56). Direct evidence indicating that disruption of ER function could result in neurodegeneration came from the characterization of the Woozy mutant mice, where disruption of a BiP co-chaperone triggers neuronal dysfunction associated with spontaneous protein aggregation in the brain (57). However, most of the evidence supporting the involvement of ER stress in neurodegeneration is correlative, and manipulation of the UPR in vivo was required to define the actual contribution of the pathway to the disease process.

Here we describe the generation of a brain-specific XBP-1–deficient mouse model. To our surprise, XBP-1 deficiency did not result in spontaneous neurological dysfunction, as measured by the absence of signs of trembling, paralysis, body weight loss, breeding abnormalities, spontaneous death, muscle strength, or motor performance. We hypothesized, however, that the IRE1α–XBP-1 pathway might contribute to conditions of chronic stress, rather than basal neuronal function. Thus, we challenged XBP-1−/− mice with murine prions. This disease model evokes several central features of diverse neurodegenerative diseases, such as the accumulation of misfolded protein aggregates, neuronal loss, and progressive appearance of neurological-disease signs leading to death of the animal. More importantly, expression of XBP-1−/− in prion-infected mice correlates with an ER stress response involving the up-regulation of ER chaperones and other UPR-related genes (28). Prion infection led to XBP-1 mRNA splicing and the up-regulation of ER chaperones, such as PDI and Grp58; phosphorylation of the stress kinases, JNK and ERK; and the induction of proapoptotic genes PUMA, BIM, and caspase-12 processing. Unexpectedly, infection of XBP-1−/− mice with prions did not affect the appearance of any of these ER stress markers, nor did it affect prion misfolding, replication, or animal survival. Similarly, we recently described that genetic manipulation of the apoptosis program does not affect prion pathogenesis (58). Based on the known role of XBP-1 as a survival factor against ER stress in other experimental systems, we predicted an enhancement of PrD pathogenesis in the absence of XBP-1 expression in the CNS. Our results suggest that the occurrence of ER stress in neurodegenerative disorders may be an epiphenomenon associated with irreversible cellular damage and not causal of the disease. Alternatively, one may speculate that the up-regulation of ER chaperones is due to specific regulation of their promoters and not UPR activation. In mammals, the UPR is not restricted to the IRE1α–XBP-1 pathway, and activation of other UPR pathways may compensate XBP-1 deficiency in our PrD model. However, our data suggest that this may not be the case because we did not observe any significant alteration in the activation of ATF6 or the PERK pathway, as measured by processing of ATF6, eIF2α phosphorylation, or the expression of ATF4 and CHOP at late stages of the disease. The possible effects of ATF6 and PERK on prion pathogenesis in vivo by using genetic manipulation remain to be determined.

Our data suggest that XBP-1 may be dispensable for certain functions of the CNS under basal and pathological conditions. XBP-1 expression is relevant to ERAD, a pathway that mediates the degradation of abnormally folded proteins spontaneously generated during the protein-folding process at the ER. Disruption of other protein-clearance pathways in the CNS, such as autophagy, results in spontaneous neurodegeneration due to the accumulation of ubiquitinated intracellular protein aggregates, leading to neuronal apoptosis, motor dysfunction, and early animal death. Spontaneous disease was not observed in XBP-1−/− mice, suggesting that ERAD may not be drastically altered in this model. In summary, our study presents the unexpected finding that XBP-1, the most conserved arm of the UPR-signaling pathway, does not influence the occurrence of a neurodegenerative condition associated with PrP misfolding and aggregation despite predictions that such diseases are related to ER stress and irreversible neuronal damage.

Materials and Methods

Generation of Brain-Specific XBP-1 Conditional KO Mice. We designed the targeting vector so that exon 2 is flanked by two loxP sites (see SI Materials and Methods). XBP-1+/+ mice were crossed with mice expressing Cre recombinase under the control of the Nestin promoter (NesCre+1) to achieve deletion of XBP-1 in the CNS (XBP-1−/−). To define the efficiency of XBP-1 deletion in the CNS, genomic DNA was isolated from different brain regions and the spinal cord and was analyzed by Southern blotting. Near total deletion of xbp-1 in this tissue was observed (Fig. 18).

Analysis of Neurological Functions. Several assays were used to monitor XBP-1−/− performance, including rotarod, grip strength, and the inverted grill. Detailed methods are described in SI Materials and Methods.

In Vivo Scrapie Prion Infection. Animals were injected stereotaxically in the right hippocampus with 1 μl of RML-infected brain homogenate as previously described (30). Brains and other tissues were extracted and analyzed histologically.

SDS/PAGE, Western Blot, and PrP Analysis. Cell lysates or tissue homogenates were prepared in RIPA buffer [0.15 mM NaCl, 0.5 mM Tris-HCl (pH 7.2), 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS] as previously described (33). Western blotting was performed as previously described (33). The profile of PK sensitivity for in vivo-generated PrPw was studied by subjecting samples to incubation for 60 min at 45°C with different concentrations of PK ranging from 0 to 1,500 μg/ml. The digestion was stopped by adding an electrophoresis sample buffer. Complete methodological details are described in SI Materials and Methods.

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