MATERIAL AND METHODS

Construction of transgenic mice. The original half-genomic mouse PrP plasmid, pHGPrP, (1) kindly provided by Professor Charles Weissmann, was used to create the transgene (Figure S1). At Rocky Mountain Laboratories (RML), C-terminal sequences encoding the GPI signal sequence were removed from a serine codon at residue 233 to an SfoI site approximately 400 bases downstream, and these were replaced by a stop codon and useful restriction sites. This was accomplished by generating a 0.15kb PCR fragment from the BstEII site in the mouse PrP open reading frame up to the PrP codon for amino acid residue 232, which was then followed by a stop codon and MluI and XhoI restriction sites. This sequence was used to create plasmid p16-3 by replacing the normal mouse PrP sequence from BstEII to an XhoI site in the Bluescript cloning vector just downstream from SfoI and EcoRI sites in the PrP cDNA clone p1-5, containing the 1.3kb 5’ EcoRI fragment from mouse PrP cDNA in a Bluescript KS+ vector (2). The “half-genomic” mouse PrP plasmid, pHGPrP, was digested with BamHI and religated to remove 5kb of unsequenced DNA in the promoter region. This vector was digested with SalI and XhoI and religated to eliminate these sites. This vector was digested with AgeI and SfoI, and an oligonucleotide polylinker was inserted preserving these sites, inserting a new XhoI site and eliminating the original PrP intervening sequences. This vector was digested with NotI and BspEI and a 6.2kb fragment from the original pHGPrP was inserted to replace the original unsequenced DNA from the promoter. This vector was digested with AgeI and XhoI, and an AgeI to XhoI fragment from the GPI-negative PrP plasmid, p16-3, was
inserted to create the GPI-negative mouse PrP plasmid (p52-3). This plasmid was digested with Not I and SbfI to remove the bacterial plasmid sequences, and the mouse PrP DNA fragment was used to inoculate eggs to generate transgenic mice. Transgenic mice were generated at The Scripps Research Institute (TSRI) using PrP(-/-) mice derived from mice originally obtained from Dr. Jean Manson at NPU in Edinburgh. Several founder lines were identified by DNA PCR and were screened for expression of brain PrP mRNA by a real-time RT-PCR assay.

At TSRI, Tg lines were maintained by crossing Tg mice with non-Tg PrP (-/-) mice, and Tg-positive and Tg-negative littermates were identified and used. C57BL/6 mice were used as scrapie-susceptible mouse PrP (+/+ ) positive controls. At RML, Tg lines were maintained by crossing Tg mice with non-Tg mice heterozygous for mouse PrP (+/-) on the C57BL/10 background, which were obtained by six serial backcrosses of PrP(+/-) mice together with selection using microsatellite DNA markers to obtain individuals with greater than 98% C57BL genes. From the cross between GPIneg PrP Tg (+/-) mice and mouse PrP (+/-) mice, four genotypes were obtained with or without the transgene or the endogenous mouse PrP gene. Mice were identified using three DNA PCR tests on tail DNA. Test for GPIneg transgene: upper oligo 624
(5’AACCCTACCCCTCAGGGTG3’), lower oligo 2037
(5’CAGGGCGCCTCGAGACGCTCA3’). Reaction for PrP null allele identified the inserted Neo resistance gene sequences using upper oligo 1179
(5’GATGGATTGCACGCAGGTTC3’) and lower oligo 1180
(5’TTCAGGCCTGCGACAGTTTTG3’) (3). Reaction for endogenous mouse PrP, but negative for GPIneg mouse PrP and negative for the PrP(-/-) allele with the Neo inserted
at KpnI was targeted to the region of the KpnI site and to 3’ PrP sequences deleted in the 
GPI-negative construct: upper oligo 2057(5’CCAAGGAGGGGTACCCAT3’), lower 
oligo 2038 (5’TCCCACGATCAGGAAGATGAG3’). Conditions for all three PCR 
reactions were as previously described (3).

Flotation assay. Floatation assays for detergent-resistant membranes (DRMs) were 
performed using a combination of previously published methods (4, 5). Briefly, a 10% 
(w/v) brain homogenate in cold citrate-buffered saline (CBS) (25 mM citrate, pH 6.0, 
0.137 M NaCl, 1 mM EDTA) was diluted 1:1 with CBS + 2% Triton X-100 and 
incubated on ice for 30 min. The lysate was adjusted to 26% Optiprep (in CBS) and 
overlaid with an Optiprep step gradient (in CBS) with 350 µl/ step of 23, 16, 8, and 2.5% 
Optiprep. The tubes were spun in a Beckman TLS-55 rotor at 25000 rpm for 90 min at 
4°C. Nine 200 µl fractions were collected from the top of the tubes and proteins from 
each fraction were methanol precipitated and immunoblotted with anti-PrP antibody D13 
(6).

PrP Detection by Immunofluorescence Staining. To analyze cell surface PrP, mouse 
fibroblast cells were transduced with retroviral vectors to express either 3F4 epitope-
tagged wild-type mouse PrP or GPI-negative PrP. Cells were grown on glass chamber 
slides, washed with PBS, then incubated with mouse monoclonal anti-PrP antibody, 3F4, 
(7) for 30 min at room temperature (RT). Antibody was then washed from the slide with 
PBS, cells were fixed in neutral buffered 3.7% formaldehyde for 20 min, RT, blocked 
with 0.1M glycine in PBS for 5 min at RT, then incubated with rabbit anti-mouse IgG 
conjugated to Alexa Fluor 488 (Molecular Probes) diluted 1/500 in PBS containing 2%
BSA. To analyze intracellular PrP, cells were washed with PBS, fixed with neutral buffered 3.7% formaldehyde for 20 min at RT, blocked in 0.1 M glycine in PBS for 5 min, then permeabilized using 0.4% Triton X-100 in PBS for 10 min, RT. Cells were washed followed by incubation with anti-PrP antibody, 3F4, for 30 min, RT. Slides were washed with PBS and incubated with secondary antibody as described above. The nuclei were counterstained with DAPI (diluted 1/1500 in methanol). Slides were mounted with Vectashield mounting medium for fluorescence (Vector Laboratories, Inc.). Fluorescence patterns were visualized with a Nikon Microphot SA epi-fluorescence microscope. Images were captured with a Hamamatsu C5985 chilled CCD video camera.

**Scrapie inoculation of mice.** Mice were inoculated intracerebrally at 6 weeks of age with 0.03 or 0.05 ml of a 1% suspension of brain derived from mice infected with scrapie strain ME7, 22L or RML/Chandler. Inocula contained 0.7-1.0 x 10^6 ID50 units of infectious scrapie agent.

**Analysis of PrP expression on hippocampal neurons.** Hippocampal neurons were isolated from saline-perfused mice and processed for analysis by flow cytometry as previously described (8). PrP detection used anti-PrP antibody D18 (6). Live cells were analyzed directly to detect cell surface expression, and cells fixed in 4% paraformaldehyde for 10 min followed by permeabilization using 0.1% saponin for 3 min were analyzed for intracellular PrP expression.

**Neuropathology, immunohistochemistry and Thioflavin S staining.** Mice were
lightly anesthetized, exsanguinated by axillary bleeding and brains were immediately removed and placed in neutral buffered 3.7% formaldehyde 3-5 days. Brains were dehydrated, embedded in paraffin and cut into 4 micron sections using a Leica microtome. Slides were deparaffinized and rehydrated to Ventana APK rinse, a Tris-HCl pH7.6 buffer. Immunohistochemical staining was done on the Ventana Nexus stainer. GFAP staining for activated astrocytes used rabbit anti-GFAP (DAKO) diluted 1/1000 in 1% normal goat serum (NGS) in APK buffer, followed by Vector biotinylated goat anti-rabbit IgG diluted 1/250 in 1% NGS in APK buffer, followed by exposure to avidin-conjugated alkaline phosphatase and development with Ventana V-red (Fast Red A and B). Thioflavin S staining was done using 1% wt/volume of Thioflavin S [MP Biomedicals]. PrP-res was detected using R30 anti-PrP peptide (residues 89-103) antibody (9). To expose antigenic epitopes of PrP-res, slides were immersed in Tris-HCl, pH7.6 APK buffer and heated 20 minutes at 120°C at 20psi [Biocare Medical Decloaking Chamber]. Slides were then cooled and stained overnight at 4°C with R30 anti-PrP serum diluted 1/1500. Slides were then rinsed with buffer and stained on the automatic stainer with biotinylated goat anti-rabbit IgG (Vector) diluted 1/250, followed by Supersensitive Strepaavidin diluted 1/3 and developed with Ventana amionethylcarbazol as previously described (10).

**Immunoblotting analysis of PrP.** For detection of PrP-sen by immunoblotting, tissue homogenates (20% w/v) were prepared in PBS with protease inhibitors. The samples were vortexed for 1 min followed by sonication for 2 min in a cup-horn sonicator. Insoluble debris was removed by centrifugation at 2700 g for 10 min at 4°C. Total protein concentration in the cleared post-nuclear supernatants was determined by BCA
assay (Pierce). An aliquot of each supernatant was mixed with 1 volume of 2X SDS-PAGE sample buffer and immediately boiled for 5 min. Samples were stored at -20°C until needed. Freshly boiled samples containing known amounts of total protein were electrophoresed on 10% Bis-Tris NuPAGE gels in MES running buffer. Proteins were transferred to PVDF membranes and visualized by enhanced chemifluorescence using the anti-PrP antibody D13 (InPro Biotechnology) (6) and anti-human Fab-specific-alkaline phosphatase secondary antibody (Sigma).

To detect PrP-res by immunoblotting, brain homogenates (20%) were made in 0.01M Tris-HCl, pH7.4 with 0.005M MgCl₂ using disposable Konex microcentrifuge tubes and pestles. After sonication for 2 min, DNase (1mg/g starting tissue) was added and homogenates were incubated 1h at 37°C. An equal volume of 20% Sarkosyl in Tris-HCl buffer was added, and the suspension was vortexed and incubated 1h at room temperature. Suspensions were clarified by centrifugation at 10,000g for 30min at 10°C, and supernatant was centrifuged 215,000g for 2h at 10°C. The final pellet was resuspended by sonication in water (1ml/200mg starting tissue), digested with Proteinase K (25 micrograms/ml) for 30min at 37°C. The reaction was stopped by addition of phenylmethysulfonylfluoride and cooling on ice. After centrifugation at 215,000g for 1h at 10°C, pellets were resuspended by sonication in sample buffer, sonicated, boiled 5 min and run on SDS-PAGE gels. PrP-res bands were detected using rabbit anti-PrP peptide sera, R30 (residues 89-103) and/or R20, (residues 218-231) (11).

**Tissue processing and electron microscopy.** Mice were lightly anesthetized and perfused with saline followed by 2% glutaraldehyde + 2% paraformaldehyde in 0.15M
cacodylate buffer. Brains were then removed, fixed and processed as previously described (12). For ultrastructural analysis, vibratome sections were postfix fixed with 2% glutaraldehyde/0.1% osmium tetroxide in 0.1M sodium cacodylate buffer and fragments from the frontal cortex were embedded in Epoxy. Blocks were sectioned with an Ultracut E ultramicrotome (Leica, Nussloch, Germany) and analyzed with a Zeiss EM10 electron microscope (Carl Zeiss, Oberkochen, Germany) (13). From each case a total of 20 electron micrographs were obtained, 10 at a final magnification of 5000x and 10 at 15,000x.

**In situ hybridization.** PrP mRNA was detected by in situ hybridization using a digoxigenin-labeled riboprobe annealed at 60°C on paraffin-embedded brain sections as previously described (14). The mouse PrP-reactive 379 base riboprobe was synthesized by transcription from the T3 promoter of HaeII digested p16-3 (described above) using digoxigenin-labeled UTP.

**Legends for Supplementary Figures**

**Figure S1.** Diagram comparing features of pHGPrP versus plasmid, p52-3, used to generate GPIneg mouse PrP transgenic mice. Region from amino acid residue 232 to SfoI site 0.4 kb downstream from stop codon was removed and replaced by an oligonucleotide encoding a stop codon in place of residue 232 plus several restriction sites including SfoI. Transgene was excised with NotI and SbfI to obtain DNA for inoculation of eggs. PrP encoded by transgene lacks the GPI anchor, but has the same amino acids (23-231) as wild-type PrP after removal of the signal peptide (SP) and the C-terminal GPI signal sequences.
Figure S2. Wild-type PrP but not GPIneg PrP is enriched in DRMs. Brain homogenate from Tg mouse expressing both wild-type and GPIneg PrP was extracted with cold Triton X-100 and subjected to floatation on an Optiprep gradient to assay for association of the two types of PrP with rafts/detergent-resistant membranes (DRMs). PrP in the gradient fractions (1-9) was detected by immunoblotting. The asterisks represent the various bands associated with wild-type (top four asterisks) and GPIneg (bottom asterisk) PrP. Note that wild-type PrP is enriched in the low density DRM fractions (4-6) while GPIneg PrP is detected in the high density fractions (8-9). TgGPIneg/wt(+/+), brain homogenate from Tg mouse expressing wild-type and GPIneg PrP; wt, brain homogenate from wild-type mouse; TgGPIneg, brain homogenate from Tg mouse expressing GPIneg PrP; purified rMoPrP, purified recombinant MoPrP. Molecular mass markers are indicated in kDa on the left.

Figure S3. Immunofluorescence staining of 3F4-positive PrP in cells expressing wild-type PrP (GPI-positive) or GPI-negative PrP. Permeabilized fixed cells show both GPI-positive and GPI-negative intracellular PrP mainly in a perinuclear location consistent with the Golgi. Live cell staining reveals that the GPI-positive PrP is expressed on the cell surface, whereas GPI-negative PrP is undetectable. Thus, the GPI-negative PrP lacks attachment to the plasma membrane and is secreted into the surrounding medium.

Figure S4. PrP-res in brain after 22L infection, comparison of mice expressing only GPIneg PrP (A and C) versus mice expressing GPIneg PrP plus wild-type PrP (B and D). (A) Plaque-like PrP-res accumulations at 194 days post-infection in cerebellum of mouse
expressing only GPIneg PrP. (B) Both diffuse and plaque-like PrP-res plus severe vacuolation at 162 days post-infection in cerebellum of mouse expressing GPIneg PrP plus wild-type PrP. (C) Plaque-like PrP-res accumulations at 194 days post-infection in corpus callosum, cerebral cortex and needle track in mouse expressing only GPIneg PrP, similar to early descriptions of amyloid deposition after intracerebral scrapie inoculation (15) (D) Higher magnification of PrP-res deposited in both a plaque-like and a diffuse pattern at 162 days post-infection in corpus callosum and hippocampus of mouse expressing both GPIneg PrP plus wild-type PrP. The diffuse PrP-res is seen in both the hippocampus and in the cortex (upper left), whereas the plaque-like material is mostly in the corpus callosum.

**Figure S5.** In situ hybridization showed detection of PrP mRNA by red staining in both hippocampal (Hip) and cortical (Ctx) neurons of GPIneg PrP transgenic (Tg) mice as well as normal C57BL/6 (B6) controls, but not in negative control PrP-knock-out (KO) mice. There was no evidence for increased expression of PrP mRNA in brain endothelial cells (arrow). Therefore, overexpression of the Tg in endothelial cells did not appear to account for the endothelial localization of PrP-res in this model.
Table S1. Regional distribution of PrP-res in brains of GPI-negative PrP transgenic mice.

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<thead>
<tr>
<th>Brain region</th>
<th>Scrapie strains</th>
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<td></td>
<td>RML</td>
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<td>Corpus callosum</td>
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<td>Olfactory bulb</td>
<td>+</td>
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<tr>
<td>Forebrain</td>
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<td>Cerebellum</td>
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<td>Brainstem</td>
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Data are from mice examined at 194-400 days post-inoculation.

Reference List


FigS1

The diagram illustrates the gene structure and protein expression for two different constructs: phgPrP and p52-3 transgene.

- **phgPrP**
  - Gene structure with restriction enzyme sites (Not I, Sfo I, Sbf I, Sal I).
  - Coding regions marked as SP (Signal Peptide) and PrP.
  - Stop codon indicated.
  - Excised sequence denoted.

- **p52-3 transgene**
  - Similar gene structure with Not I and Sfo I sites.
  - Coding regions marked as SP (Signal Peptide) and PrP, with a note that the protein does not have a GPI anchor.
Fig. S2
Fig. S5