**Prion Strain Discrimination Based on Rapid *In Vivo* Amplification and Analysis by the Cell Panel Assay**

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**Abstract**

Prion strain identification has been hitherto achieved using time-consuming incubation time determinations in one or more mouse lines and elaborate neuropathological assessment. In the present work, we make a detailed study of the properties of PrP-overproducing *Tg*a20 mice. We show that in these mice the four prion strains examined are rapidly and faithfully amplified and can subsequently be discriminated by a cell-based procedure, the Cell Panel Assay.

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**Introduction**

Transmissible spongiform encephalopathies (TSEs) or prion diseases are characterized by the accumulation in the brain and sometimes in the lymphoid tissues [1,2] of an abnormally structured form (PrPSc) of the host prion protein (PrPSc) [3]. PrPSc is thought to be the only [4] or the major [5–7] constituent of the infectious agent, the prion. PrPSc is precipitable by sodium phosphotungstate (NaPTA) and thought to comprise a proteinase K-resistant and a proteinase K-sensitive fraction [8]. Prions occur in the form of diverse strains exhibiting specific biological and biochemical characteristics [9,10]. It is important to discriminate between prion strains because they exhibit distinct interspecies transmission properties, and in particular different pathogenicity for humans [11,12]. Discrimination between strains has hitherto been time-consuming and cumbersome, relying on the incubation time (usually 5 months or more) in a panel of inbred mouse lines and the determination of the lesion profile by semi-quantitative assessment of vacuolation in nine regions of the brain grey matter [13]. This method has been and still is the gold standard for strain identification in murine models. However, the neuropathological analysis is very demanding and relies on the experience of the operator. The present study investigates the suitability of faster approaches to the identification of prion strains. Some 15 years ago it was shown that knocking out *Prnp*, the gene encoding PrP, rendered mice resistant to prion infection and incapable of propagating prions [14]. Importantly, introducing PrP-encoding transgenes into the knockout mice restored susceptibility to prions, a finding that enabled reverse genetics of *Prnp* [15]. The murine *Prnp* transcription unit consists of 3 exons, of which the first and second are separated by a 2-kb, and the second and third by a 10-kb intron. The coding sequence is contained entirely in the third exon [16]. Because the transcription unit, even without the 5’ flanking region, is 8–14 kb long [16,17], a construct devoid of the large intron was prepared in order to facilitate modifications of the transgene. Introduction of the resulting so-called pHG-PrP (”half-genomic PrP”) construct into PrP knockout mice resulted in lines with various copy numbers of the transgene which expressed PrP at different levels, not necessarily in proportion to the copy number. Mice overexpressing PrP exhibited shorter incubation times than wild-type mice upon RML inoculation, but there was no direct correlation with the level of expression. Of particular interest was the line designated *Tg*a20, which in the homozygous state contained 60 copies of pHG-PrP (ORF, haplotype a [18]), expressed PrP at about 10 times the wild type level and succumbed to intracerebral (i.c.) inoculation with RML prions, administered at a high dose (1% brain homogenate of a terminally sick wild-type mouse) within 60+/−4 days, compared to 131+/−9 days for wild-type mice [15].

Because of the reduced incubation times, *Tg*a20 mice have been extensively used for prion bioassays, both by the incubation time method as well as by endpoint titration [19–22]. It has been shown that PrP overexpression favors the intraneural spread of prions [23] and the clinical expression of scrapie [22], and that the RML and ME7 scrapie strains show different incubation periods in *Tg*a20 mice [24]. However, many features of *Tg*a20 mice and their response to prion infection have not been investigated, limiting their use for accelerated strain identification. For example, it is not known whether in these mice PrP is overexpressed in proportion to wild-type levels in various brain regions. In particular, it is not known whether different prion strains are propagated faithfully in *Tg*a20 mice, and if they give rise to patterns of PrPSc deposition and histopathological changes similar to those in the C57BL/6 mouse widely used for strain typing.

In the present study we compared the expression pattern of PrPSc in the brain of *Tg*a20 mice and C57BL/6 qualitatively by immunohistochemistry (IHC) and quantitatively by western blot analysis. Because PrPSc is the substrate for the disease-associated PrPSc, and because *Tg*a20 mice succumb more rapidly to prion...
disease, we compared various biochemical characteristics of PrP<sup>C</sup> and PrP<sup>Sc</sup> in wild-type and Tga20 mice. We then determined if prion strains are propagated faithfully in Tga20 mice by the Cell Panel Assay (CPA) [25]. We also investigated if different prion strains can be distinguished by neuropathological criteria, in particular whether vacuolation, gliosis and PrP<sup>Sc</sup> deposition patterns are similar to those in C57BL/6 mice.

**Materials and Methods**

**Mouse bioassays**

The use of animals was conducted according to institutional guidelines after review of the protocol by the Institutional Animal Care and Use Committee. Mice were anesthetized by isoflurane and inoculated in the prefrontal cortex area with 20 μl of 1% brain homogenate. They were euthanized by CO<sub>2</sub> followed by cervical dislocation when they showed pronounced clinical signs, manifested by ataxia, a hunched back, loss of appetite, and a matted hair coat, prior to terminal symptoms.

**Blinded study**

Two or three sections of Tga20 mouse brain, uninfected or infected with RML, ME7, or 22L, were prepared and coded by AS. Immunohistochemical staining as described below and strain identification were performed blind by YK.

**Histological and immunochemical procedures**

All procedures were carried out at room temperature unless indicated otherwise.

**General procedures.** Brains and spleens were fixed in Carnoy’s fixative for 24 h and stored in butanol until paraffin embedding. Tissue sections (4–7 μm) were dried at 60°C for 1 h, deparaffinized in xylene, rehydrated in graded alcohols and washed in distilled water. Routine staining was with Mayer’s hemalaun and cosin.

**Immunofluorescent detection of PrP<sup>C</sup> (Fig. 1).**

Rehydrated sections were treated with 10 μg/ml proteinase K (PK; Roche) in 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA for 5 min, in order to enhance the immunoreactivity of PrP, rinsed in cold water, and treated with 4 M guanidinium thiocyanate for 10 min. Sections were then washed twice in distilled water, twice in PBS, once in PBST (0.1% Triton X-100 in PBS) and incubated for 2 h with D18 monoclonal anti-PrP antibody [46] which was directly labeled with Alexa 555 fluorophore (5 μg/ml; Alexa Fluor 555 monoclonal antibody labeling kit, Invitrogen) in PBS. After washing twice in PBS, sections were mounted in Vectashield (Vector). Images were taken through an epifluorescence Zeiss microscope (AxioZeiss, Imager.A1) (http://www.zeiss.com/) equipped with an AxioCam MRc camera, using AxoVision software version 14, and processed with Adobe Photoshop (CS2, Version 9.0.2)

![Image](image_url)

**Figure 1.** The PrP<sup>C</sup> expression pattern in different brain regions of Tga20 mice differs from that in C57BL/6 mice. PrP<sup>C</sup> expression in six brain regions was monitored using Alexa 555-labeled D18 anti-PrP antibody in six brain regions: A, cortex; B, cerebellum; C, brain stem; D, striatum; E, thalamus. All photos except the ones labeled “C57+” were taken at the same settings. “C57+” are photos of the same area as “C57”, but taken at longer exposures. F,G: PrP<sup>S</sup> expression in Purkinje cells. PrP<sup>S</sup> and calbindin in the cerebellum were immuno-labeled using Alexa 555-tagged D18 anti-PrP antibody (red) and Alexa 488-tagged anti-calbindin rabbit polyclonal antibody (green), respectively. F: C57BL/6 mouse cerebellum, G: Tga20 mouse cerebellum. PrP<sup>S</sup> is stained red, calbindin green. The overlay on the right shows PrP<sup>S</sup>-expressing Purkinje cells in yellow. M, molecular layer; G, granular layer of the cerebellum; P, Purkinje cell layer. Bars represent 20 μm.

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Colocalization of PrP^C and calbindin in Purkinje cells. For fluorescence double-labeling studies rehydrated sections were pretreated and stained with Alexa 555-labeled D18 monoclonal anti-PrP antibody as described above, followed by 2 h incubation with anti-calbindin rabbit polyclonal antibody (Sigma), labeled with Alexa 488 fluorophore using the Zenon kit (Zytox, West Pico, CA) and exposure to X-ray film (GE Healthcare, Piscataway, NJ), and quantified by UVP (BioSpectrum AC Imaging System).

Proteinase K digestion of PrP^Sc. PrP^Sc was precipitated with 22L, RML and ME7-infected brains by the NaPTA procedure [47]. NaPTA pellets obtained from 500 µl of 10% brain homogenates were resuspended in 10% PrP^0/o brain homogenate (containing on average 10 mg/ml of protein) and digested with 50 µg PK/ml (final concentration) for 1 h at 56°C and 450 rpm in a Thermomixer R (Eppendorf), to yield PrP27-30. Aliquots of PrP27-30, were incubated with PK at concentrations ranging from 100 to 3,200 µg/ml for 1 h at 37°C. PrP27-30 was quantified by western blot analysis.

Cell Panel Assay

Cell lines were maintained and the Cell Panel Assay was performed as described in [25] except that the assays with LD9 cells were under conditions that rendered them susceptible to 30IC (S.P.M. and C.A.Demczyk, unpublished results).

Results

PrP^C expression levels

Western blot analysis showed that PrP^C levels in Tg20 relative to C57BL6 mice were approximately 5 to 20-fold higher, depending on the brain region (Table 1). Immunohistochemical analysis revealed a higher intensity of staining of intracellular PrP^C, PrP^D and PrP^E of the neurupil and PrP^E associated with the white matter tracts (Fig. 1 A–E). Strikingly, cerebellar Purkinje cells of Tg20 mice fail to express PrP^C (Fig. 1 B, G), reflecting the absence of PrP mRNA reported earlier [14]. Purkinje cells of C57BL/6 and CD1 mice (Figs. 1 B, F and data not shown) express PrP^C, albeit not in all areas of the cerebellum [26].

Biochemical properties of PrP^C and PrP^Sc in Tg20 compared to C57BL6 mice

To compare the resistance to proteinase K (PK) of PrP^C from uninfected Tg20 and wild type mice, we diluted Tg20 brain homogenates 1:3 into PrP^0/o brain homogenate to achieve the same concentration of PrP^C as in homogenates of C57BL/6 mice (Fig. 2 A1). The homogenates (containing on average 10 mg protein/ml) were digested with PK at 40 µg/ml for various times...
Figure 2. Biochemical analyses of PrP\(^\text{C}\) and PrP\(^\text{Sc}\) from Tg\(^{20}\) and C57BL/6 brains. All samples were analyzed by western blotting and the quantification of the blots is summarized in Table 2. A. PK digestion of PrP\(^\text{C}\). A1: Uninfected Tg\(^{20}\) brain homogenates were diluted 1:8 into PrP\(^{\text{Po/o}}\) brain homogenate to adjust PrP\(^\text{C}\) to the same levels as in C57BL/6 homogenates. Serial 1:2 dilutions of the two preparations, compared by western blot analysis, showed indistinguishable PrP\(^\text{C}\) levels.

A2: C57BL/6 and 1:8 diluted Tg\(^{20}\) brain homogenates were digested for the times indicated with 40 µg PK/ml at 56°C.

A3: The same samples as in (A2) were digested with PK at the concentrations indicated at 37°C for 60 min.

B: Thermolysin digestion of brain homogenates from C57BL/6 or Tg\(^{20}\) mice infected with 22L, RML or ME7. Homogenates were treated with 25 µg TL/ml for one hour at 70°C and

D: PK resistance of PrP\(^{\text{30}}\)
more resistant to PK than PrPC from C57BL/6 mice in both experiments. Maybe PrPSc produced at high levels aggregates, perhaps in the form of aggresomes, and thereby become less susceptible to PK digestion. Cytoplasmic, toxic forms of PrP have been shown to accumulate in aggresomes [27–29]. In contrast, PrPSc (resPrPSc), from Tg20 and C57BL/6 mice infected with the various strains, showed no difference in resistance to further digestion with PK, at concentrations up to 3,200 μg/ml (Fig. 2 D).

Brain homogenates from Tg20 mice infected with 22L, RML and ME7 contained 2.4, 1.5 and 1.5 times, respectively, more total PrP, i.e. PrPSc plus PrPSc, than those from C57BL/6 mice (Fig. 2 B, C, data not shown and Table 2), which is not in proportion to the eightfold PrPSc overexpression in uninfected Tg20 mice. The increase of total PrP in infected C57BL/6 mice is most likely due to a doubling of the PrPSc level [30] in addition to the accumulation of PrPSc; in infected Tg20 mice there is little PrPSc (see below) and possibly no increase of PrPSc, hence less increase of total PrP.

PrPSc comprises PK-sensitive PrPSc (senPrPSc) and PK-resistant PrPSc (resPrPSc) moieties [30]. SenPrPSc was claimed to be resistant to thermolysin (TL) treatment under conditions allowing complete PrPSc digestion [31,32]. We analyzed by western blot the proportions of TL- and PK-resistant PrP in the brains of C57BL/6 as compared to C57BL/6 mice (Fig. 2 A, A2) or for 1 h with PK concentrations ranging from 5 to 50 μg/ml (Fig. 2, A3). Interestingly, PrPSc from Tg20 mice was more resistant to PK than PrPSc from C57BL/6 mice in both experiments. Maybe PrPSc produced at high levels aggregates, perhaps in the form of aggresomes, and thereby become less susceptible to PK digestion. Cytoplasmic, toxic forms of PrP have been shown to accumulate in aggresomes [27–29]. In contrast, PrPSc (resPrPSc), from Tg20 and C57BL/6 mice infected with the various strains, showed no difference in resistance to further digestion with PK, at concentrations up to 3,200 μg/ml (Fig. 2 D).

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The specific infectivities of resPrPSc, as determined by the SSCA using LD9 cells [25], differ between strains and mouse lines (Table 2). This underlines the previously described observation that resPrPSc is an unreliable indicator of infectivity [33–36].

**Faithful propagation of prion strains**

Different strains of prions exhibit cell-specific tropism that can be assessed in *vivo* by the Cell Panel Assay (CPA) [25]. This assay is based on a sensitive, accurate and rapid cell-based procedure for quantification of prion infectivity, the Standard Scrapie Cell Assay (SSCA) [37]. Cells are exposed to prions, passaged for three splits, immunostained for PrPSc, and individual, PrPSc-containing cells are counted using automated imaging equipment. The response of a cell line to a prion strain is expressed by the Response Index (RI), the concentration of the sample required to give a designated proportion of infected cells (usually taken as 1.5%) under standardized conditions. Prion strains can be characterized by the ratio of their RIs on four cell lines, PK1, CAD5, LD9 and R33; this panel allows discrimination of RML, ME7, 301C and 22L prions [25].

C57BL/6 and Tg20 mice inoculated with these four strains (using 20 μl of 1% brain homogenates of terminally sick wild-type mice) were sacrificed when they showed advanced clinical symptoms. Table 3 shows that incubation times were shortened for Tg20 mice, but remarkably to different extents for the various strains, ranging from a reduction of 85 days or 59% for RML to only 36 days or 22% for 301C. Brain homogenates were subjected to the CPA: as shown in Fig. 3, the RI values on PK1, CAD5 and R33 cells relative to those on LD9 cells were the same for C57BL/6 and Tg20, within the limits of error. Thus, the four strains we examined had the same CPA characteristics whether they were propagated in Tg20 or in C57BL/6 mice. Interestingly, the RI values were 3 to 12 times lower for strains propagated in Tg20 than for C57BL/6 brains (Fig. 3 and Supp. Table) except for 301C, which had an equal or higher RI in Tg20 than in C57BL/6 mice; this may be due to the relatively long incubation time for 301C in Tg20, allowing for more extensive replication.

**Comparison of disease-associated PrP deposition and histopathology in Tg20 and C57BL/6 mice**

The neuropathological and immunohistochemical phenotype elicited by the three prion strains ME7, RML and 22L in Tg20 differed from that in C57BL/6 mice. This is exemplified by PET-BLOT images of brain sections which showed strikingly lower cortical, hippocampal and cerebellar PrPSc signal intensities in 22L-infected Tg20 than in C57BL/6 mice (Fig. 4 F). Although overexpressing PrPSc, Tg20 mice accumulated less PrPSc, in this context defined as PrP stained immunohistochemically in infected brain under conditions where there is no or only faint background staining in uninfected controls (Fig. 4 A–E and Fig. S1). This is in good agreement with the results of the biochemical PrP analyses described above. However, there were strain-dependent differences between the different brain regions. The brainstem (Fig. 4 B) and thalamus (Fig. S1) exhibited PrPSc deposition of similar intensity in Tg20 and C57BL/6 mice, which was found with all the strains examined. Hallmarks of the ME7 strain in Tg20 mice

![Image](58x24 to 76x41)
were the dense PrPSc plaques in the cortex and hippocampus. A characteristic of 22L infection in Tgα20 mice was the presence of plaque-like PrPSc deposits in the granular layer of the cerebellum (Table 4). None of those features were observed in the four RML-infected Tgα20 brains examined, which exhibited diffuse PrPSc deposits in the cortex, hippocampus and brainstem.

### Table 2. PrP levels in prion-infected brains of C57BL/6 and Tgα20, and specific infectivities of PK-resistant PrP.

| PrP in 22L-infected mouse brains (arbitrary units/μg protein) | Preparation | C57BL/6 (%) | Tgα20 (%) | Tgα20/C57BL/6 |
|-------------------------------------------------------------|-------------|-------------|-----------|--------------|
| Total PrP                                                   | 18471 (100) | 45243 (100) | 2.4       |
| TL-resistant PrP                                           | 2050 (11)   | 1606 (4)    | 0.8       |
| PK-resistant PrP                                           | 4137 (22)   | 742 (2)     | 0.18      |
| Ratio TL/PK                                                 | 1:2         | 2.2:1       |           |

| PrP in RML-infected mouse brains (arbitrary units/μg protein) | Preparation | C57BL/6 (%) | Tgα20 (%) | Tgα20/C57BL/6 |
|-------------------------------------------------------------|-------------|-------------|-----------|--------------|
| Total PrP                                                   | 15140 (100) | 23392 (100) | 1.5       |
| TL-resistant PrP                                           | 2169 (14)   | 1823 (8)    | 0.84      |
| PK-resistant PrP                                           | 1384 (9)    | 1258 (5)    | 0.91      |
| Ratio TL/PK                                                 | 1:6:1       | 1:4:1       |           |

| PrP in ME7-infected mouse brains (arbitrary units/μg protein) | Preparation | C57BL/6 (%) | Tgα20 (%) | Tgα20/C57BL/6 |
|-------------------------------------------------------------|-------------|-------------|-----------|--------------|
| Total PrP                                                   | 17411 (100) | 23200 (100) | 1.3       |
| TL-resistant PrP                                           | 1184 (7)    | 271 (1)     | 0.23      |
| PK-resistant PrP                                           | 5032 (29)   | 951 (4)     | 0.19      |
| Ratio TL/PK                                                 | 1:4:3       | 1:3:5       |           |

| Specific infectivity of PK-resistant PrP (RI/resPrPSc)*     | Strain      | C57BL/6 (%) | Tgα20 (%) | Tgα20/C57BL/6 |
|-------------------------------------------------------------|-------------|-------------|-----------|--------------|
| 22L                                                         | 56          | 90          | 1.6       |
| RML                                                         | 66          | 15          | 0.2       |
| ME7                                                         | 16          | 14          | 0.9       |

The PrP content of brain homogenates was assessed by western blot and quantification of immunoreactive bands in arbitrary pixel units by UVP (BioSpectrum3AC Imaging System). Each measurement was adjusted for gel loading to provide the pixel value per μg of sample protein. All gels have been normalized to each other allowing the comparison of PrP values. TL- and PK-resistant PrP values have been calculated using regression curves of signal intensities obtained on serial dilutions of each sample (Fig. 2B and C and Fig. S3).

*The specific infectivity of resPrPSc is herein defined as the ratio of the RI measured in LD9 cells (susceptible to all three prion strains) relative to resPrPSc (pixels per ng of brain protein, see above).

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### Table 3. Characteristic parameters of various prion strains in C57BL/6 and Tgα20 mice.

| Survival times* | Average in days ± SD (n) | Titer | Log LD50 units/g brain wet weight |
|-----------------|--------------------------|-------|---------------------------------|
|                 | C57BL/6 | Tgα20 | C57BL/6 | Tgα20 | C57BL/6 |
| RML             | 144±5.5 (48) | 59 (8) | 85 | 0.41 | 8.75 |
| 22L             | 135±1.5 (45) | 83 (3) | 52 | 0.61 | 8.26 |
| ME7             | 143±6.6 (49) | 94 (3) | 49 | 0.68 | 8.26 |
| 301C            | 162±1.4 (15) | 126 (3) | 36 | 0.78 | n.d. |

*Mice were inoculated with 20 μl of 1% brain homogenate and euthanized when they showed pronounced clinical signs, rather than at terminal stage of disease. The average incubation period for ME7 in C57BL/6 mice is shorter than the 155–160 days described in some other studies [24,48,49]. This might be due to variations in the criteria for euthanasia, the number of passages of the strain and/or variations between different C57BL/6 mouse lines.

*Because of the very short symptomatic period in Tgα20 mice, all mice in a group were killed on the same day.

*By endpoint titration in C57BL/6 mice (6 mice per group, 22L and ME7; 5 mice per group, RML) [50]. The inocula were homogenates of brains from terminally sick C57BL/6 mice infected with 22L, ME7 or 301C, or from terminally sick CD1 mice infected with RML.

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Figure 3. The Cell Panel Assay of four prion strains propagated in Tga20 or wild-type mice results in similar relative Response Indices on four cell lines. CAD5 (red), PK1 (blue), R33 (green) and LD9 (violet) cells were exposed to 1:3 serial dilutions of 0.1% brain homogenates infected with 22L (a,b), RML (c,d), ME7 (e,f) or 301C (g,h) propagated in Tga20 mice (a,c,e,g) or wild-type mice (b, f, h, C57BL/6; d, CD1). The number of PrPSc-positive cells ("spots") is plotted against log[dilution] of the brain homogenate. The Response Index (RI1.5%/3) of a cell line for a prion strain (vertical dotted line) is the concentration (reciprocal of the homogenate dilution) that yields 300 spots per 20'000 cells (1.5% PrPSc-positive cells) after the 3rd split (horizontal dotted line). The "RI ratio" of a strain is the RI on a cell line relative to that on LD9; it was very similar for each strain regardless of whether it was propagated in wild-type or Tga20 mice (see Table S1 for RI values).

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Figure 4. PrP<sup>Sc</sup> deposition pattern in C57BL/6 and Tg<sub>a</sub>20 mice infected with three scrapie prion strains. Although PrP<sup>C</sup> levels are higher in all brain regions of Tg<sub>a</sub>20 as compared to C57BL/6 mice, PrP<sup>Sc</sup> levels were lower in Tg<sub>a</sub>20 mice except for the brainstem and thalamus (see also Fig. S1). PrP<sup>Sc</sup> was detected by IHC using anti-PrP mouse monoclonal antibody Bar 233 (SpiBio) and secondary anti-mouse IgG antibody, as described in the methods section. Bar 233 stains PrP<sup>Sc</sup> deposits with various morphologies in the brains of C57BL/6 and Tg<sub>a</sub>20 mice infected with ME7, 22L and RML scrapie strains. Only faint diffuse cytoplasmic and neuropil staining was seen in uninfected mouse brains (Fig. S1). The secondary antibody alone stains only plasma in the blood vessels, as can be seen in large vessels in the last pictures on the right of A, D and E. A, striatum; B, brain stem; C, cortex; D, cerebellum; E, hippocampus. Table 4 summarizes the findings. Bars represent 50 µm. F: PET-BLOTS of horizontal brain sections from Tg<sub>a</sub>20 and C57BL/6 mice infected with the 22L strain and the corresponding uninfected control mice. Arrows show the cortices, hippocampi and cerebella. doi:10.1371/journal.pone.0005730.g004
The vacuolation scores of the different brain regions of Tg20 mice varied to some extent for each prion strain. However, they were not distinctive enough to identify strains with confidence using conventional lesion profiles (Table 5). On the other hand, PrPSc deposition profiles in Tg20 mice were substantially different between the strains 22L, ME7 and RML and did not correlate with the level of PrPC overexpression in these mice (Tables 1, 5 and Fig. 5). A blinded analysis of a panel of slides by one of us (Y.K.) demonstrated that it was possible to reliably distinguish these strains on the basis of PrPSc immunohistochemistry.

In conclusion, 22L, ME7 and RML prions propagated in Tg20 mice retain their characteristic tropism for cultured cells, allowing their distinction by the Cell Panel Assay. While conventional lesion profiling seems not to be feasible, immunohistochemistry reveals characteristic differences in the location and pattern of PrPSc deposits. Thus, Tg20 mice allow not only accelerated infectivity

**Table 4.** Strain-specific diagnostic features in Tg20 mice.

|                | 22L | ME7 | RML |
|----------------|-----|-----|-----|
| Plaque-like PrPSc deposits in the granular layer of the cerebellum | +   | −   | −   |
| Dense PrPSc plaques in the cortex and hippocampus | −   | +   | −   |
| PrPSc staining of cerebellum | +   | −   | −   |
| Diffuse PrPSc deposits in cortex and hippocampus | +   | −   | +   |

**Table 5.** Comparative neuropathology in Tg20 and C57BL/6 mice infected with three prion strains.

|               | C57BL/6 | Tg20 |
|---------------|---------|------|
| **ME7**       |         |      |
| Spongiosis    | +++     | +    |
| Gliosis       | +++     | +    |
| PrPSc         | +++     | +    |
| Cortex        | +++     | +    |
| Hippocampus   | +++     | +    |
| Cerebellum    | +++     | +    |
| Brainstem     | +++     | +    |
| Thalamus      | +++     | +    |
| Striatum      | +       | +    |
| Septum        | +       | +    |
| **22L**       |         |      |
| Spongiosis    | +++     | +    |
| Gliosis       | +++     | +    |
| PrPSc         | +++     | +    |
| Cortex        | +++     | +    |
| Hippocampus   | +++     | +    |
| Cerebellum    | +++     | +    |
| Brainstem     | +++     | +    |
| Thalamus      | +++     | +    |
| Striatum      | +       | +    |
| Septum        | +       | +    |
| **RML**       |         |      |
| Spongiosis    | +++     | +    |
| Gliosis       | +++     | +    |
| PrPSc         | +++     | +    |
| Cortex        | +++     | +    |
| Hippocampus   | +++     | +    |
| Cerebellum    | +++     | +    |
| Brainstem     | +++     | +    |
| Thalamus      | +++     | +    |
| Striatum      | +       | +    |
| Septum        | +       | +    |

The severity of spongiform changes, astrogliaosis and PrPSc deposition are indicated as follows: −, none; +, slight; ++, moderate; ++++, severe; ++++, very severe. PrPSc is defined as PrP immunoreactive material which is not observed in similarly processed sections from uninfected mice, and its overall intensity was visually assessed regardless of the morphology of the deposits (described in Table 4).
determination, but also strain discrimination, at least for the strains examined in the present study.

Discussion

Our endeavor to characterize Tga20 mice in terms of their PrP expression and their response to prion infection led us to a number of interesting observations.

We found that overexpression of PrPC in Tga20 brains is not homogeneous, in contrast to its expression in the brains of C57BL/6 mice. While some regions, in particular brainstem, highly overexpress PrPC, others, such as cortex and striatum, express only moderately higher levels of PrPC (Fig. 5). However we found no detectable expression in Tga20 cerebellar Purkinje cells, in agreement with the previously reported absence of PrP mRNA [15]. The expression of PrP by Purkinje cells in wild-type mice has been the subject of discordant reports [26,38–41]. We confirm that, as described previously [26,41], wild-type mice express PrP in Purkinje cells, albeit not homogeneously throughout the cerebellum. The PrP expression pattern does not correspond to a clear anatomical distribution [39] and the reason for the heterogeneity of Purkinje cells with regard to PrP expression in wild-type mice remains unknown. The absence of PrP and PrP mRNA in Purkinje cells as well as the variable overexpression levels of PrP in different brain areas of Tga20 mice remain unexplained. PrP expression was also markedly different in the spleens of Tga20 and wild-type mice; we detected PrP only in the red pulp but not in the follicular germinal centers of the transgenic animals, whereas in wild-type mice PrP is localized in the germinal centers (Fig. S2 and [42]). One hypothesis is that the PrP expression pattern in Tga20 mice is altered due to the deletion of the large intron from the PrP gene construct. Alternatively, or in addition, the integration site of the transgenes could modulate expression. It should also be noted that the pHG expression construct used in these transgenic mice is, lamentably, heterogeneous in regard to its constituent segments: the promoter region, the 1st exon as well as the intron and its flanking splice regions are derived from I/LN mice (Prnpb), most of the 5’ and 3’ non-coding regions as well as the ORF are from NMR1 mice (unknown Prnp haplotype) and the 3’ part of the 3’ non-coding and the 3’ flanking region are from NZW/lac mice (Prnpa) [15].

The CPA provides an alternative, novel and rapid method for strain discrimination [25]. It is based on strain-specific tropism that enables prions to chronically infect some cell lines but not others. The molecular basis for this strain-specific characteristic might be similar to that underlying the brain tropism trait that is exploited to identify strains on the basis of the lesion profiles [43]. We determined that the four strains examined in this study retained their characteristic behavior in the CPA after being replicated in Tga20 mice, despite the fact that the lesion and PrP deposition profiles were different than in wild-type mice. In addition, the PrP deposition profiles in Tga20 mouse brains were sufficiently different for 22L, ME7 and RML prions to allow clear discrimination between these three strains.

As shown in Table 3, the time elapsing between inoculation and terminal disease (“incubation time”) is shorter in Tga20 than in C57BL/6 mice, albeit not to an equal extent for the different prions.
strains. The duration of the clinical phase was also shortened, from about 2 weeks in wild-type mice to a few days in Tga20 mice. Our studies and those of others [15,24] rule out overall higher levels of cerebral PrPSc as a reason for accelerated pathogenesis and disease, and support the hypothesis that a specific viral brain region ("clinical target area") [44,45] may be preferentially affected. In fact, as shown by immunohistochemistry, PrPSc accumulates at lower levels in all brain regions of Tga20 except for the brainstem and thalamus, which show intense PrPSc staining with all prion strains examined (Fig. 4 and Fig. S1), suggesting that one or both of these may be the clinical target areas.

It remains to be explained why PrPC, which is abundantly present in most brain regions of Tga20 mice, largely fails to be converted into PrPSc. One suggestion has been that PrPSc generated in Tga20 mice may be sensitive to PK digestion [24], another, that the increased rate of synthesis of PrPC results in improperly folded, perhaps aggregated PrP which is not suitable for conversion to PrPSc, or which accumulates in an inappropriate compartment, such as the aggresome [27–29]; our finding that PrPSc in uninfected Tga20 brains is more resistant to digestion by PK than its wild-type counterpart may provide some support for the latter view. A more likely explanation, however, is that less PrPSc and infectivity accumulate in Tga20 mice because of their shortened survival time. This hypothesis is supported by the fact that lower infectivities in Tga20 mice were observed by the CPA for RML, 22L and ME7, which showed strong reductions in incubation times, but not for 301C, which showed only moderate reduction.

Because of the inhomogenous overexpression of PrPC in different brain regions, Tga20 mice provided a unique opportunity to study the relationship between PrPSc expression levels and PrPSc accumulation. Interestingly, except for the presumed clinical target areas brainstem and thalamus, PrPSc accumulation patterns do not follow PrPC overexpression (Fig. 5). For example, the cerebellum expresses higher PrPC levels than the cortex in Tga20 mice, yet there is virtually no PrPSc deposition for two of three strains. This once more raises the question as to the factors underlying the different tropism exhibited by the various strains.

Finally, of practical importance, the prion strains we examined retain their identity after propagation in Tga20 mice and can be discriminated by the CPA as well as by immunohistochemical analysis of PrPSc deposits, reducing the time required for analysis by months.

**Supporting Information**

**Figure S1** PrPSc deposition pattern in the thalamus of C57BL/6 and Tga20 mice infected with three scrapie prion strains and PrPC staining in the thalamus of uninfected control mice. PrPC and PrPSc were detected by IHC using anti-PrP mouse monoclonal antibody Bar 233 (SpiBio) and secondary anti-mouse IgG antibody. Bar 233 stains PrPSc deposits with various morphologies in the brains of C57BL/6 and Tga20 mice infected with ME7, 22L and RML scrapie strains. Only faint diffuse cytoplasmic and neuropil staining was seen in uninfected mouse brains. Bars represent 20 μm (ME7) or 50 μm (22L, RML and uninfected).

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**Figure S2** PrPSc in spleens of C57BL/6 and Tga20 mice. PrPSc was stained using the monoclonal antibody D18. General procedures were similar to those described for the processing of brain tissue. The differences were that tissue pretreatment consisted only of exposure to guanidinium thiocyanate without PK treatment, and D18 anti-PrP antibody was used at 5 μg/ml for 2 h followed by washes with PBS and incubation with secondary anti-human IgG linked to HRP (Southern Biotech) at 1 μg/ml for 2 h. A, C: PrPC in the spleens of C57BL/6 is seen in the germinal centers of follicles. B, D: PrPC is not detectable in the follicles of Tga20 mouse spleens, but can be seen in the interfollicular zones (red pulp). Bars represent 100 μm (upper panels) or 20 μm (lower panels).

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**Figure S3** Quantification of TL- and PK-resistant PrP. UVP quantification data of PrP immunoreactive bands, expressed in arbitrary pixel units, were plotted against the sample concentration. Regression curves show good signal linearity over the dilution series. The ratio of PK- or TL-resistant PrP in C57BL/6 versus Tga20 mice is calculated from the ratio of the slopes of the corresponding regression lines.

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**Table S1** RIs

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**Author Contributions**

Conceived and designed the experiments: YK PS SM CW CIL. Performed the experiments: YK PS SM GFS AS NS CW CIL. Analyzed the data: YK PS SM NS CW CIL. Wrote the paper: CW CIL.

**References**

1. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216: 136–144.
2. Doi S, Ito M, Shinagawa M, Sato G, Isomura M, et al. (1988) Western blot detection of scrapie-associated fibril protein in tissues outside the central nervous system from preclinical scrapie-infected mice. J Gen Virol 69: 955–960.
3. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, et al. (1995) Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 90: 10962–10966.
4. Legname G, Baskakov IV, Nguyen HO, Riesner D, Cohen FE, et al. (2004) Synthetic mammalian prions. Science 303: 675–678.
5. Dickinson AG, Ounram GW, Taylor DM, Foster JD (1989) Further evidence that scrapie agent has an independant genome. In: Court LA, Dormont D, Brown P, Kingsbury DT, eds. Unconventional virus diseases of the central nervous system. pp 446–459.
6. Couzin J (2004) Biomedicine. An end to the prion debate? Don’t count on it. Science 305: 389.
7. Weissmann C (1994) A ‘unified theory’ of prion propagation. Nature 352: 679–683.
8. Safar J, Wille H, Itri V, Groth D, Serban H, et al. (1998) Eight prion strains have PrPSc molecules with different conformations. Nat Med 4: 1157–1165.
9. Bruce ME (1993) Scrapie Strain Variation and Mutation. Br Med Bull 49: 622–638.
10. Besen RA, Marsh RF (1992) Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. J Virol 66: 2096–2101.
11. Will RG, Ironside JW, Zeieller M, Cousens SN, Estibeiro K, et al. (1996) A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 347: 921–925.
12. Lasmézas CI, Fourmigue J, Nouvel V, Boe H, Marcé D, et al. (2001) Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: Implications for human health. Proc Natl Acad Sci USA 98: 4142–4147.
13. Fraser H, Dickinson AG (1968) The sequential development of the brain lesions of scrapie in three strains of mice. J Comp Pathol 78: 301–311.
14. Biebler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, et al. (1993) Mice devoid of PrP are resistant to scrapie. Cell 73: 1339–1347.
15. Fischer M, Rutledge T, Raeber A, Sailer A, Moser M, et al. (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. Embo J 15: 1255–1264.
16. Biebler K, Oesch B, Scott M, Westaway D, Walchli M, et al. (1986) Scarpie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 46: 417–429.
17. Westaway D, Cooper G, Turner S, Costa MD, Carlson GA, et al. (1994) Structure and polymorphism of the mouse prion protein gene. Proc Natl Acad Sci USA 91: 6418–6422.
18. Segade F, Claudino E, Wrobel K, Ramos S, Lazo PS (1995) Isolation of nine gene sequences induced by silica in murine macrophages. J Immunol 154: 2343–2392.
19. Klein MA, Frigg R, Fleisch E, Raeber AF, Kalinke U, et al. (1997) A crucial role for B cells in neuroinvasive scrapie. Nature 390: 687–690.
20. Montrasio F, Cozzi A, Fleisch E, Rossi D, Klein MA, et al. (2001) B lymphocyte-restricted expression of prion protein does not enable prion replication in prion protein knockout mice. Proc Natl Acad Sci U S A 98: 4034–4037.
21. Seeger H, Heikenwalder M, Zeller N, Kranich J, Schwarz P, et al. (2005) Coincident scrapie infection and nephritis lead to urinary prion excretion. Science 310: 324–326.
22. Thackray AM, Klein MA, Bujblosso R (2003) Subclinical prion disease induced by oral inoculation. J Virol 77: 7991–7998.
23. Glazet M, Aguzzi A (2000) PrP(C) expression in the peripheral nervous system is a determinant of prion neuroinvasiveness. J Gen Virol 81: 2013–2021.
24. Thackray AM, Klein MA, Aguzzi A, Bujblosso R (2002) Chronic subclinical prion disease induced by low-dose inoculum. J Virol 76: 2510–2517.
25. Mahal SP, Baker CA, Demczyk CA, Smith EW, Julius C, et al. (2007) Prion strain discrimination in culture: the cell panel assay. Proc Natl Acad Sci U S A 104: 20908–20913.
26. Haeberle AM, Ribaut-Barassin C, Bombard G, Mariani J, Hunsmann G, et al. (2000) Synaptic prion protein immunoreactivity in the rodent cerebellum. Microsc Res Tech 50: 66–75.
27. Grenier C, Bussommete C, Vowell L, Roussou X (2006) Molecular morphology and toxicity of cytoplasmic prion protein aggregates in neuronal and non-neuronal cells. J Neurochem 97: 1456–1466.
28. Ma J, Wollmann R, Lindquist S (2002) Neurotoxicity and neurodegeneration with PrP accumulates in the cytosol. Science 290: 1781–1785.
29. Cohen E, Taraboulos D (2005) Scarpie-like prion protein accumulates in aggresomes of cyclosporin A-treated cells. Embo J 22: 404–417.
30. Biebler H, Raeber A, Sailer A, Fischer M, Aguzzi A, et al. (1994) High prion and PrPsc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol Med 1: 19–30.
31. Owen JP, Maddison BC, Whitelam GC, Gough KC (2007) Use of thermolysin in the diagnosis of prion diseases. Mol Biotechnol 35: 161–170.
32. Grenier C, Gross N, Tattum MH, Jackson GN, Clarke AR, et al. (2000) Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin. Biochem J.
33. Lasnieraz CI, Dedy JP, Robain O, Jaegly A, Beringue V, et al. (1997) Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 275: 402–405.
34. Manson J, Jamieson E, Baybutt H, Tuzi N, Barron R, et al. (1999) A single amino acid alteration (101L) introduced into murine PrP dramatically alters incubation time of transmissible spongiform encephalopathy. EMBO J 18: 6653–6664.
35. Bavec PC, Stolze L, Fleisch E, Enari M, Weissmann C (2003) A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions. Proc Natl Acad Sci U S A 100: 11666–11671.
36. Liu T, Zwangman T, Li R, Pan T, Wong BS, et al. (2001) Differential expression of cellular prion protein in mouse brain as detected with multiple anti-PrP monoclonal antibodies. Brain Res 896: 118–129.
37. Ford MJ, Burton LJ, Li H, Graham CH, Frobert Y, et al. (2002) A marked disparity between the expression of prion protein and its message by neurons of the CNS. Neuroscience 111: 533–551.
38. Watts JC, Drisaldi B, Ng V, Yang J, Strome B, et al. (2007) The CNS glycoprotein Shadoos have PrP(Sc)-like protective properties and displays reduced levels in prion infections. EMBO J 26: 4030–4050.
39. Laine J, Marc ME, Sy MS, Axelrad H (2001) Cellular and subcellular morphological localization of normal prion protein in rodent cerebellum. Eur J Neurosci 14: 47–56.
40. Brown KL, Ritchie DL, McBride PA, Bruce ME (2000) Detection of PrP in extraneural tissues. Microsc Res Tech 50: 40–45.
41. Fraser H, Dickinson AG (1973) Scrapie in mice: agent-strain differences in the distribution and intensity of grey matter vacuolation. J Comp Pathol 83: 29–40.
42. Dickinson AG, Outram GW (1979) The scrapie replication-site hypothesis and its implication for pathogenesis. In: Prusiner SB, Hardy WU, eds. Slow transmissible diseases of the nervous system. New-York: Academic Press. pp 13–31.
43. Kimberlin RH, Walker CA (1986) Pathogenesis of scrapie (strain 263K) in hamsters infected intracerebrally, intraperitoneally or intracranially. J Gen Virol 67: 253–263.
44. Williamsam RA, Peretz D, Puulla C, Hall H, Bastidas RB, et al. (1998) Mapping the prion protein using recombinant antibodies. J Virol 72: 9413–9418.
45. Wadsworth JD, Joiner S, Hill AF, Campbell TA, Deshrasalas M, et al. (2001) Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. Lancet 358: 171–180.
46. Bruce ME, Boyle A, Couzens S, McConnell I, Foster J, et al. (2002) Strain characterization of natural sheep scrapie and comparison with BSE. J Gen Virol 83: 695–704.
47. Dell'Omo G, Vannoni E, Vyssotski AL, Di Bari MA, Nonno R, et al. (2002) Strain specificity of scrapie in C57BL/6 mice: evidence of strain-specific differences in congenital susceptibility. J Gen Virol 83: 695–704.