Prion clearance in bigenic mice

Jiri G. Safar,1,2 Stephen J. DeArmond,1,3 Katarzyna Kociuba,1 Camille Deering,1 Svetlana Didorenko,1 Essia Bouzamondo-Bernstein,3 Stanley B. Prusiner1,2,4 and Patrick Tremblay1,2†

Institute for Neurodegenerative Diseases1 and Departments of Neurology2, Pathology3 and Biochemistry and Biophysics4, University of California, 513 Parnassus Ave, San Francisco, CA 94143, USA

The clearance of prions from the brain was investigated in bigenic mice designated Tg(tTA::PrP+/+), in which expression of the cellular prion protein (PrPc) was regulated by oral doxycycline administration. With suppression of PrPc expression, the incubation time for RML prions was prolonged almost threefold from ~150 to ~430 days. To determine the clearance rate of disease-causing PrPSc, bigenic mice were given oral doxycycline beginning 98 days after inoculation with RML prions and sacrificed at various time points over the subsequent 56 days. The half-life (t1/2) for PrPSc was ~1-5 days in mouse brain, in reasonable agreement with the apparent t1/2 of 30 h that was determined in a separate study for scrapie-infected mouse neuroblastoma (ScN2a) cells in culture. Both protease-sensitive and -resistant conformers of PrPSc were cleared at the same rate. The t1/2 value for PrPc clearance from brain was ~18 h, which was considerably longer than the t1/2 of 5 h found in ScN2a cells. The capability of the brain to clear prions raises the possibility that PrPSc is normally made at low levels and continually cleared, and that PrPSc may have a function in cellular metabolism. Moreover, these bigenic mice make it possible to determine both components of PrPSc accumulation, i.e. the rates of formation and clearance, for various strains of prions exhibiting different incubation times.

INTRODUCTION

In prion diseases, the cellular prion protein, designated PrPc, is converted into the disease-causing isoform, designated PrPSc. PrPc is composed of three α-helices and two β-strands, while PrPSc is a β-sheet-rich protein that is likely to contain a β-helix (Donne et al., 1997; Pan et al., 1993; Riek et al., 1997; Safar, 1996). Recent electron crystallography and molecular modelling studies suggest that the infectious monomer may be composed of a PrPSc trimer (Govaerts et al., 2004; Wille et al., 2002).

Prions in both mammals and yeast multiply by inducing the precursor protein to adopt the conformation of the protein in the prion state. In fungi, polymerizing the prion precursor proteins into amyloid fibrils is sufficient to create infectious particles (Maddelein et al., 2002; Sparrer et al., 2000). In mammals, the task seemed more complex until recently (Legname et al., 2004).

In attempting to produce mammalian prions de novo, we initially employed transgenic (Tg) mice expressing high levels of MoPrP(P101L), which harbours the analogous mutation causing Gerstmann–Sträussler–Scheinker syndrome in humans. These mice developed neurodegenerative disease spontaneously (Hsiao et al., 1999) and brain extracts from these mice transmitted prion disease to Tg mice expressing low levels of MoPrP(P101L), designated Tg196 mice (Hsiao et al., 1994; Telling et al., 1996). Such experiments were plagued by the inability to demonstrate protease-resistant (r)PrPSc in the brains of the spontaneously ill Tg mice, as well as of the inoculated Tg196 mice. Subsequently, we used the P101L mutation to guide the folding of a 55 aa peptide composed of MoPrP residues 89–143 into a β-rich conformation that polymerized into fibrils. The fibrils produced neurological dysfunction ~1 year after inoculation into Tg196 mice, while the non-β-rich form of this peptide did not (Kaneko et al., 2000). Brain extracts prepared from ill Tg196 mice serially transmitted disease to Tg196 recipients with a similar incubation time, but did not cause disease in wild-type (wt) mice (Tremblay et al., 2004).

Impressed by the amyloid deposition in the brains of Tg196 mice inoculated with β-rich fibrils (Tremblay et al., 2004), we investigated larger, recombinant (rec)PrPs polymerized into amyloids, which represent a subset of β-rich structures. Using recMoPrP(89–230) produced in Escherichia coli to form amyloid fibrils, we inoculated the fibrils intracerebrally into Tg mice expressing MoPrP(89–231) (Supattapone et al.,

1Present address: Neurochem Inc., 275 boul. Armand-Frappier, Laval, Quebec, Canada H7V 4A7.

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established that PrPSc is the sole component of the mammalian prion. While Tg mice studies have demonstrated that the rate of PrPSc accumulation in brain is directly proportional to the level of PrPC expression (Prusiner et al., 1990), little is known about the factors that govern the rates of PrPSc formation and clearance. The availability of bsgenic mice, designated Tg(TA: PrP+/0), in which PrP expression can be reversibly regulated (Tremblay et al., 1998), offers an opportunity to investigate the formation and clearance of PrPSc in vivo. Tg(TA: PrP+/0) mice were inoculated with RML prions and developed neurological dysfunction ∼150 days later. When PrP expression was suppressed by ∼95% by administration of doxycycline, the onset of neurological deficits was delayed to ∼430 days (Tremblay et al., 1998). Using these bsgenic mice, we measured the clearance as well as the rates of accumulation of both rPrPSc and protease-sensitive (s)PrPSc in brain. In the brains of these prion-infected mice, the half-life (t1/2) for clearance of PrPSc was ∼1.5 days, in good agreement with studies of scrapie-infected neuroblastoma (ScN2a) cells (Peretz et al., 2001b).

The capability of the brain to clear prions raises the possibility that PrPSc is normally made at low levels and continually cleared. The steady-state level of PrPSc must be below 10^5 molecules per ml of 10% (w/v) brain homogenate (BH) since homogenates prepared from uninfected animals do not transmit disease to inoculated recipients (Prusiner et al., 1983). The hypothesis that PrPSc might be present at low levels in normal animals raises the question as to whether PrPSc has an as-yet-unidentified metabolic function. Moreover, this conjecture suggests that prion diseases may arise from dysregulation in the concentration of PrPSc rather than the formation of a new conformer.

**METHODS**

**Source and care of laboratory animals.** Tg(Pnp-tTA)F959/FVB/Prnp^0/0 mice were crossed with Tg(tetO-PrP)E6740/FVB/Prnp^0/0 mice to produce bsgenic Tg(TA: PrP+/0) mice, in which PrP expression was under the control of the doxycycline-inducible promoter as described previously (Tremblay et al., 1998). Tg mice were obtained by microinjection of FVB/Prnp^0/0 N4F1 obtained by crossing Prnp^+/+ with FVB animals for four generations before interbreeding to homozygosity. Breeding and screening of Tg mice were performed as described previously (Scott et al., 1989).

**Prion inoculum.** Anaesthetized animals were inoculated at 8 weeks (±4 days) of age with 20 μl RML prions (1% BH in PBS) and observed for signs of neurological dysfunction three times a week as described previously (Carlson et al., 1994; Scott et al., 1989). Mice with fully developed clinical symptoms were euthanized and their brains harvested for further studies.

**Administration of doxycycline and care of laboratory animals.** For breeding, animals were kept on 0.02 mg doxycycline ml^{-1} in their drinking water, which was ceased at 3 weeks of age to allow high levels of PrP expression (Tremblay et al., 1998). After animals were inoculated intracerebrally with RML prions, doxycycline administration was initiated at different time points post-inoculation to suppress PrPSc expression. In dose-dependency experiments, doxycycline at 0-002, 0-02, 0-2 or 2 mg ml^{-1} was added to the drinking water with 5% sucrose to mask the bitter taste. In clearance experiments, an initial single dose of 25 mg doxycycline kg^{-1} was administered intraperitoneally, followed by 2 mg doxycycline ml^{-1} in the drinking water.

**Treatment of BHs.** For biochemical analysis only, 10% BH samples were prepared in Ca^2+ /Mg^2+-free PBS by homogenization (three times for 15 s each) with a PowerGen 125 homogenizer (Fisher Scientific). Homogenates were clarified by centrifugation at 500 g for 5 min on a tabletop centrifuge. Supernatants were subjected either to sodium phosphotungstate (PTA) precipitation or to proteinase K (PK; Gibco-BRL) treatment followed by PTA precipitation (Safar et al., 1998). For PK digestion, 5% BH samples were incubated with 25 μg PK ml^{-1} for 1 h at 37°C. PK activity was blocked using a protease inhibitor (PI) cocktail, composed of 2 μg aprotinin and leupeptin (A/L) ml^{-1} and 0.2 mM PMSF. Samples were diluted with one volume of 4% Sarkosyl/PBS and analysed by Western blotting or by conformation-dependent immunoassay (CDI) (see below).

For PK digestion followed by PTA precipitation, 10% BH was treated with PK and the reaction was blocked using the PI cocktail. The samples were diluted with one volume of 4% Sarkosyl/PBS and then PTA precipitated. For PTA precipitation, 1 ml aliquots of the samples were adjusted to 0-31% PTA, 2-6 mM MgCl2, incubated at 37°C for 1–16 h and centrifuged at 16000 g for 30 min. Pellets were resuspended in PBS with PI and 0-2% Sarkosyl and immediately analysed by Western blotting or CDI (see below).

**Western blotting.** Volumes (500 μl) of 5% (w/v) homogenates were prepared in PBS containing 2% (w/v) Sarkosyl and the protein concentration was measured using the bicinchoninic acid assay (Pierce). For digested samples, aliquots were treated with 20 μg PK ml^{-1} at a ratio of 1:50 [PK : protein (w/w)] for 1 h at 37°C. Digestion was stopped with 5 mM PMSF. PK-treated samples were mixed with an equal volume of SDS loading buffer and boiled for 5 min; 30 μl samples were analysed by 12% SDS-PAGE using precast gels (Bio-Rad). For undigested samples, aliquots were mixed with an equal volume of SDS loading buffer and boiled for 5 min; 10 μl samples were analysed by 12% SDS-PAGE using precast gels. Western blots were developed with 1 μg recFab D13 ml^{-1} and peroxidase-labelled anti-Fab secondary antibody, and detected using the enhanced chemiluminescence system as described previously (Peretz et al., 2001a).

**Detection of sPrPSc and rPrPSc by CDI.** Samples were processed for CDI using a time-resolved fluorescence (TRF) method as described previously (Safar et al., 1998). Samples were split into two aliquots; one was kept untreated (native) and the other (denatured) was treated with one volume of 8 M guanidinium hydrochloride and heated at 80°C for 5 min. Both aliquots were diluted 20-fold with water containing 0.5 mM PMSF and 2 μg A/L ml^{-1}. Samples were loaded in triplicate on 96-well polystyrene microplates (OptiPlate HTF-96; Packard) that had been pre-coated with recFab D18 (Peretz et al., 1997) for the sandwich-formatted CDI or pre-activated with glutaraldehyde (0-2% in PBS, pH 7-4); 2 h) for direct CDI. The plates were incubated for 2 h at room temperature and blocked overnight at 4°C with Tris-buffered saline (TBS; 20 mM Tris/HCl pH 7-5; 1% BSA; 6% (w/v) sorbitol). The next day, a-PrP europium-labelled, chimeric human–mouse (HuM) recFab D13
(Peretz et al., 1997) at a concentration of 0·55 µg ml⁻¹ was added to the plates and incubated for 2 h at room temperature. The (D–N) difference, which is the binding of antibody to native and denatured samples, is directly proportional to the concentration of PrPSc. The concentration may be determined by a mathematical model that was developed previously to calculate the amount of β-sheet in PrP (Safar et al., 1998). The concentration of PrP in the samples was calculated from calibration curves present on each plate and generated with β-rich recMoPrP(89–231) (Mehlhorn et al., 1996) serially diluted into BH of Pn0/0 transgenic mice (Büeler et al., 1992). The concentration of sPrPSc was calculated according to the formula: [sPrPSc] = [PrPSc] – [rPrPSc], in which [PrPSc] is the concentration of PrPSc before PK treatment and [rPrPSc] is the concentration of the protease-resistant fraction after PK treatment.

Neuropathology. Brains were rapidly removed from animals and either perfusion fixed in 10 % buffered formalin or snap frozen. Immunohistochemical localization of rPrPSc was accomplished on formalin-fixed, paraffin-embedded tissue sections by the formic acid-hydrated autoclaving method (Muramoto et al., 1992). The concentration of rPrPSc with 3 M guanidine isothiocyanate for 10 min at room temperature. Histoblot sections by the histoblot procedure (Taraboulos et al., 1992a). HuM D13 and HuM D18, which recognize MoPrP(97–106) and MoPrP(132–156), respectively (Peretz et al., 1997), were used as primary antibodies.

Cryostat sections were pressed on to wet nitrocellulose paper and allowed to air dry. Sections were exposed to 200 µg PK ml⁻¹ for 1 h at 37 °C to eliminate PrPSc, followed by denaturation of the remaining rPrPSc with 3 M guanidine isothiocyanate for 10 min at room temperature. Histoblot sections were incubated overnight at 4 °C with D13 or D18 diluted 1:100. Following rinsing, histoblot sections were incubated with the secondary antibody, alkaline phosphatase-conjugated anti-human IgG (Promega), for 1 h at room temperature and color was developed with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Promega) after additional rinses.

Table 1. Levels of PrP expression and incubation times in bigenic mice after inoculation with RML prions

<table>
<thead>
<tr>
<th>Tg lines*</th>
<th>PrP expression (fold)†</th>
<th>Doxycycline (mg ml⁻¹)‡</th>
<th>n/n0§</th>
<th>Mean incubation period ± SEM (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(tetO-PrP⁺/⁰)</td>
<td>0·1</td>
<td>–</td>
<td>12/12</td>
<td>431 ± 5·3</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>0·1</td>
<td>2</td>
<td>3/3</td>
<td>432 ± 7·2</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>ND</td>
<td>0·2</td>
<td>7/7</td>
<td>424 ± 12·7</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>ND</td>
<td>0·02</td>
<td>7/7</td>
<td>398 ± 23·6</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>ND</td>
<td>0·002</td>
<td>8/8</td>
<td>274 ± 16·1</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>2</td>
<td>–</td>
<td>12/12</td>
<td>149 ± 8·4</td>
</tr>
<tr>
<td>Tg(tTA : PrP⁺/⁰)</td>
<td>4</td>
<td>–</td>
<td>18/18</td>
<td>81 ± 4·2</td>
</tr>
</tbody>
</table>

* Tg(tetO-PrP⁺/⁰)E6740/FVB/Pn0/0 mice were crossed with Tg(PrnP+tTA)F959/FVB/Pn0/0 mice to produce bigenic Tg(tTA : PrP)3/FVB/Pn0/0 mice. Bigenic mice were either heterozygous (+/0) or homozygous (+/+ +) for the tetO-PrP transgenes.
† PrP expression compared with PrPSc in the brains of uninoculated wild-type FVB mice, which were normalized to onefold. ND, Not determined.
‡ Concentration of doxycycline in the drinking water beginning 7 days before intracerebral inoculation with RML prions.
§ Number of mice developing prion disease divided by the number inoculated with RML prions.

RESULTS

Suppression of PrPSc expression lengthens incubation times

Tg(PrnP+tTA)F959/FVB/Pn0/0 mice were crossed with Tg(tetO-PrP⁺/⁰)E6740/FVB/Pn0/0 mice to produce bigenic Tg(tTA : PrP)3 mice, in which PrPSc expression was under the control of a doxycycline-responsive promoter (Tremblay et al., 1998). The concentration of PrPSc in Tg(tetO-PrP⁺/⁰)E6740/FVB/Pn0/0 mice was ~5 % of that observed in Tg(tTA : PrP⁺/⁰)3 mice (Table 1). Tg(tTA : PrP⁺/⁰)3 mice are heterozygous for the tetO-PrP transgene. When the Tg(tTA : PrP⁺/⁰)3 mice were bred to homozygosity for the PrP transgene, the expression level was found to double. The level of PrPSc expression in Tg(tTA : PrP⁺/ +)3 mice was fourfold of that found in wt FVB mice brain, which was normalized to onefold.

When Tg(tTA : PrP⁺/⁰)3 mice were given 2 mg doxycycline ml⁻¹ in the drinking water, PrPSc expression decreased to 0·1-fold, similar to that found in Tg(tetO-PrP⁺/⁰)E6740/FVB/Pn0/0 mice. The incubation times for RML prions in Tg(tTA : PrP⁺/⁰)3 mice given 2 mg doxycycline ml⁻¹ and Tg(tetO-PrP⁺/⁰)E6740/FVB/Pn0/0 mice were ~430 days (Fig. 1, Table 1). When Tg(tTA : PrP⁺/⁰)3 mice were inoculated with RML prions and not given doxycycline, the incubation time was ~150 days. Tg(tTA : PrP⁺/ +)3 mice inoculated with RML prions exhibited incubation times of ~80 days (Fig. 1, Table 1). Doubling PrPSc expression decreased the incubation time by a factor of two (Table 1), in accordance with earlier studies using Tg mice expressing different levels of Syrian hamster PrPSc (Prusiner et al., 1990).
Kinetics of PrPSc accumulation in the brains of FVB mice exhibiting incubation times of Tg(tTA : PrP+)

The levels of PrP expression could be regulated in a dose-dependent fashion by administration of doxycycline. Tg(tetO-PrP) mice treated with higher doses of doxycycline displayed longer incubation periods. To suppress PrP expression, animals received various concentrations of doxycycline in the drinking water beginning 7 days before intracerebral inoculation with RML prions. Bigenic Tg(tTA : PrP+)
mice displayed longer incubation periods. To suppress PrP expression, animals received various concentrations of doxycycline in the drinking water beginning 7 days before intracerebral inoculation with RML prions. Bigenic Tg(tTA : PrP+)
mice showed progressively lengthening of the incubation time. The addition of 0.002 mg doxycycline ml⁻¹ resulted in a two-fold increase in the incubation time from ~150 to ~275 days (Fig. 1). Addition of 0.02 mg doxycycline ml⁻¹ produced incubation times of ~400 days; higher levels of doxycycline failed to produce any substantial increase in the incubation time. Tg(tetO-PrP) mice harbouring only the target transgene exhibited incubation times of ~430 days. Prion-inoculated FVB mice receiving 2 mg doxycycline ml⁻¹ developed disease in ~130 days compared with control FVB mice exhibiting incubation times of ~125 days.

**Kinetics of PrPSc accumulation in the brains of Tg(tTA : PrP+/0) mice**

To study the clearance of PrPSc from the brains of Tg(tTA : PrP+/0) mice, we first determined the kinetics of PrPSc accumulation after intracerebral inoculation with RML prions. Tg(tTA : PrP+/0) mice were sacrificed at various intervals after inoculation and PrPSc levels determined by CDI (Supplementary Fig. S1a). PrPSc levels peaked at 98 days post-inoculation and gradually decreased over the next 40 days. By 142 days post-inoculation, the PrPSc level in the brains of these asymptomatic mice was ~80% of the level found at 98 days. From this point, the mice remained well for another week until neurological dysfunction ensued (Fig. 1). In contrast to the biphasic accumulation of PrPSc, the accumulation of rPrPSc was monophasic and exponential with a maximum level found at 142 days, approximately 1 week before the onset of neurological symptoms (Supplementary Fig. S1b). We did not measure the levels of rPrPSc during the clinical phase of disease; some investigators have argued that prion titres rise during this period, while others have found no evidence to support such a contention (Brown et al., 1982; Oesch et al., 1983).

**Doxycycline modulates PrPSc levels in Tg(tTA : PrP+/0) mice**

At five different times after inoculation of RML prions, doxycycline was administered to Tg(tTA : PrP+/0) mice. Starting doxycycline at 98 or 126 days after inoculation demonstrated that readily measurable reductions in PrPSc could be achieved (Supplementary Fig. S2a). Similarly, reductions in rPrPSc could be achieved by doxycycline administration beginning at both 98 and 126 days after inoculation, as well as at 84 days (Supplementary Fig. S2b). Administration of doxycycline at the midpoint of the incubation time, at 70 and 77 days after inoculation, did not create conditions for measuring decrements in the level of either PrPSc or rPrPSc, and the mice were asymptomatic at 290 days post-inoculation. For comparison, mice expressing PrPSc inoculated with RML prions developed disease at ~150 days (Fig. 1).

**Clearance of sPrPSc and rPrPSc**

Based on the clearance curves in Supplementary Fig. S2, we administered doxycycline to Tg(tTA : PrP+/0) mice beginning 98 days after inoculation with RML prions. At six different time points, we sacrificed Tg(tTA : PrP+/0) mice and measured PrPSc and PrPSc levels in their brains.

PrPSc levels in the brains of uninoculated Tg(tTA : PrP+/0) mice declined to ~40% of the initial level within 1 day of beginning doxycycline treatment and to ~5% after 7 days (Figs 2 and 3a). This low level of PrPSc persisted for the remainder of the 56 day period. From CDI measurements (Fig. 2) and densitometric scans of Western blots (Fig. 3a) of PrPSc levels in the brains of uninfected Tg(tTA : PrP+/0) mice, we estimated that the apparent t½ of PrPSc in the brains of these mice was ~18 h.

After doxycycline administration was initiated, rPrPSc levels declined more slowly than PrPSc levels in Tg(tTA : PrP+/0) mice. By Western immunoblotting, rPrPSc was found to reach the minimum level to be detectable after ~7 days and remained at that level for at least another 3 weeks (Fig. 3b and c). In the brains of Tg(tTA : PrP+/0) mice sacrificed at...
154 days post-inoculation, after being on doxycycline for 56 days, the levels of rPrPSc were rising. Using densitometric scanning of Western blots, we estimated the $t_{1/2}$ of rPrPSc in the brains of these mice to be $\sim 5$ days (Fig. 3c). Using CDI, we measured the levels of PrPSc, which is composed of both sPrPSc and rPrPSc, in the brains of Tg(tTA : PrP +/0)3 mice that began receiving doxycycline at 98 days after inoculation with RML prions. We estimated the $t_{1/2}$ of rPrPSc and sPrPSc in the brains of these mice to be $\sim 5$ days (Fig. 4a). At 98 days after inoculation when doxycycline administration commenced, rPrPSc represented $\sim 15\%$ of PrPSc in the brains of RML-inoculated mice. After 56 days of doxycycline administration, the proportion of rPrPSc doubled, reaching $\sim 30\%$ (Fig. 4b).

Direct measurement of PrPSc by CDI revealed that up to $\sim 50\%$ of total PrP in the brains of RML-infected Tg(tTA : PrP +/0)3 mice was PrPSc. Both Western blotting and CDI revealed the rapid clearance of rPrPSc from the brains of RML-inoculated Tg(tTA : PrP)3 mice. The CDI measures the kinetics of rPrPSc clearance and rebound more rapidly than Western blots (Figs 3c and 4a). While the CDI has a detection range of more than three orders of magnitude and detects all conformers of PrPSc after guanidinium hydrochloride denaturation, SDS-PAGE and Western blots depend on incomplete denaturation in SDS and the signal is proportional to only those unfolded monomers that are transblotted on to the filter. We conclude that observed differences are likely to reflect the different clearance and formation rates of different conformers detected by each method.

**Suppression of PrPSc results in clearing of rPrPSc from grey matter**

To examine the impact of PrPSc suppression on the distribution of rPrPSc in the brain, Tg(tTA : PrP +/0)3 mice were inoculated with RML prions, administered doxycycline at 98 days post-inoculation and sacrificed 58 days later. Animals on doxycycline were well when sacrificed, in
contrast to inoculated Tg(tTA:PrP<sup>+/0</sup>)<sup>3</sup> mice not receiving doxycycline, which exhibited neurological dysfunction at 156 days post-inoculation. The ill animals exhibited rPrP<sub>Sc</sub> accumulation throughout the brain, with the most intense immunostaining in the white matter tracts of the thalamus (Fig. 5a) and white matter of the corpus callosum and cerebellum (Fig. 5b). rPrP<sub>Sc</sub> was also found in grey matter, where immunostaining resulted in a low-intensity signal that enabled the neocortex, hippocampus and cerebellar cortex to be delineated on the histoblots. In contrast, the mice receiving doxycycline beginning at 98 days post-inoculation and sacrificed at 156 days post-inoculation exhibited little or no rPrP<sub>Sc</sub> in grey matter (Fig. 5c and d). Residual rPrP<sub>Sc</sub> was found almost exclusively in white matter, particularly in the corpus callosum (Fig. 5c). In the cerebellum, rPrP<sub>Sc</sub> immunostaining was seen primarily at the interface of the white matter and cerebellar cortex (Fig. 5d).

**Fig. 4.** Clearance of sPrP<sub>Sc</sub> and rPrP<sub>Sc</sub> in the brains of Tg(tTA:PrP<sup>+/0</sup>)<sup>3</sup> mice infected with RML prions. (a) Concentration of rPrP<sub>Sc</sub> and sPrP<sub>Sc</sub> in the brains of RML-infected bigenic mice. (b) Relative levels of sPrP<sub>Sc</sub> and rPrP<sub>Sc</sub> in Tg mice receiving doxycycline beginning at 98 days after RML inoculation and for the indicated durations: 10 animals were euthanized and their brains taken for analysis by CDI. To measure rPrP<sub>Sc</sub>, samples were first digested with PK and then precipitated with PTA; the proportion of sPrP<sub>Sc</sub> was calculated as described previously (Safar et al., 1998; Tremblay et al., 2004). Data points and bars represent the mean ± SEM obtained from three to five brains in three independent measurements.

**Fig. 5.** Histoblots of brain sections demonstrate that clearance of rPrP<sub>Sc</sub> after blockage of PrP<sub>C</sub> expression is more efficient from grey matter than white matter. Coronal brain sections, at the level of the hippocampus and thalamus (a, c) and at the cerebellum and pons (b, d), were taken from two Tg(tTA:PrP<sup>+/0</sup>)<sup>3</sup> mice 156 days after inoculation with RML prions. One animal developed clinical symptoms (a, b), while the other, in which PrP<sub>C</sub> expression was blocked beginning at 98 days post-inoculation, remained well (c, d). In the brain of the mouse with blocked PrP<sub>C</sub> expression (c, d), substantially less PrP<sub>Sc</sub> was present, most of which appeared to be in the white matter. To suppress PrP<sub>C</sub> expression, doxycycline was administered at 98 days post-inoculation in an initial single dose of 25 mg kg<sup>−1</sup> intraperitoneally, followed by 2 mg ml<sup>−1</sup> in the drinking water. CC, Corpus callosum; Hp, hippocampus; NC, neocortex; Th, thalamus.

**PrP<sub>C</sub> suppression protects nerve cells and reduces rPrP<sub>Sc</sub> deposits**

Using the hydrated autoclaving method on brain sections, we were able to localize the deposition of rPrP<sub>Sc</sub>. In three Tg(tTA:PrP<sup>+/0</sup>)<sup>3</sup> mice that showed neurological dysfunction at 156 days after inoculation, we found punctate deposits of rPrP<sub>Sc</sub> in both the grey and white matter. In the hippocampus, intense rPrP<sub>Sc</sub> deposits were found in the molecular layer of the dentate gyrus and in the lacunosum moleculare and stratum oriens of Ammon’s horn (Fig. 6a–c), indicating advanced stages of prion neuroinvasion. Granular rPrP<sub>Sc</sub> deposits were located in the overlying corpus callosum. Severe loss of pyramidal neurons in the hippocampus CA1 region was also apparent (Fig. 6b and c). In contrast, when PrP<sub>C</sub> expression was suppressed beginning at 98 days post-inoculation, histological sections taken at 156 days post-inoculation showed no evidence of hippocampal nerve cell loss (Fig. 6d and e) and substantially less PrP<sub>Sc</sub>. rPrP<sub>Sc</sub> in the brains of these doxycycline-administered mice was confined largely to the corpus callosum (Figs 5c and 6d and f).

In the cerebellum of Tg(tTA:PrP<sup>+/0</sup>)<sup>3</sup> mice at 156 days post-inoculation, PrP<sub>Sc</sub> was found throughout the molecular and granule cell layers of the cerebellar cortex.
A substantial proportion of PrPSc in the molecular layer appeared to be in Bergmann radial glia and their processes (Supplementary Fig. S3b, arrow). Purkinje cells adjacent to Bergmann glia were immunonegative. When PrPC expression was suppressed by doxycycline beginning at 98 days post-inoculation, no PrPSc was found in the molecular layer in the brains of these mice at 156 days post-inoculation; however, focal PrPSc deposits were found in the granule cell layer and the immediate underlying white matter in the lateral cerebellar cortex (Supplementary Fig. S3c and d). Little or no PrPSc was found in grey matter of the mice given doxycycline and most of the remaining PrPSc appeared in white matter, with overall less intense staining for PrPSc when compared with mice not given doxycycline. In both mice receiving doxycycline and mice not receiving doxycycline, many PrPSc deposits were seen surrounding blood vessels in the cerebellum (Supplementary Fig. S3b and d). Similar perivascular deposits were also found in other brain regions (data not shown).

DISCUSSION

Despite suppressing PrPC expression by almost 95%, bigenic mice eventually developed neurological dysfunction
(Fig. 1). Clearly, this low level of PrP<sup>C</sup> expression is sufficient to support prion replication at a level that eventually results in neurological disease. These findings argue that therapeutic strategies depending on small interfering (si)RNAs are unlikely to work, since siRNAs are generally incapable of diminishing protein expression by more than 90–95%. Gene therapy that uses antisense RNA to suppress PrP<sup>C</sup> expression is equally unlikely to be successful. Nevertheless, the ability of the brain to clear PrP<sup>Sc</sup> indicates that highly efficacious pharmacotherapeutics capable of abolishing nascent PrP<sup>Sc</sup> formation should lead to complete removal of prions from the central nervous system and thus a cure for prion diseases.

## Inducible transgenes and PrP<sup>Sc</sup> clearance from the brain

Using Tg(tTA : PrP<sup>+</sup>/0)3 mice, we demonstrated that PrP<sup>Sc</sup> can be cleared from the brain following doxycycline suppression of PrP<sup>C</sup> expression between 84 and 126 days after inoculation (Supplementary Fig. S2b). Our studies demonstrate that, in brain, PrP<sup>Sc</sup> is not only being formed but is also being removed. The t<sub>1/2</sub> value of clearance of both sPrP<sup>Sc</sup> and rPrP<sup>Sc</sup> was ~1.5 days (Table 2). That both sPrP<sup>Sc</sup> and rPrP<sup>Sc</sup> have similar t<sub>1/2</sub> values was unexpected, since rPrP<sup>Sc</sup> is resistant to proteolytic degradation and thus would be expected to have a longer half-life than sPrP<sup>Sc</sup>. It is possible that the actual t<sub>1/2</sub> might be shorter due to the lag phase necessary for doxycycline to reach equilibrium in the brain and for complete inhibition of PrP<sup>C</sup> transgene transcription. The lag phase for this inducible transgene system in neonatal mice has been estimated to be <1 h (Gossen & Bujard, 2002). However, the contribution of continuing PrP<sup>Sc</sup> conversion from residual PrP<sup>C</sup> expression, which remains at ~5% of the level in Tg(tTA : PrP<sup>+</sup>/0)3 mice, might increase the t<sub>1/2</sub> of PrP<sup>Sc</sup>. We do not believe that this low level of nascent PrP<sup>Sc</sup> formation would substantially change the t<sub>1/2</sub> for PrP<sup>Sc</sup>.

Our findings differ from those reported by others who knocked out the PrP gene at ~12 weeks of age using Cre-mediated PrP depletion (Mallucci et al., 2003). In those studies, Tg(MloxP)Pr<sup>0/0</sup> mice were crossed with Tg(NFH-Cre)Pr<sup>0/0</sup> mice to produce bigenic mice. The Tg(NFH-Cre) mice expressed Cre recombinase under the control of the neurofilament heavy chain (NFH) promoter, which becomes active at 10–12 weeks of age, resulting in Cre-mediated recombination of PrP-coding transgenes containing loxP sequences in neuronal cells, but not in astrocytes or other cells. Tg(NFH-Cre/MloxP) mice were inoculated with RML prions at 3–4 weeks of age, allowing prion replication to proceed until Cre-mediated neuronal PrP<sup>C</sup> depletion occurred. Upon depletion of PrP<sup>C</sup>, early spongiform change was reversed and progression to clinical disease prevented, but extraneuronal PrP<sup>Sc</sup> continued to accumulate up to levels found in terminally ill wt animals. The results with Tg(NFH-Cre/MloxP) mice are consistent with earlier studies, showing that PrP<sup>Sc</sup> produced in brain grafts did not cause pathological changes in the brains of host Prnp<sup>0/0</sup> mice (Brandner et al., 1996). Moreover, in contrast to our studies, in which doxycycline suppression of PrP<sup>C</sup> in Tg(tTA : PrP<sup>C</sup>) mice prevented nascent PrP<sup>Sc</sup> formation and clearance of existing PrP<sup>Sc</sup> was measured, Tg(NFH-Cre/MloxP) mice could not be used to investigate PrP<sup>Sc</sup> clearance.

### Table 2. Half-life (t<sub>1/2</sub>) of PrP isoforms in cultured ScN2a cells and in Tg(tTA : PrP<sup>+</sup>/0)3 mice inoculated with RML prions

<table>
<thead>
<tr>
<th>ScN2a cells</th>
<th>Tg(tTA : PrP&lt;sup&gt;+&lt;/sup&gt;/0)3 mice</th>
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<tr>
<td></td>
<td>sPrP&lt;sup&gt;Sc&lt;/sup&gt;</td>
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<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>2–6–7–0 h</td>
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*Data from Borchelt & Prusiner (1988); Borchelt et al. (1990); Caughey et al. (1989); Peretz et al. (2001b).

## Clearance of PrP isoforms from cultured cells

Anti-PrP antibodies have been used to determine the apparent t<sub>1/2</sub> for PrP<sup>Sc</sup> in ScN2a cells (Peretz et al., 2001b). A t<sub>1/2</sub> value of ~30 h was calculated for the clearance of PrP<sup>Sc</sup> in ScN2a cells (Table 2), which is similar to the 36 h calculated for PrP<sup>Sc</sup> in brain. It is noteworthy that scrapie-infected cell cultures probably represent a mixture of infected cells and uninfected revertants. Some evidence supports the proposition that infected cells in culture eventually die when their PrP<sup>Sc</sup> levels rise above a threshold level (Schätzl et al., 1997; Tatzelt et al., 1996). Whether the t<sub>1/2</sub> value of 30 h in ScN2a cells overestimates the actual t<sub>1/2</sub> due to the unknown rate of inhibition of PrP<sup>Sc</sup> formation in cells continuously expressing PrP<sup>C</sup> remains to be established (Peretz et al., 2001b; Supattapone et al., 2001b).

Most studies of PrP<sup>C</sup> turnover and cellular metabolism have relied on cultured cells (Borchelt et al., 1990; Caughey et al., 1989; Drisaldi et al., 2003; Gilch et al., 2001), in which glycosylated and glycolipidated PrP<sup>C</sup> appears on the cell surface. Whether PrP<sup>C</sup> reaches the plasma membrane or axon terminal in vivo remains to be determined (Borchelt et al., 1994). The deduced t<sub>1/2</sub> of PrP<sup>C</sup> derived from experiments using ScN2a cells is relatively short, ranging from 2.6 to 7 h, depending on the experimental design and PrP sequence (Borchelt et al., 1990; Caughey et al., 1989; Drisaldi et al., 2003; Nunziante et al., 2003). In contrast, the apparent t<sub>1/2</sub> of PrP<sup>C</sup> expressed in mouse brain presented here was ~18 h (Table 2) (Borchelt et al., 1994; Daude et al., 1997; Peters et al., 2003).

The t<sub>1/2</sub> value for PrP<sup>C</sup> represents the mean of different turnover times in diverse cell types in many different brain regions. Some additional factors include the turnover of PrP<sup>C</sup> in different subcellular compartments of mature neurons, such as PrP<sup>C</sup> that migrates by fast axonal transport to the nerve terminals, PrP<sup>C</sup> recently documented in cytosol...
and PrP$\text{C}$ internalized in endosomal compartments via caveolae (Borchelt et al., 1994; Mironov et al., 2003; Peters et al., 2003). Although PrP mRNA and PrP$\text{C}$ have consistently short $t_{1/2}$ values in ScN2a cells, it is likely that PrP mRNA in the brains of Tg mice has an extended $t_{1/2}$, which contributes to the extended $t_{1/2}$ of PrP$\text{Sc}$ observed in the brain (Borchelt et al., 1990; Muller et al., 1997). Moreover, $t_{1/2}$ measurements of PrP$\text{Sc}$ in ScN2a cells under non-differentiating conditions do not reflect the expected complexity of mRNA and PrP$\text{C}$ turnover in a variety of fully differentiated neurons and glial cells in the mature mouse brain in our experiments (Borchelt et al., 1990; Caughey et al., 1989; Drisaldi et al., 2003; Gilch et al., 2001). The bigenic mouse system described in this paper now permits us to determine PrP mRNA and PrP$\text{C}$ turnover at a cellular level in the brain.

**Factors modulating PrP$\text{Sc}$ levels**

The findings reported here and previously demonstrate that PrP$\text{Sc}$ levels in brain are determined by both the rates of formation and clearance. In earlier studies, we demonstrated that PrP$\text{C}$ expression is directly proportional to the rate of PrP$\text{Sc}$ formation (Prusiner et al., 1990), but inversely related to the length of the incubation time: the greater the level of PrP$\text{C}$ expression, the greater the rate of PrP$\text{Sc}$ formation and the shorter the incubation time. In general, PrP$\text{Sc}$ formation as reflected in shorter incubation times occurs when the sequences of PrP$\text{C}$ and PrP$\text{Sc}$ are the same (Prusiner et al., 1990; Scott et al., 1989). As an exception, a particular strain of prion may preferentially interact with PrP$\text{C}$ of another sequence. For example, variant CJD prions composed of HuPrP$\text{Sc}$ are more readily transmitted to Tg(BoPrP) mice expressing bovine PrP than to Tg(MHu2M) mice expressing chimeric mouse–human PrP (Korth et al., 2003; Scott et al., 1999). This contrasts with sporadic CJD, familial CJD(E200K) and iatrogenic CJD prions, all of which more readily transmit to Tg(MHu2M) mice than to Tg(BoPrP) mice (Scott et al., 2005; Telling et al., 1995). These findings argue that the tertiary structure of PrP$\text{Sc}$ reflecting the particular prion strain governs the interaction with PrP$\text{C}$ during prion replication.

Whether strains of prions are cleared or formed at different rates remains to be established. In one example, mice expressing MoPrP-A or MoPrP-B, which differ at positions 108 and 109, replicated prions at different rates (Westaway et al., 1987).

**Mechanism and kinetics of PrP$\text{Sc}$ clearance from the brain**

The mechanism of PrP$\text{Sc}$ clearance from the brain is unknown. From cultured cell studies, it seems likely that PrP$\text{Sc}$ is hydrolysed in acidic endosomes as well as lysosomes (Caughey et al., 1990; Taraboulos et al., 1992b). The clearance of PrP$\text{Sc}$ was accelerated in ScN2a cells by branched polyamines, presumably by diminishing the resistance of rPrP$\text{Sc}$ to proteolysis at acidic pH (Supattapone et al., 1999, 2001b).

In Tg(tTA:PrP) mice given 2 mg doxycycline ml$^{-1}$ in the drinking water, PrP$\text{C}$ expression was reduced by ~95 %. The residual PrP$\text{C}$ was sufficient to support PrP$\text{Sc}$ replication, albeit at a slow rate. These bigenic mice eventually developed central nervous system degeneration at ~430 days after inoculation (Table 1). The slow accumulation of PrP$\text{Sc}$ is reflected in studies of $t_{1/2}$ for clearance measured both by CDI and Western blot, in which PrP$\text{Sc}$ rapidly declined after administration of doxycycline and eventually began to accumulate again (Figs 3c and 4a). The extremely rapid response of Tg(tTA:PrP) mice to oral doxycycline in suppressing PrP mRNA transcription followed by PrP$\text{Sc}$ degradation suggests that the $t_{1/2}$ measurements are likely to reflect the actual rates of PrP$\text{Sc}$ clearance.

**Therapeutics for prion disease**

The data presented here demonstrate that PrP$\text{Sc}$ can be cleared from the brain. This is encouraging since it argues that drugs that abolish PrP$\text{Sc}$ formation or enhance clearance can rid the brain of prions. However, the efficacy of such drugs will depend on how well they accomplish either task: PrP$\text{Sc}$ formation must be abolished completely or clearance enhanced to remove all PrP$\text{Sc}$.

As in earlier studies (Brandner et al., 1996), our data indicate a critical role of PrP$\text{C}$ in neurodegeneration caused by PrP$\text{Sc}$. We found that residual rPrP$\text{Sc}$ persisting in some brain areas after suppression of PrP$\text{C}$ expression produced few or no signs of neurodegeneration in surrounding cells (Fig. 6). In the presence of PrP$\text{C}$, a severe loss of pyramidal neurons was observed in the hippocampal CA1 region; moreover, the remaining neurons were shrunken, suggesting that they were also undergoing degeneration. In contrast, no obvious nerve cell loss was detectable in animals in which PrP$\text{C}$ expression was suppressed. Therefore, the structural transition that PrP$\text{C}$ undergoes should prove to be an effective drug target.

**Does PrP$\text{Sc}$ have a cellular function?**

Studies demonstrating the clearance of PrP$\text{Sc}$ raise the possibility that PrP$\text{Sc}$ is normally made at low levels and continually removed. Such a proposal posits that PrP$\text{Sc}$ may have an as-yet-unidentified function and that prion diseases are disorders of PrP$\text{Sc}$ metabolism.

If we assume that PrP$\text{Sc}$ is formed in normal cells, then we would argue that prion diseases arise from the dysregulation of PrP$\text{Sc}$ metabolism. Thus, PrP$\text{Sc}$ begins to accumulate when the rate of formation exceeds the rate of clearance. As the net accumulation of PrP$\text{Sc}$ continues to increase, a point is reached when a cell can no longer tolerate the level of PrP$\text{Sc}$ and it begins to malfunction. Such a scenario seems particularly appealing in the spontaneous and inherited forms of prion disease.
Explaining how the seemingly wide variety of PrPSc conformations, each of which represents a different strain, might participate in regulating the metabolism of normal cells poses a conundrum. However, determining whether prion strains with different incubation times show different rates of PrPSc clearance might prove informative. Defining the rates of both formation and clearance for different prion strains would seem to be a fundamental issue in prion biology, which is now amenable to investigation using the bigenic mouse system described here.

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