Trans-species amplification of PrP\textsuperscript{CWD} and correlation with rigid loop 170N

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Chronic wasting disease (CWD) is an efficiently transmitted spongiform encephalopathy of cervids. Whether CWD could represent a threat to non-cervid species remains speculative. Here we show that brain homogenates from several CWD-susceptible non-cervid species, such as ferrets and hamsters, support amplification of PrP\textsuperscript{CWD} by sPMCA, whereas brain homogenates from CWD-resistant species, such as laboratory mice and transgenic mice expressing human PrP\textsuperscript{C} [Tg(HuPrP) mice], do not. We also investigated whether several North American species that share the environment with cervids would support amplification of PrP\textsuperscript{CWD} by sPMCA. Three native rodent species, including voles and field mice, supported PrP\textsuperscript{CWD} amplification, whereas other species (e.g. prairie dog, coyote) did not. Analysis of PrP sequences suggests that an ability to support amplification of PrP\textsuperscript{CWD} in trans-species sPMCA is correlated with the presence of asparagine at position 170 of the substrate species PrP. Serial PMCA may offer insights into species barriers to transmission of CWD.

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Introduction

Chronic wasting disease (CWD) of deer, elk and moose is a prion disease first identified in the Rocky Mountain region and now recognized in 15 states, Canada, and one Asian country (Williams, 2005; Williams and Young, 1980). Like other transmissible spongiform encephalopathies (TSEs) such as ovine scrapie, bovine spongiform encephalopathy (BSE) and human Creutzfeldt–Jakob Disease (CJD), CWD is caused by the conversion of normal, protease-sensitive PrP\textsuperscript{C} protein to a misfolded, protease-resistant PrP\textsuperscript{RES} which accumulates in the central nervous and lymphoid systems and leads to wasting and spongiform encephalopathy (Sigurdson et al., 2002, 1999; Spraker et al., 2002).

The facile spread of CWD is different from most TSEs and may be mediated by differences in PrP\textsuperscript{C} sequence, prion strain, and other still unknown factors (Bartz et al., 1995; Lucassen et al., 2003). We have demonstrated efficient transmission of infectious prions from the saliva and excreta of infected cervids (Mathiason et al., 2006; Safar et al., 2008). While the known natural host range for CWD is limited to cervids, some non-cervid species, e.g. ferrets and hamsters, can be infected experimentally (Bartz et al., 1998; Harrington et al., 2008; Raymond et al., 2007; Sigurdson et al., 2008a). Trans-species transmission of prion diseases is infrequent due to the species barrier phenomenon, which may be given species brain substrate to support PrP\textsuperscript{C} to PrP\textsuperscript{RES} conversion (Jones et al., 2007; Saa et al., 2006; Soto et al., 2005). When PrP\textsuperscript{C} and PrP\textsuperscript{RES} from the same species are used, in vitro amplification preserves the biochemical characteristics, infectivity and species barriers of the seed PrP\textsuperscript{RES} (Rossier et al., 1997; Castilla et al., 2008, 2005; Kocisko et al., 1995; Lucassen et al., 2003). We have demonstrated efficient amplification of CWD PrP\textsuperscript{RES} (PrP\textsuperscript{CWD}) by serial protein misfolding cyclic amplification (sPMCA) using transgenic mice [Tg(CerPrP)] over-expressing cervid PrP\textsuperscript{C} as brain substrate (Green et al., 2008; Kurt et al., 2007; Meyerrett et al., 2008).

The plausibility of trans-species sPMCA is supported by cell-free conversion studies which have shown that some conversion may...
occur when combining PrP<sup>C</sup> and PrP<sup>RES</sup> from different species (Kocisko et al., 1994; Piening et al., 2006; Priola et al., 2001; Raymond et al., 2000). Here we apply sPMCA to demonstrate that brain homogenates from species shown to be susceptible to CWD infection in vivo also support amplification of CWD prions in vitro (e.g. ferrets and hamsters) whereas relatively resistant species [e.g. laboratory mice (Mus spp.)] (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006; Williams and Young, 1980) do not. We extended this approach to include species abundant in North America likely to be exposed to CWD in the wild and that therefore have potential to serve as reservoirs or laboratory models for CWD. Interestingly, we found that all tested species that expressed asparagine at Prp position 170 supported trans-species amplification of PrP<sup>CWD</sup>. All but one species that expressed serine at PrP position 170 failed to support trans-species amplification of PrP<sup>CWD</sup>.

Results

Species susceptible to CWD infection in vivo

Deer, Tg(CerPrP) mouse, and ferret brain homogenates support PrP<sup>CWD</sup> amplification

To determine whether in vitro PrP<sup>CWD</sup> amplification is demonstrable in a susceptible species, we first performed sPMCA using normal-brain homogenates (NBH) from white-tailed deer (Odocoileus virginianus), a natural host for CWD. For these experiments, CWD-positive deer brain 104 was diluted 1:10 into NBH from white-tailed deer and subjected to sPMCA with 1:2 dilutions into fresh NBH at each subsequent round for a total of 4 rounds. We previously reported (Kurt et al., 2007), and here confirm, that deer brain homogenates support ~5-fold increases in PrP<sup>RES</sup> in sPMCA (Fig. 1A, left panel), thus NBH from the native CWD-susceptible species will support PrP<sup>C</sup>-to-PrP<sup>RES</sup> conversion in vitro. We extended this work using NBH from Tg(CerPrP)1536<sup>−/−</sup> mice, which express cervid PrP<sup>C</sup> at ~4-fold the concentration of that in deer brain. Using Tg(CerPrP)1536<sup>−/−</sup> NBH and the CWD-positive deer brain D10, PrP<sup>RES</sup> amplification was at least 100 to 250-fold per round of PMCA (Kurt et al., 2007) and amplification was consistently achieved with starting dilutions up to 1:16,000, whereas the equivalent un-amplified dilutions (−PMCA samples) were not detectable by Western blot (Fig. 1A, right panel).

To initiate trans-species sPMCA studies, we first used NBH from ferrets (Mustela putorius furo), a species that is susceptible to CWD (Bartz et al., 1994; Sigurdson et al., 2008a), as a PrP<sup>C</sup> conversion substrate. Ferret NBH supported amplification at starting dilutions of up to 1:16,000 of D10 (Fig. 1B).

In control experiments, D10 added to PrP-null mouse (PrP<sup>0/0</sup>) brain homogenate did not amplify, indicating that the majority of PrP<sup>C</sup> which is converted in sPMCA comes from the NBH vs. the PrP<sup>RES</sup> seed material (not shown).

Species relatively less-susceptible to CWD infection in vivo

Hamster brain homogenates have varying ability to support PrP<sup>CWD</sup> amplification

Raymond et al. (2007) have demonstrated that Syrian golden (Mesocricetus auratus), Chinese (Cricetulus griseus) and Armenian (Cricetulus migratorius) hamsters are variably susceptible to intracerebral inoculation of CWD. We have recently confirmed the in vivo susceptibility of Syrian golden hamsters to CWD (100% infected after inoculation with D10, Hoover lab, unpublished). To investigate differences in the ability of hamster species to support CWD amplification in vitro, we harvested NBH from Armenian, Chinese and Syrian golden hamsters for sPMCA. We found that in three experiments, Syrian golden hamster NBH supported amplification of 1:8000–1:16,000 dilutions of PrP<sup>CWD</sup> (Fig. 2A). Chinese hamster NBH consistently supported amplification of up to 1:2000 dilutions of mule deer PrP<sup>CWD</sup> (Fig. 2B) and Armenian hamsters supported amplification of up to ~1:1000 dilutions of D10 (Fig. 2C).

Mink brain homogenates did not support PrP<sup>CWD</sup> amplification

American mink (Mustela vison) are closely related to ferrets and differ from the latter by very few residues in PrP amino acid sequence (Bartz et al., 1994), however, recent studies suggest that a relatively strong species barrier exists restricting CWD transmission to mink by even the intracranial route (Harrington et al., 2008). In our experiments mink NBH did not support amplification of PrP<sup>CWD</sup> even when a high concentration (a 1:10 dilution) of D10 seed was used in order to provide as much seed material as possible (Fig. 3). Higher concentrations of D10 were not feasible due to the difficulty in distinguishing potentially new PrP<sup>RES</sup> from input seed. In these experiments Western blot PrP<sup>CWD</sup> signals degraded with successive rounds of sPMCA (Fig. 3).

Species relatively resistant to CWD infection in vivo

BALB/c mouse brain failed to support PrP<sup>CWD</sup> amplification

Common laboratory mouse (Mus) strains are considered to be resistant to CWD (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006). Therefore we evaluated NBH from BALB/c mice (selected as a common laboratory mouse strain expressing wild-type
Mus PrPC) as a potential negative control in trans-species sPMCA. BALB/c NBH did not support amplification of PrPCWD, even when a 1:10 starting dilution of D10 seed was used (Fig. 4A). As with other NBH that did not support amplification, Western blot PrPCWD signals degraded with each successive round.

Human PrP transgenic mouse brain failed to support PrPCWD amplification in vitro

We attempted to amplify PrPCWD using NBH from two strains of transgenic mice hemizygous for transgenes expressing human PrP. We generated Tg mice expressing human PrP encoding either M or V at codon 129 by microinjection of fertilized embryos from FVB/Prnp0/0 mice. The resulting founders were mated to FVB/Prnp0/0 mice to produce lines that were hemizygous for the transgene array. Expression of HuPrP in the CNS of mice was examined by Western blotting with mAb 6H4. Mice from the Tg(HuPrP-M129)6816+/- and Tg(HuPrP-V129)7823+/- lines expressed HuPrP in the CNS at approximately 16- and 5-fold the level of PrP in the brains of wild-type mice. Neither strain NBH supported amplification of 1:10 or 1:50 starting dilutions of D10 (Fig. 4B).

Species for which CWD susceptibility is unknown

Prairie dog brain homogenates failed to support PrPCWD amplification

Prairie dogs (Cynomys ludovicianus) are ground-dwelling rodents prevalent in the Western United States, including within CWD-
enzytotic areas, and would likely be exposed indirectly to CWD. Prairie dog brain did not support PrP\(^{\text{CWD}}\) to PrP\(^{\text{RES}}\) conversion when seeded with 1:10 or 1:50 dilutions CWD-positive brain (Fig. 5A).

**Domestic cat and coyote brain homogenates did not support PrP\(^{\text{CWD}}\) amplification**

Canid and feld carnivore species may be directly exposed to CWD by predation and scavenging. Domestic cats (Felis catus), in addition to exotic felids, are susceptible to BSE, leading to cases of feline spongiform encephalopathy in several European countries (Pearson et al., 1992; Sigurdson and Miller, 2003; Wells and McGill, 1992). However, their susceptibility to CWD has not been determined. Interestingly, cat NBH did not support PrP\(^{\text{CWD}}\) amplification (Fig. 5B).

We harvested NBH from one coyote (Canis latrans) and performed trans-species sPMCA. Coyote NBH also did not support amplification of PrP\(^{\text{CWD}}\) (Fig. 5C). We subjected NBH that did not support sPMCA, such as from cat and coyote, to up to 8 rounds of PMCA and no change in results was produced (see cat, Fig. 5B).

**Macaque brain homogenates did not support PrP\(^{\text{CWD}}\) amplification**

At least one species of non-human primate is susceptible to CWD (Marsh et al., 2005). We obtained NBH from three rhesus macaques (Macaca mulatta) for trans-species sPMCA. Macaque NBH did not support amplification of PrP\(^{\text{CWD}}\) (Fig. 5D).

Prairie vole and field mouse brain homogenates support PrP\(^{\text{CWD}}\) amplification

In our search for non-cervid species susceptible to CWD we examined PrP\(^{\text{CWD}}\)-to-PrP\(^{\text{RES}}\) conversion using NBH from several North American rodents. These studies were prompted in part by the work of Chandler (1971), Chandler and Turfrey (1972) and Nonno et al. (2006) demonstrating that field voles (*Microtus agrestis*) and bank voles (*Myodes glareolus*) are susceptible to scrapie. We therefore assessed the prairie vole (*Microtus ochrogaster*), a common North American species, for its capacity to amplify PrP\(^{\text{CWD}}\) in trans-species sPMCA. In three experiments, we found that prairie vole NBH consistently supported amplification of PrP\(^{\text{CWD}}\) at starting dilutions of up to 1:50 to 1:100 D10 brain within four rounds of sPMCA (Fig. 6A). These results were similar to those obtained using ferret, Chinese hamster and Tg1536 mouse NBH, and suggested to us that prairie voles may be susceptible to CWD, a hypothesis we are currently testing in ongoing infectivity studies. Concurrently, we became aware of the work of Johnson, Heisey and colleagues who have reported ongoing in vivo studies indicating that other North American vole species are susceptible to CWD (C. Johnson, D. Heisey and colleagues, personal communication).

We next examined two species of common North American field mice, *Peromyscus leucopus* and *Peromyscus maniculatus bairdii*, by trans-species sPMCA. *P.m. bairdii* in particular has a geographic range that overlaps that of CWD-positive cervids (Baker, 1983). Both species of *Peromyscus* mice consistently supported amplification of dilutions D10 up to 1:100 (Fig. 6B). Studies examining the in vivo susceptibility of *Peromyscus* mice to CWD are also in progress. Again separately, Heisey, Johnson and colleagues have gathered data that suggest both of these *Peromyscus* species are susceptible to CWD (C. Johnson, D. Heisey and colleagues, personal communication).

Cyclic amplification of PrP\(^{\text{CWD}}\) correlated with position 170 of the substrate species PrP

Host PrP primary structure is associated with susceptibility to particular TSEs (Jewell et al., 2005; Laplanche et al., 1993; O’Rourke

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**Fig. 5.** Prairie dog, domestic cat, wild coyote and macaque monkey NBH do not support amplification of PrP\(^{\text{CWD}}\) (A) For attempts to amplify PrP\(^{\text{CWD}}\) in prairie dogs, CWD-positive brain was diluted 1:10 (2 replicates, lanes 4–5) or 1:50 (2 replicates, lanes 6–7) into the NBH and subjected to 4 rounds of sPMCA. Lanes 2–3: Dilutions (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Prairie dog NBH only, showing PrP\(^{\text{F}}\) not digested with PK. (B) For the cat experiment shown (representative of 3 cats), CWD-positive brain was diluted 1:50 into the NBH and subjected to 8 rounds of sPMCA (4 replicates, lanes 4–7). Lane 2: A dilution (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Cat NBH only, showing PrP\(^{\text{F}}\) not digested with PK. (C) For attempts to amplify PrP\(^{\text{CWD}}\) in coyote, CWD-positive brain was diluted 1:10 (2 replicates, lanes 4–5) and 1:50 (2 replicates, lanes 6–7) and subjected to 4 rounds of sPMCA. Lanes 2–3: Dilutions (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Coyote NBH only, showing PrP\(^{\text{F}}\) not digested with PK. (D) For attempts to amplify PrP\(^{\text{CWD}}\) in macaque NBH, CWD-positive brain was diluted 1:50 (4 replicates, lanes 4–7) into the NBH and subjected to 3 rounds of sPMCA (4 replicates, lanes 4–7). Lane 2: A dilution (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Macaque NBH only, showing PrP\(^{\text{F}}\) not digested with PK.

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**Fig. 6.** NBH from prairie vole and *Peromyscus* mites support amplification of PrP\(^{\text{CWD}}\). (A) For amplification of PrP\(^{\text{CWD}}\) in prairie voles, CWD-positive brain was diluted 1:50 (2 replicates, lanes 4–5) or 1:100 (2 replicates, lanes 6–7) into the NBH and subjected to 3 rounds of sPMCA. Lanes 2–3: Dilutions (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: PrP\(^{\text{F}}\) not digested with PK. (B) For amplification of PrP\(^{\text{CWD}}\) in *Peromyscus* mice, CWD-positive brain was diluted 1:50 (4 replicates, lanes 4–7) into the NBH and subjected to 3 rounds of sPMCA. Lanes 2–3: Dilutions (labeled – PMCA) frozen at –70 °C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: PrP\(^{\text{F}}\) not digested with PK.
et al., 2004; Westaway et al., 1994). We compared the PrP primary structures of the animals we used for sPMCA (sequencing of prairie dog Prnp has not been successful) in an effort to find amino acids that whilst others do not. Of particular interest was the loop between the β2 strand and α2 helix (PrP residues 166–175), which is more stable in elk than mice (Gorfe and Caflisch, 2007; Gossert et al., 2005) and may contribute to the transmission barrier between these species (Sigurdson et al., 2008b). Of the species we studied, all of those that express asparagine at PrP position 170 (i.e. Tg(CerPrP)1536 mouse, Peromyscus mouse, prairie vole, Syrian, Chinese and Armenian hamster) supported amplification of PrP<sup>CWD</sup> by sPMCA (Fig. 7). In contrast, only one (ferret) of the eight species (ferret, Mus mouse, two strains of Tg(HuPrP) mouse, cat, coyote, mink, macaque) that express serine at position 170 supported amplification of PrP<sup>CWD</sup> (Fig. 7), indicating that 170N facilitates PrP<sup>CWD</sup> amplification. We did not find correlations at any other PrP amino acid positions.

Discussion

Here we demonstrate that CWD prions can be amplified in normal-brain homogenates (NBH) from several outbred species whose habitat overlaps with that of free-ranging cervids. Our findings complement and extend recent studies that suggest sPMCA results may correlate with ability or inability to support PrP<sup>CWD</sup> amplification and species barriers to conversion may be affected by prion strain, other CWD inocula (Raymond et al., 2007). Corresponding results have generally correlated. However, in vivo and in vitro results have generally correlated. However, in vivo and in vitro species barriers to conversion may be affected by prion strain, infectious titer of the inoculum, the genetic background of the recipient species, and PrP<sup>C</sup> expression levels. One of our PrP<sup>CWD</sup> sources (D10) amplified in NBH from several hamster species, which is consistent with the higher attack rates of this inoculum in Syrian golden hamsters (100%, Hoover lab, unpublished) than reported with other CWD inocula (Raymond et al., 2007). Corresponding in vivo studies in many non-cervid species (e.g. felids, canids) would require lengthy observation periods before definitive conclusions regarding susceptibility could be made.

PrP<sup>C</sup> observation has also been shown to play a role in determining the ability to support amplification of PrP<sup>CWD</sup> (Kurt et al., 2007). We estimated the PrP<sup>C</sup> concentration of brain homogenates from several species by Western blot and did not find differences that could explain our results (data not shown). Some of the species used, e.g. the transgenic HuPrP mice, express high levels of PrP<sup>C</sup> and still did not support amplification.

PrP sequence also influences the efficiency of in vitro prion conversion and species barriers to disease transmission (Bossers et al., 1997; Nonno et al., 2006; Piening et al., 2006; Priola et al., 2001). We sequenced the PrP gene from each of the species examined (sequencing of prairie dog Prnp has not yet been successful) for which published data were not available, deduced the primary structures, and compared these with published sequences for the other species. The amino acids present at position 222 (225 in deer) and 92 (96 in deer), both of which are associated with susceptibility to CWD in cervids (Jewell et al., 2005; O’Rourke et al., 1999, 2004), did not correlate with ability or inability to support amplification.
We then examined the L1 loop (residues 166–175), which is more stable in elk than mice primarily due to the presence of asparagine rather than serine at position 170 (Gorfe and Cafisch, 2007; Gossert et al., 2005). Of the animals we used, all of those that express asparagine at PrP position 170 (i.e. Tg(CerPrP)1536 mouse, Peromyscus mouse, prairie vole, Syrian, Chinese and Armenian hamsters) supported amplification of PrPCWD by sPMCA. In contrast, only one (ferret) of the eight animals (laboratory mouse, both Tg(HuPrP) mice, cat, coyote, ferret, mink, macaque) that express serine at position 170 supported amplification of PrPcWD. The mobility of the loop is influenced by other residues such as Phe175 (Gorfe and Cafisch, 2007), and the unique presence of leucine, rather than phenylalanine (and the corresponding phenyl ring), at position 175 of ferret PrP may induce greater stability via side chain hydrogen bonding. This might explain the greater PrPcWD-to-PrPCWD conversion observed when we used ferret NBH, despite the presence of Ser170. The loop region, including residues 170 and sheep polymorphism 171, is thought play a role in determining PrP conversion/susceptibility in several species (Bosser et al., 1997; Christen et al., 2008; Gorfe and Cafisch, 2007; Piening et al., 2006; Sigurdson et al., 2008b; Westaway et al., 1994). We did not find correlations with any other PrP amino acid positions, including 155 (Piening et al., 2006; Priola et al., 2001).

In our experiments, mind brain homogenates did not support sPMCA even though this species exhibits some susceptibility to CWD in vivo (Harrington et al., 2008). This clearly supports the premise that other factors in the brain and periphery besides PrP sequence (Deleault et al., 2005, 2003; Sigurdson et al., 2002, 1999) affect susceptibility in vivo. This premise is supported by studies that show host genetic background affects TSE susceptibility (Lloyd et al., 2001) and demonstrates that extrapolations from negative in vitro conversion results should be made with caution.

This study demonstrates successful trans-species sPMCA using a diverse range of animal species. Many of these findings corroborate data from other studies. For instance, Bartz et al. (1998) and Sigurdson et al. (2008a) demonstrated that ferrets are susceptible to CWD in vivo. We found that ferret NBH supported amplification of high dilutions of input PrPCWD. Raymond et al. (2007) demonstrated that hamsters are variably susceptible to CWD in vivo. We found that the capacity of hamster NBH to amplify PrPCWD depended on the species: Syrian golden and Chinese hamster NBH supported robust amplification of PrPCWD, and Armenian hamster NBH supported minimal amplification. As might have been anticipated, wild-type laboratory mouse (Mus) NBH did not support sPMCA, just as these animals fail to support CWD amplification in vivo (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006; Williams and Young, 1980). Finally, we were not able to amplify PrPCWD using NBH from HuPrP transgenic mice, a result which is consistent with findings from the in vivo studies of Kong et al. (2005) and Tamguney et al. (2006) using transgenic mice expressing human PrP.

We are mindful that susceptibility to prion disease is affected by dose, route of inoculation, host lifespan and other factors, and therefore is difficult to quantify. Although our sPMCA results (with the exception of mink) were consistent with in vivo studies demonstrating susceptibility or resistance to CWD in the same species, in vivo studies are still the gold standard for determining TSE susceptibility. Thus we have initiated in vivo studies in a subset of species that support sPMCA as described here, in an effort to explore whether sPMCA can actually be used to predict in vivo susceptibility.

Materials and methods

Transgenic mice encoding cervid PrP

Transgenic mice expressing cervid PrP were generated in the Telling lab (Browning et al., 2004) and have been used previously in sPMCA (Green et al., 2008; Kurt et al., 2007; Meyerett et al., 2008).

Production of transgenic mice encoding human PrP

To generate transgenic (Tg) mice expressing human PrP encoding either methionine (M) or valine (V) at codon 129, referred to as HuPrP-M129 and HuPrP-V129 respectively, coding sequences were excised from the cloning vector pSP72 using Sall and XhoI and inserted into the XhoI restriction site in MoPrP/Xho (Borchelt et al., 1996), a derivative of the ‘half genomic’ Prnp expression vector (Fischer et al., 1996). The Nol fragments encompassing the HuPrP expression cassettes were purified by electrophoresis in low melting temperature agarose in the absence of ethidium bromide. DNA was recovered by phenol and chloroform extraction followed by dialysis against microinjection buffer (Tris–Cl 10 mM, pH 7.5/EDTA 0.1 mM). After estimation of the DNA concentration by quantitative gel electrophoresis, the DNA was diluted to a final concentration of 2 ng/μl for pronuclear microinjection of fertilized FVB/Prp<sup>−/−</sup> oocytes. Founder mice were identified by tail biopsy and extraction of genomic DNA using a Beckman Biomek FX robotics station followed by PCR screening for the presence of the transgene. Approximately 1 cm of tail tissue was digested overnight at 55 °C with proteinase K (0.5 mg/ml final concentration) in 50 mM Tris pH8.0, 100 mM EDTA, 0.5% SDS, the DNA extracted with phenol and chloroform and concentrated by ethanol precipitation. Estimates of the levels of PrP expression in the CNS of F1 offspring were determined by semi-quantitative immuno dot blotting. Aliquots of Tg brain homogenates containing equal amounts of total protein, determined by bicinchoninic acid assay (BCA) (Pierce, Rockford, IL), were subjected to two-fold serial dilutions and bound to Whatman PrP monoclonal antibody (mAb) 6H4 (Prionics), developed using enhanced chemiluminescencence (ECL-Plus) and exposed to X-ray film. Levels of PrP expression were estimated by comparison to PrP levels in brain extracts of wild-type mice. Transgenic F1 offspring were mated with FVB/Prp<sup>−/−</sup> to establish individual Tg lines. In all cases, Tg mice were maintained on a FVB/Prp<sup>−/−</sup> background. Tg(HuPrP-M129)6816/+ and Tg(HuPrP-V129)7823/− mice were used in these studies.

Sources and preparation of brain homogenates

Donor animals were euthanized according to ACUC approved protocols and immediately perfused (except prairie dogs), to remove as much blood as possible, with phosphate buffered saline (PBS) plus 5 mM EDTA. At least two individuals (in most cases three) of each species, and one coyote, were used. Deer brain was provided by David Osborn (Warnell School of Forestry, University of Georgia). Tg(CerPrP)1536<sup>+</sup>/− mice (Browning et al., 2004) were housed at CSU. Ferrets were obtained from Marshall Farms Inc. BALB/c mice were provided by James Perry and Anne Avery at CSU. Prairie deer mice and white-footed mice (Peromyscus maniculatus bairdii and P. leucopus, respectively) were obtained from the University of South Carolina Genetic Stock Center and were housed at CSU. Prairie voles (Microtus ochrogaster) were obtained from Thomas Curtis (University of Oklahoma) and were housed at CSU. Coyote brain (Canis latrans) was obtained from the National Wildlife Research Center Utah Field Station with the help of John Shivik and Stacey Brummer. Mink brain was a gift from Jason Bartz (Creighton University). Syrian Golden hamsters were obtained from Harlan Labs. Armenian and Chinese hamsters were a gift from Greg Raymond, Richard Race, Brent Race and Byron Caughey at the Laboratory of Persistent Viral Diseases, Rocky Mountain Veterinary Branch, NIAID, NIH. Cat brain was harvested from animals that were involved in other studies being conducted in the CSU pathogen-free facility and were made available by Sue VandeWoude. Prairie dogs were obtained from the Black-Footed Ferret Conservation Center, US Fish and Wildlife Service, thanks to Paul Marini. Macaque monkey brain was obtained from Tulane Primate Research Center. Transgenic mice expressing human PrP...
prion protein, referred to as Tg(HuPrP)6816+/- and Tg(HuPrP)7823+/-, were housed at the University of Kentucky/Telling lab.

Preparation of brain homogenates

Normal-brain homogenates (NBH) were prepared as previously described (Kurt et al. 2007).

CWD-positive brain homogenates were prepared as follows: (1) D10 was prepared from a CWD-infected mule deer (generously provided by Michael Miller, Colorado Division of Wildlife), and (2) 104 was prepared from a white-tailed deer experimentally infected with CWD source LAO1 (Colorado Division of Wildlife) and housed at CSU (Mathiason et al., 2006). The CWD-positive brain homogenates were prepared at a final concentration of 20% (w/v) and were not subjected to centrifugation.

Serial PMCA procedure

To eliminate possible contamination, NBH was thawed on ice and loaded into 96 well plates (TempPlate III, USA Scientific) in a laboratory that was never used for prion research. The plate was then transported to the prion research laboratory where CWD-positive brain homogenate was diluted into the NBH to comprise a total volume of 50 μl (unseeded, NBH-only controls also comprised 50 μl). Non-amplified dilutions (— PMCA in figures) were frozen at −70 °C for the duration of the experiment for comparison with amplified (+ PMCA) samples. The PrP<sup>CWD</sup> concentrations of —PMCA samples were equivalent to corresponding + PMCA samples after sPMCA. The plate was placed in a Misonix 3000 sonicator containing 200 ml distilled water, leaving 2−3 mm between the horn and the plate bottom. The plate was subjected to 40 s bursts at power level 7 followed by 30 min incubations at 37 °C for 48 h (this comprising 1 round of PMCA), and the samples were diluted 1:2 into fresh NBH for each new round (control dilutions were diluted identically at the start of the experiment and were then frozen, not amplified). These settings yielded the most efficient amplification of PrP<sup>CWD</sup> in our experiments.

Electrophoresis and Western blotting

After sPMCA, samples were digested with proteinase K (PK) (Invitrogen) before transfer and blotting. Due to innate differences in protease sensitivity of PrP<sup>+</sup> between each substrate species, equivalent amounts of parallel non-seeded samples were used as a guide to assure complete PrP<sup>+</sup> digestion. Samples using Tg(CerPrP)1536+/- mouse, prairie dog, mink, coyote and cat NBH as substrate were brought to a final SDS concentration of 0.25% prior to digestion with 100 μg/ml PK for 30 min at 37 °C followed by 10 min at 45 °C. Ferret, Tg (HuPrP)6816+/- and Tg(HuPrP)7823+/- substrates were digested similarly but without incubation at 45 °C. Deer, hamster, vole, Peromyscus and BALB/c mouse substrates were digested with 100 μg/μl PK, and macaque with 50 μg/μl PK, for 30 min at 37 °C. All samples (including unseeded, NBH-only controls) had a final volume of 10 μl after addition of PK.

Electrophoresis and transfer to PVDF membranes were performed as previously described (Kurt et al., 2007). We screened each species to find optimal antibodies based on PrP<sup>+</sup> detection (Table 1). Antibodies used for manuscript figures are as follows: for detection of PrP<sup>+</sup> in Tg(CerPrP)1536+/-, cat, coyote, ferret, prairie dog, mink, vole and deer samples, membranes were incubated in Bar224 mAb (a gift from Jacques Grassi, CEA, Saclay, France) conjugated directly to horse radish peroxidase (HRP) for at least 1 h. For detection of hamster, Peromyscus and BALB/c Mus samples, membranes were incubated in mAb SAF83 (Cayman Chemical) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab (Jackson Labs) diluted 1:20,000. For detection of PrP in Tg(HuPrP)6816+/- and Tg(HuPrP)7823+/- mouse samples, membranes were incubated in mAb 12F10 (Cayman Chemical) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab diluted 1:20,000. Macaque samples were incubated in mAb 7D9 (Abcam) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab diluted 1:20,000. All membranes were washed several times in dH2O containing 0.2% Tween-20 before application of ECL-plus™ chemiluminescent reagents (Amersham). Data were generated using a digital Fuji-Doc™ gel documentation system (Fuji) with automated detection of saturation limits, and densitometric analyses were performed with ImageGauge™ quantification software. Successful amplification was indicated by increases in PrP<sub>CWD</sub> relative to — PMCA samples or by the presence, after sPMCA, of protease-resistant bands in samples containing otherwise undetectable concentrations of PrP<sub>CWD</sub> concurrent with the absence of PrP<sub>CWD</sub> in non-seeded, NBH-only control samples. We analyzed samples after 3 or 4 rounds of sPMCA and performed up to eight rounds of sPMCA in attempts to obtain conversion in those species that did not support PrP<sub>CWD</sub> amplification. Data shown are representative of multiple experiments using 2−3 individuals of each species and one coyote.

PCR and PRNP sequencing

DNA was extracted from NBH of coyote, Peromyscus mice and prairie voles by addition of 500 μl chloroform−phenol isoamyl-alcohol (IAA) to 1 ml NBH. The samples were then inverted 10 times and centrifuged at 12,000 × g for 5 min at room temperature before the aqueous layer was removed and combined with IAA and these steps repeated as described. The aqueous layer was then removed and mixed with 0.1 volumes of 3M sodium acetate, followed by 2.5 volumes of 100% ethanol. Samples were stored at −20 °C for 48 h before centrifugation at 14,000 × g for 30 min at 4 °C, at which time the fluid was decanted and the pellet allowed to dry. The DNA was re-suspended in 50 μl of 1× TE buffer. For Polymerase Chain Reaction (PCR), the following forward and reverse primers were used for each species: (1) Microtus ochrogaster: GTGGAAACACCGCATTAGC AAAATGGTATGCTCAGCAATTCGTG, ATGGCACTGGTCTGCTTGCAAA and CGTGCAGAAGGTGTTCTGTGTTG, (2) Peromyscus bairdii: AATCCTTGGTCCTAGGCTGACCTA and ACGGCCGCGTAGATGACATC, (3) L. leucopus: AACTTGTGGTCCTAGGCTGACCTA and TGACTCTGTGCCTGTCGTTGATGTA, and (4) Canis latrans: GTTTGAAAACCACTATGGCGCC and CGACGAGATGGAGAGATCCG. PCR products were excised from the gel using Invitrogen PureLink Quick Gel Extraction Kit and were cloned using the TOPO TA Cloning Kit (Invitrogen). At least two clones from each species were sequenced (Macromolecular Resources, Colorado State University) (GenBank accession nos. FJ232956, FJ232957, FJ232958 and FJ232959) and then used to deduce amino acid sequence.
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