



Published in final edited form as:

Nature. 2009 September 24; 461(7263): 529–532. doi:10.1038/nature08289.

Asymptomatic deer excrete infectious prions in feces

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Abstract

Infectious prion diseases ¹ – scrapie of sheep ² and chronic wasting disease (CWD) of several species in the deer family ^{3,4} – are transmitted naturally within affected host populations. Although several possible sources of contagion have been identified in excretions and secretions from symptomatic animals ^{5–8}, the biological importance of these sources in sustaining epidemics remains unclear. Here we show that asymptomatic CWD-infected mule deer (*Odocoileus hemionus*) excrete CWD prions in their feces long before they develop clinical signs of prion disease. Intracerebral (i.c.) inoculation of irradiated deer feces into transgenic (Tg) mice overexpressing cervid PrP revealed infectivity in 14 of 15 fecal samples collected from 5 deer at 7–11 months before the onset of neurological disease. Although prion concentrations in deer feces were considerably lower than in brain tissue from the same deer collected at the disease terminus, the estimated total infectious dose excreted in feces by an infected deer over the disease course may approximate the total contained in brain tissue. Prolonged fecal prion excretion by infected deer provides a plausible natural mechanism that might explain the high incidence and efficient horizontal transmission of CWD within deer herds ^{3,4,9}, as well as prion transmission between susceptible deer species.

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The authors declare no competing financial interests.

Author contributions

G.T., M.W.M., and S.B.P. designed the transgenic mouse studies; G.T., M.W.M., L.L.W., T.M.S., C.P., A.L., and S.J.D. performed various aspects of the research on mule deer or transgenic mice; G.T., M.W.M., D.V.G., S.J.D., and S.B.P. analyzed the data; G.T., M.W.M., S.J.D., and S.B.P. wrote the paper. All authors discussed the results and commented on the manuscript.

Supplementary information

Supplementary information accompanies this paper. Supplementary information is linked to the online version of the paper at www.nature.com/nature.

Prions are transmissible, proteinaceous agents that cause fatal neurodegenerative diseases¹. In cervids, including deer (*Odocoileus* spp.), elk (*Cervus elaphus nelsoni*), and moose (*Alces alces shirasi*), prions cause CWD^{4,10}. The incidence of CWD can be remarkably high in both captive and wild herds^{3,4,11} and epidemiologic data argue that efficient horizontal transmission drives epidemic dynamics^{9,11,12}. Although cervids can be infected orally^{13,14} and seem to be able to contract CWD from contaminated environments¹⁵, precisely how and when CWD prions are shed into the environment have not been described. Previous studies have identified CWD prions in saliva, blood, urine, antler velvet, and muscle, lymphoid, and other tissues of symptomatic cervids with late-stage disease^{5-7,14,16}. These sources of CWD prions may contribute to the spread of CWD, but none parsimoniously explains natural CWD transmission both within and between species in the deer family. In order to fit observed patterns, a natural CWD transmission mechanism must be effected within biologically realistic limits of the carrier medium, cannot require “cannibalism”^{9,15}, and should be indirect to explain both environmental persistence and spread among multiple host species^{4,10,12,15}. Because empirical data¹³⁻¹⁵ and modeling¹² suggested fecal excretion of prions throughout much of the disease course as potentially important to CWD transmission, we investigated whether prions are shed in feces from mule deer during the course of CWD infection.

To obtain a standard based on which we could determine the infectivity of tissues and excrement containing CWD prions, we first performed endpoint titrations of the Elk1 CWD isolate¹⁷ by i.c. inoculation into transgenic mice overexpressing elk prion protein [Tg(ElkPrP) mice] using 10 ten-fold dilutions of a 10% brain homogenate ranging from 10^{-1} to 10^{-10} (Supplementary Fig. 1A). Based on the method of Spearman-Kärber¹⁸, we determined the titer of this homogenate to be 7.4 ± 0.24 log infectious ($ID_{50} \pm SE$) U/ml; using Cox regression analysis¹⁹, which estimates the ID_{50} based on Kaplan-Meier survival times and accounts for censored events, the titer was 7.5 log ID_{50} U/ml with a 95% confidence interval (CI) between 7.0 and 7.9 log ID_{50} U/ml. Next, we correlated the ID_{50} values with the respective incubation times for dilutions 10^{-1} to 10^{-6} (<50% of the mice developed disease for dilutions 10^{-7}) (Supplementary Fig. 1B) as a basis for determining unknown titers using the incubation time assay²⁰. The dynamic range of the incubation time assay strongly depends on the combination of animal model and prion strain used. In our system, dilutions of the Elk1 inoculum 10^{-3} showed similar incubation times, whereas dilutions $>10^{-7}$ did not cause disease, suggesting the dynamic range of this assay to be between 10^{-3} – 10^{-7} dilutions with median incubation times between 140–400 days (Supplementary Fig. 1A).

We assessed prion excretion in feces collected from 5 mule deer before and every 3–6 months after oral infection with CWD prions until the animals died or developed signs of CWD and were euthanized at 16–20 months (Supplementary Table 1). The presence of CWD prions in these mule deer was confirmed at ~250 dpi by positive tonsil and rectal mucosa biopsies (Supplementary Table 1)²¹. Tg(ElkPrP) mice are highly susceptible to CWD prions of deer and seem to pose no species barrier despite one amino-acid difference at codon 226: elk express glutamate and deer PrP harbors glutamine^{17,22}. Therefore, we used bioassays to estimate CWD titers in brain tissue from these 5 deer (Fig. 1) as well as

other orally infected deer with longer incubation times (Supplementary Fig. 2A), by i.c. inoculation into Tg(ElkPrP) mice. We observed median incubation times of 131–207 days and estimated prion doses between 2.9–5.7 logID₅₀ units (Supplementary Table 2); titer estimates based on Cox regression analysis were similar (Supplementary Table 2). We found a linear correlation between the incubation period in deer and prion titers accumulated in their brains (Supplementary Fig. 2B).

In addition to brain homogenates, we performed bioassays using irradiated fecal homogenates collected from infected mule deer by i.c. inoculation into Tg(ElkPrP) mice. Irradiation was used in order to damage nucleic acids and inactivate bacteria and viruses with minimal effects on prion titers²³; irradiation of the Elk1 CWD isolate did not diminish its titer when assayed in Tg(ElkPrP) mice (data not shown). Fecal samples collected from deer before oral infection and 3–4 months after infection did not transmit prion disease to Tg(ElkPrP) mice, whereas 14 of 15 fecal samples collected >4 months after inoculation transmitted disease to 37 of 126 Tg(ElkPrP) mice (29%; Fig. 2). Prion disease in mice with neurologic symptoms was confirmed by detecting proteinase K (PK)-resistant PrP^{Sc} in brain homogenates and in some cases by neuropathology (Fig. 3, Supplementary Fig. 3). Neuropathologic changes seen in symptomatic Tg(ElkPrP) mice inoculated with fecal material collected from mule deer were similar to those in Tg(ElkPrP) mice inoculated with brain homogenates from the same CWD-positive mule deer (Fig. 4).

Transmission rates of CWD prions from some irradiated fecal samples to Tg(ElkPrP) mice were as high as 62% but most fecal homogenates caused disease in less than 50% of the inoculated mice. Compared to 30 µl of a 10% brain homogenate from elk harboring –5.9 log ID₅₀ units, we estimated that 30 µl of 10% fecal homogenates contained between –1.6 and 0 log ID₅₀ units (i.e., between 0.03 and 1 ID₅₀ units) (Supplementary Table 3 and Supplementary Note). Prion titers in the feces from infected deer were variable for individual deer, but excretion of infectious prions was generally continuous until the deer became symptomatic, 16–20 months after oral infection. No trend was evident in infectivity levels in feces over time beginning at 9 months after oral infection ($p = 0.99$). This observed prion shedding pattern is consistent with the relatively early and rapid accumulation of prions in lymphoid tissue associated with the alimentary tract^{13,14}.

Fecal contamination of vegetation and soil provides a mechanism for transmitting a wide variety of parasitic bacteria, protozoa, and helminths among herbivores²⁴. Fecal prion shedding also appears to be a plausible explanation for the efficient transmission of CWD. Based on our titration results, an infected deer may shed nearly as many prions in feces over the disease course as accumulate in its brain in terminal disease. Assuming a constant infectious dose of 0 log ID₅₀ units in 3 µg of feces (equivalent to 5.5 log ID₅₀ units/g of wet feces), the cumulative total dose of prions shed during a 10-month period based on 780 g wet weight of feces produced per day²⁵ is 10.9 log ID₅₀ units, which is similar to 10.8–12.3 log ID₅₀ units found in brains of terminally sick mule deer, assuming an average brain weight of 200 g. Although prion titers in feces were relatively low, exposing deer to multiple oral doses in feces-contaminated environments could increase their overall probability of infection²⁶. Moreover, both the persistence and infectivity of prions shed in feces may be enhanced by interaction with clay soil microparticles²⁷, and mule deer may consume 8–30 g

of soil daily depending on season²⁸, thereby facilitating infection by fecal prions. The foregoing mechanism is consistent with observed conditions under which captive mule deer have shown remarkably high rates of prion infection^{3,4,9,12}, and explains how CWD could effectively transmit among mule deer in natural systems.

Our findings show that CWD prions can be shed into the environment in feces from symptomatic and, perhaps more importantly, from asymptomatic deer. These data support the fecal-oral route as a likely natural mechanism for the transmission of CWD prions among deer and other susceptible cervid species, and possibly for scrapie prions among sheep and goats²⁹. Prion shedding through much of the disease course would facilitate exposure of both conspecifics and susceptible sympatric species, as well as geographic spread as deer move between seasonal ranges. Prion contamination of forest, shrub-steppe, and grassland habitats may be largely responsible for horizontal transmission of CWD among mule deer and perhaps other species.

Methods Summary

Oral infection of mule deer

At weaning, mule deer fawns were orally infected with approximately 1 g of nonspecific, pooled, infectious brain material placed at the base of the tongue; based on previous analyses, the inoculum pool contained approximately 3 µg PrP^{Sc} per g of brain tissue³⁰ and showed prion conversion *in vitro* and infectivity *in vivo*^{13,14,30}. All infected deer surviving >250 dpi showed evidence of PrP^{Sc} accumulation in tonsil and rectal mucosa biopsies²¹, indicating successful infection. All infected deer that survived >490 dpi showed clinical signs of CWD prior to death and evidence of prion infection on postmortem examination.

Mice and inoculations

Production of Tg(ElkPrP^{+/+})12584, or Tg(ElkPrP) for simplicity, has been described previously. Tg(ElkPrP) mice do not express endogenous mouse PrP and homozygously express the *PRNP* allele encoding ElkPrP(M132) from the cosSHa.Tet cosmid vector. For i.c. inoculation, weanling mice were injected into the right parietal lobe with 30 µl of diluted sample using a 27-gauge, disposable hypodermic syringe. Inoculated animals were examined daily for their clinical status and three times weekly for neurologic dysfunction and scored for prion disease based on standard diagnostic criteria. Diseased animals were euthanized and their brains removed. One half brain was frozen and the other half immersion-fixed in 10% neutrally buffered formalin for biochemical and neuropathologic analyses, respectively. All mouse studies were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

Additional methods for care of mule deer, feces sampling and treatment, statistical analyses, Western immunoblotting, and histopathology are described in the Supplementary Information. Full Methods accompany this paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

G.T. was supported by a fellowship from the Larry L. Hillblom Foundation. This work was supported by the Colorado Division of Wildlife and grants from the US Department of Defense National Prion Research Program (NP020152) and the National Institutes of Health (AG02132). The authors thank Pierre Lessard and the staff of the Hunters Point animal facility for support with the transgenic animal experiments, Kurt Giles for screening of transgenic animals, Ana Serban for antibodies, Kristen Pomeroy for technical assistance, Jiri Safar for discussions, Hang Nguyen for editorial assistance, and Beth Williams for her early insights on CWD transmission.

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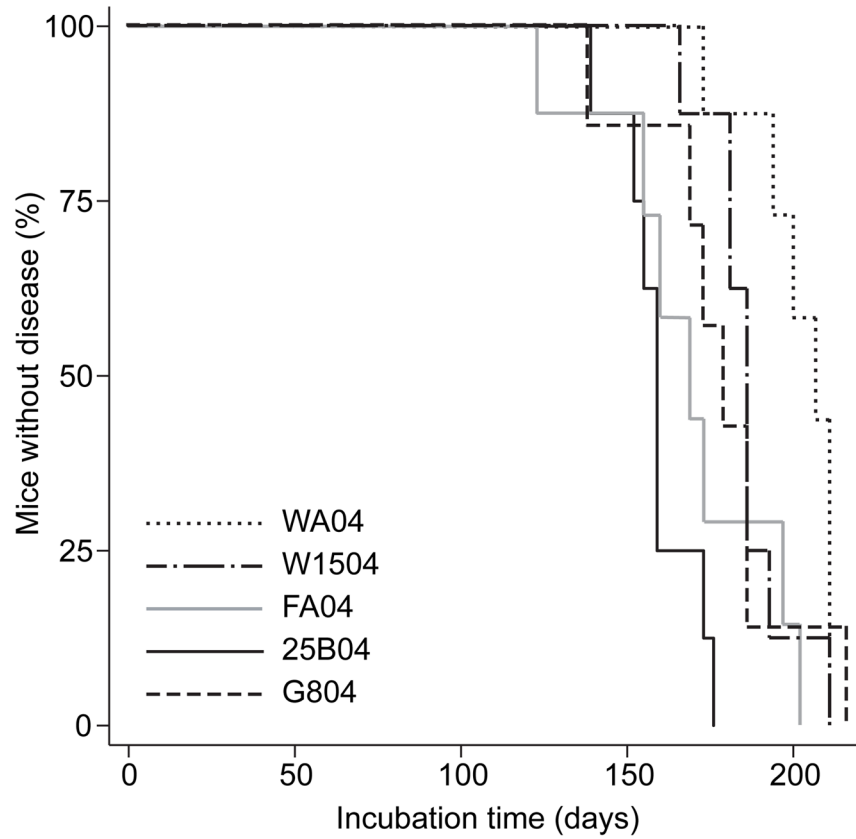


Fig. 1. Kaplan-Meier plots indicating incubation times in Tg(ElkPrP) mice after i.c. inoculation with 1% (wt/vol) brain homogenate from mule deer (ID numbers indicated) that developed CWD in 16–20 months after oral infection with CWD prions.

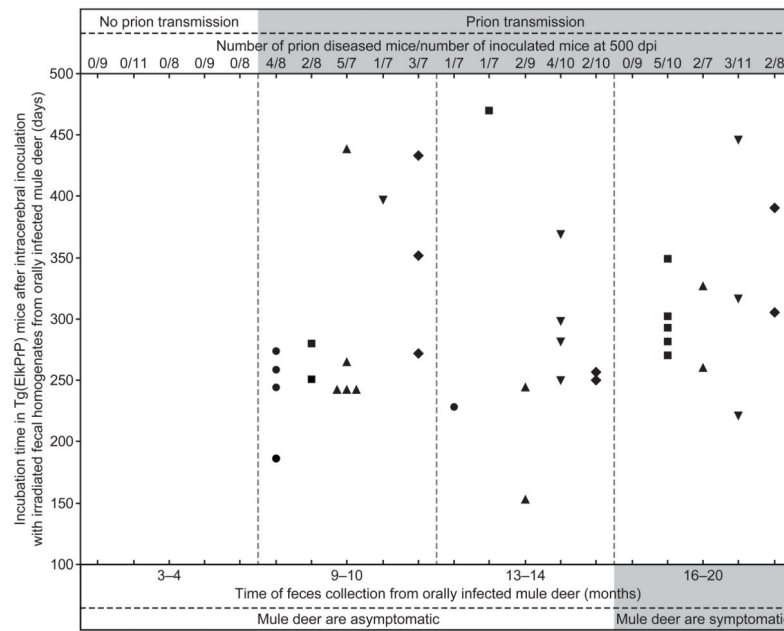


Fig. 2. Feces from 5 mule deer (WA04, circle; W1504, square; FA04, triangle; 25B04, inverted triangle; G804, diamond) were sampled before and at 4 time points following oral infection; the last collection was taken when the deer developed signs of CWD. Irradiated fecal homogenates were i.c. inoculated in Tg(ElkPrP) mice, some of which developed prion disease between 153–470 dpi when inoculated with fecal preparations collected from deer >4 months after their oral infection. Feces collected from deer before (data not shown) or at 3–4 months after oral infection did not transmit disease to Tg(ElkPrP) mice.

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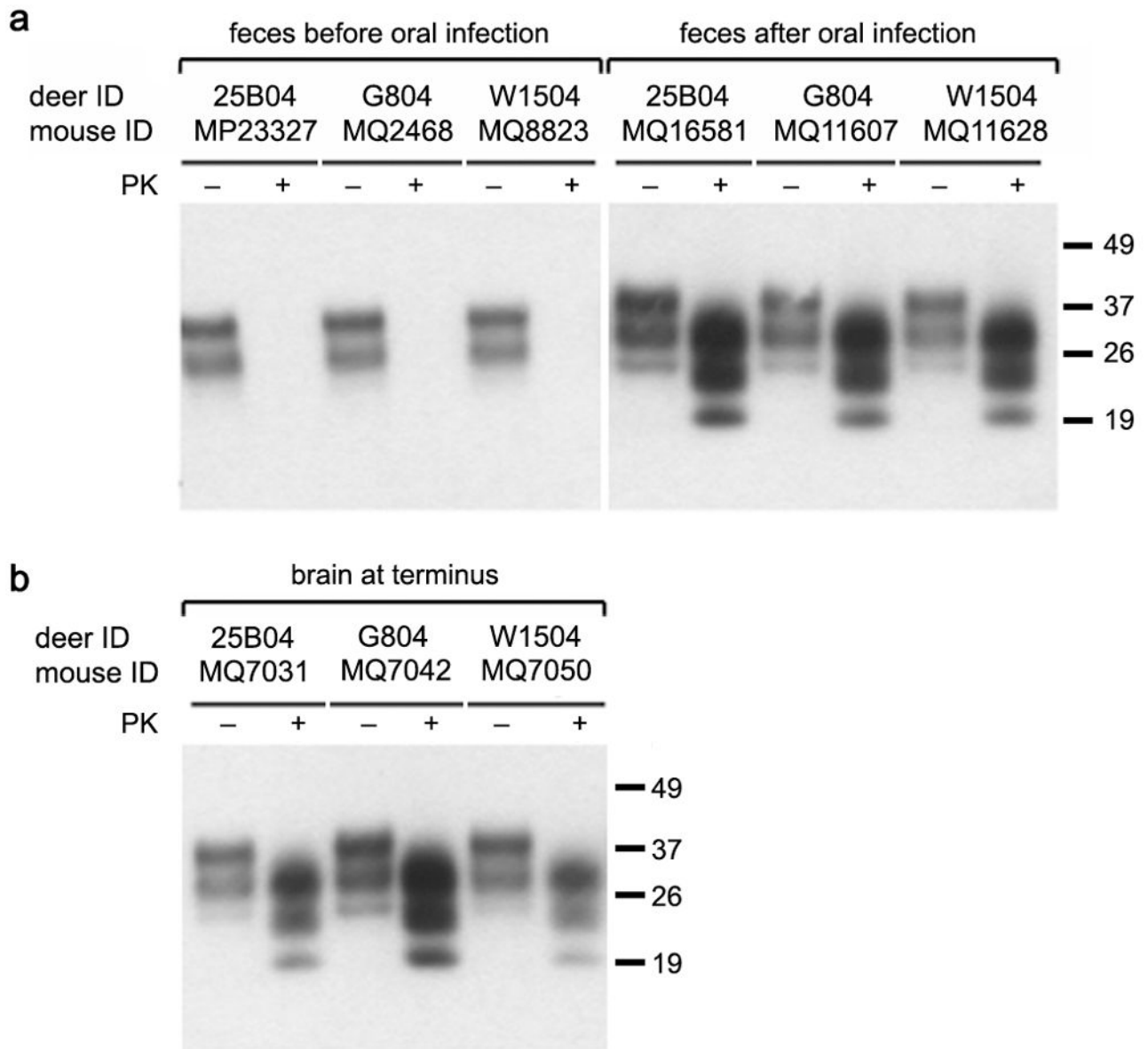


Fig. 3. Western blots of brain homogenates of Tg(ElkPrP) mice inoculated i.c. with feces (**A**) or brain homogenates (**B**) from mule deer. (**A**) Mice inoculated with mule deer feces collected before the deer were orally infected with CWD prions were sacrificed at 481 dpi and showed no PK-resistant PrP^{Sc} in their brains. In contrast, some mice inoculated with feces from CWD-infected mule deer collected at >4 months after oral infection had PrP^{Sc} in their brains. (**B**) Inoculation with brain homogenates from CWD- infected deer resulted in prion disease and PrP^{Sc} in the brains of all Tg(ElkPrP) mice. Samples were undigested (-) or digested with proteinase K (+). Molecular masses of protein standards are shown in kilodaltons (kDa).

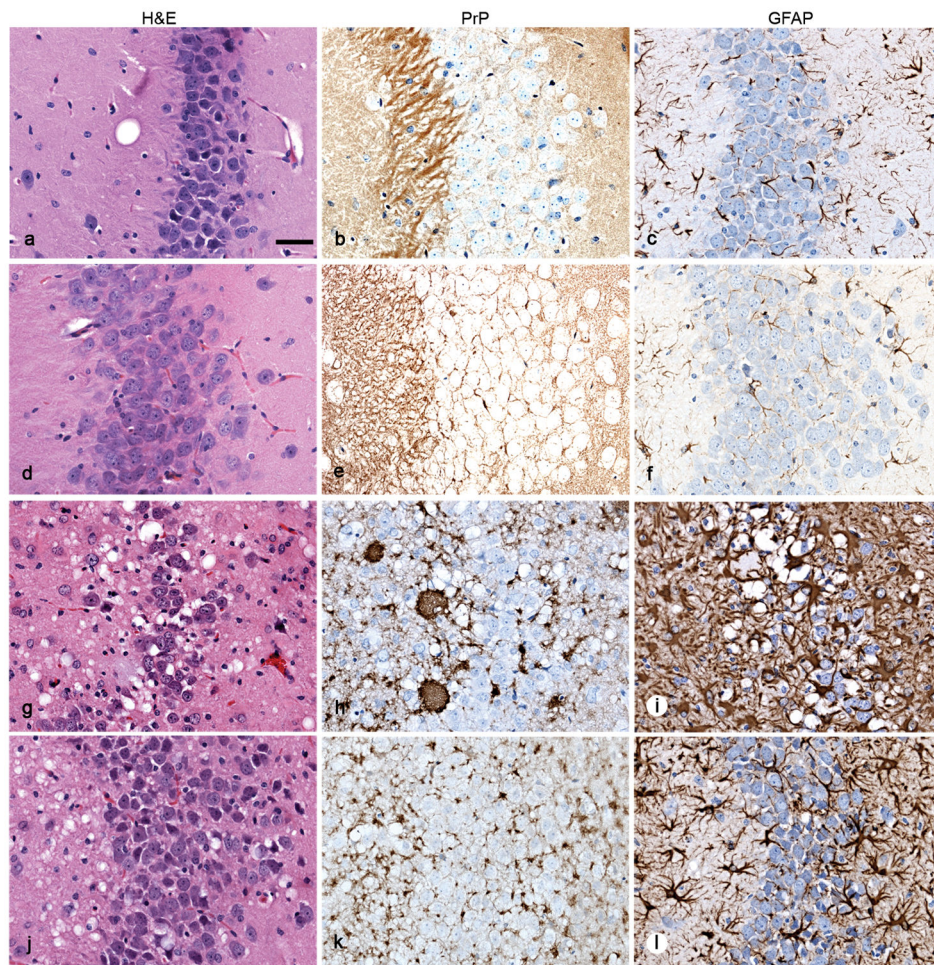


Fig. 4. Neuropathology of brain sections from Tg(ElkPrP) mice inoculated with fecal (**d–i**) or brain (**j–l**) homogenates of mule deer. Sections show vacuolation (**left**), PrP^{Sc} deposition (**middle**), and astrocytic gliosis (**right**). Uninoculated, 658-day-old, control mice showed no vacuolation (**a**), no PrP^{Sc} deposits (**b**), and mild, age-related gliosis (**c**). Mice inoculated with feces from uninfected deer remained healthy without vacuolation (**d**) or PrP^{Sc} deposits (**e**), and showed mild, age-related gliosis at 503 dpi (**f**). Mice inoculated with feces from infected deer developed neurologic symptoms in 153–438 days, showed vacuolation (**g**), PrP^{Sc} deposits (**h**), and severe gliosis (**i**) similar to mice inoculated with brain homogenates of deer with CWD (**j–l**). Bar, 25 μ m.