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Raccoons accumulate PrPSc after intracranial inoculation of the agents of chronic wasting disease or transmissible mink encephalopathy but not atypical scrapie

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Raccoons accumulate PrP^{Sc} after intracranial inoculation of the agents of chronic wasting disease or transmissible mink encephalopathy but not atypical scrapie

Abstract

Prion diseases are neurodegenerative diseases characterized by the accumulation of misfolded prion protein (PrP^{Sc}) in the brain and other tissues. Animal prion diseases include scrapie in sheep, chronic wasting disease (CWD) in cervids, and transmissible mink encephalopathy (TME) in ranch-raised mink. We investigated the susceptibility of raccoons to various prion disease agents and compared the clinicopathologic features of the resulting disease. Raccoon kits were inoculated intracranially with the agents of raccoon-passaged TME (TME^{Rac}), bovine-passaged TME (TME^{Bov}), hamster-adapted drowsy (TME^{DY}) or hyper TME (TME^{HY}), CWD from white-tailed deer (CWD^{Wtd}) or elk (CWD^{Elk}), or atypical (Nor98) scrapie. Raccoons were euthanized when they developed clinical signs of prion disease or at study endpoint (<82 mo>post-inoculation). Brain was examined for the presence of spongiform change, and disease-associated PrP^{Sc} was detected using an enzyme immunoassay, western blot, and immunohistochemistry. All raccoons inoculated with the agents of TME^{Rac} and TME^{Bov} developed clinical disease at ~6.6 mo post-inoculation, with widespread PrP^{Sc} accumulation in central nervous system tissues. PrP^{Sc} was detected in the brain of 1 of 4 raccoons in each of the CWD^{Wtd}-, CWD^{Elk}-, and TME^{HY}-inoculated groups. None of the raccoons inoculated with TME^{DY} or atypical scrapie agents developed clinical disease or detectable PrP^{Sc} accumulation. Our results indicate that raccoons are highly susceptible to infection with raccoon- and bovine-passaged TME agents, whereas CWD isolates from white-tailed deer or elk and hamster-adapted TME^{HY} transmit poorly. Raccoons appear to be resistant to infection with hamster-adapted TME^{DY} and atypical scrapie agents.

Keywords

Atypical scrapie, chronic wasting disease, mink encephalopathy, prion, raccoons

Disciplines

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Abstract. Prion diseases are neurodegenerative diseases characterized by the accumulation of misfolded prion protein (PrP^{Sc}) in the brain and other tissues. Animal prion diseases include scrapie in sheep, chronic wasting disease (CWD) in cervids, and transmissible mink encephalopathy (TME) in ranch-raised mink. We investigated the susceptibility of raccoons to various prion disease agents and compared the clinicopathologic features of the resulting disease. Raccoon kits were inoculated intracranially with the agents of raccoon-passaged TME (TME^{Rac}), bovine-passaged TME (TME^{Bov}), hamster-adapted drowsy (TME^{DY}) or hyper TME (TME^{HY}), CWD from white-tailed deer (CWD^{Wtd}) or elk (CWD^{Elk}), or atypical (Nor98) scrapie. Raccoons were euthanized when they developed clinical signs of prion disease or at study endpoint (<82 mo post-inoculation). Brain was examined for the presence of spongiform change, and disease-associated PrP^{Sc} was detected using an enzyme immunoassay, western blot, and immunohistochemistry. All raccoons inoculated with the agents of TME^{Rac} and TME^{Bov} developed clinical disease at ~6.6 mo post-inoculation, with widespread PrP^{Sc} accumulation in central nervous system tissues. PrP^{Sc} was detected in the brain of 1 of 4 raccoons in each of the CWD^{Wtd}-, CWD^{Elk}-, and TME^{HY}-inoculated groups. None of the raccoons inoculated with TME^{DY} or atypical scrapie agents developed clinical disease or detectable PrP^{Sc} accumulation. Our results indicate that raccoons are highly susceptible to infection with raccoon- and bovine-passaged TME agents, whereas CWD isolates from white-tailed deer or elk and hamster-adapted TME^{HY} transmit poorly. Raccoons appear to be resistant to infection with hamster-adapted TME^{DY} and atypical scrapie agents.

Key words: Atypical scrapie; chronic wasting disease; mink encephalopathy; prion; raccoons.

Introduction

The *transmissible spongiform encephalopathies* (TSEs) are fatal neurodegenerative diseases that include kuru and Creutzfeldt-Jakob disease in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) of cervids, and transmissible mink encephalopathy (TME). A central event in the pathogenesis of TSEs is the conversion of the cellular form of the prion protein (PrP^C), which is encoded by the *PRNP* gene, to a misfolded, pathogenic form (PrP^{Sc}). For this reason, TSEs may also be termed *prion diseases*.

When TSEs are transmitted from one species to another, the success of transmission, also known as the “transmission barrier” or “species barrier,” can be unpredictable and appears to be the result of complex interactions between the TSE agent and host factors, mainly host PrP^C.^{7,39,47} Naturally occurring TSEs have not been reported in raccoons (*Procyon lotor*), although the habitat of these ubiquitous omnivores likely overlaps with that of CWD-infected deer and

scrapie-infected sheep. In addition, raccoons have been identified as one of the primary scavengers of deer carcasses in a CWD-affected area of Wisconsin.²⁵ Previous studies demonstrated that raccoons develop clinical disease and PrP^{Sc} accumulation in tissues following intracranial (Eckroade RJ. *Neuropathology and experimental transmission to other species of transmissible mink encephalopathy (TME)*. PhD thesis. Madison, WI: University of Wisconsin, 1972)^{9,22} or oral (Eckroade RJ, 1972)⁹ inoculation of mink-origin TME, intracranial inoculation of classical sheep scrapie,²⁰ and intracranial inoculation of raccoon-passaged TME or scrapie agents.¹⁹

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TME is a rare, noncontagious prion disease of ranch-raised mink (*Mustela vison*) that is thought to be caused by a food-borne pathogen, most likely through feeding of raw tissues from BSE-affected cattle (Marsh RF, Hartsough GR. Evidence that transmissible mink encephalopathy results from feeding infected cattle. In: Proc 4th International Sci Congr Fur Anim Prod; Toronto, Canada; 1988).²⁸ We have previously reported the successful transmission of mink-origin TME to raccoons²² and demonstrated that this isolate retains its biological characteristics on second passage in raccoons.¹⁹

Inoculation of the TME agent from the Stetsonville, WI outbreak of TME in 1985 into Syrian hamsters, followed by serial passage in Syrian hamsters, resulted in the emergence of 2 clinical forms of hamster-adapted TME: the “hyper” (HY) form was characterized by hyperexcitability and cerebellar ataxia, whereas hamsters with the ‘drowsy’ (DY) form of the disease had complete lethargy and no hyperexcitability or cerebellar ataxia.²⁷ To date, the potential for raccoons to serve as a host for the HY or DY hamster-adapted TME agent is unknown.

Two forms of scrapie are recognized in sheep: classical scrapie causes disease in multiple animals within a flock, sheep develop clinical scrapie at 2–5 y of age, and affected animals have widespread spongiform change and PrP^{Sc} accumulation in the brain and lymphoid tissues. In contrast, atypical (Nor98) scrapie is characterized by sporadic incidence within a flock, disease in older animals, and lesions and PrP^{Sc} accumulation restricted to the central nervous system (CNS) tissues.^{4,5} We have previously reported the successful transmission of classical scrapie isolate US 13-7 to raccoons²⁰ and maintenance of phenotypic characteristics on second passage.¹⁹ To date, the potential for raccoons to serve as a host for atypical scrapie is unknown.

CWD is a naturally occurring contagious prion disease that affects cervid species including mule deer (*Odocoileus hemionus*),^{29,46,48} Rocky Mountain elk (*Cervus elaphus nelsoni*),^{46,49} and white-tailed deer (*Odocoileus virginianus*).^{29,46} Previous attempts to transmit CWD from mule deer to raccoons via the intracranial route were not successful.^{18,20}

We report herein the successful transmission of the agents of bovine-passaged TME, third-passage raccoon TME, CWD from elk, CWD from white-tailed deer, and hamster-adapted HY TME to raccoons. Misfolded prion protein or clinical signs were not detected following intracranial inoculation of the agents of atypical scrapie or hamster-adapted DY TME.

Materials and methods

Ethics statement

Our experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences,

Washington, DC) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL). The Institutional Animal Care and Use Committee at the National Animal Disease Center reviewed and approved the animal use protocols (protocols 2514, 3765, 3849, 3913).

Inoculum preparation and animal procedures

Inocula used to challenge raccoons were selected to represent a wide range of natural and experimental TSE isolates from different donor species. Where possible, we selected TSE isolates that had caused disease in previous animal transmission studies.

Transmissible mink encephalopathy inocula. Raccoon-passaged TME (TME^{Rac}) inoculum was prepared from the brain of a raccoon that developed clinical TSE in a prior experiment¹⁹ after inoculation of brain material from a raccoon that received the mink-passaged agent of TME²² from the Stetsonville, WI outbreak of TME in farmed mink in 1985.²⁷ The TME^{Rac} inoculum is therefore from the second passage of the mink TME agent in raccoons. Bovine-passaged TME (TME^{Bov}) inoculum was prepared from a Holstein steer that developed clinical signs of prion disease 19 mo after intracranial inoculation of mink brain homogenate from the Stetsonville farm.²⁷ The hamster-adapted drowsy (TME^{DY}) and hyper (TME^{HY}) inocula were prepared from Syrian hamsters that developed clinical TSE after intracranial inoculation of brain homogenate from hamsters in which the TME agent from the Stetsonville outbreak had been serially passed.⁶

Chronic wasting disease inocula. White-tailed deer (CWD^{Wtd}) inoculum was prepared from a pool of 11 CWD-affected white-tailed deer brainstems from Wisconsin.²³ All donor white-tailed deer were homozygous for glycine at PRNP codon 96. Elk (CWD^{Elk}) inoculum was prepared from a pool of 2 CWD-positive elk brainstems from South Dakota (equal parts MM132 and LM132 donor tissue).²³ The animals from which the inocula were prepared were all positive by immunohistochemistry (IHC) for PrP^{Sc}, and the pooled samples of both inocula were positive for PrP^{Sc} by western blot (WB).

Atypical scrapie inoculum. Atypical scrapie inoculum was prepared from a pool of cerebrum from 2 atypical scrapie-affected sheep from Norway (PRNP genotypes A₁₃₆H₁₅₄Q₁₇₁/ARH and A₁₃₆F₁₄₁R₁₅₄Q₁₇₁/AFRQ). The brains of donor sheep were positive for PrP^{Sc} using validated statutory laboratory tests and in-house WBs.

Sourcing and husbandry of raccoons has been described previously.²⁰ Briefly, 8-wk-old raccoon kits were obtained from a commercial breeder. All raccoons were vaccinated against canine distemper virus. Raccoons were assigned to

TME-inoculated ($n = 10$ TME^{Rac} [$n = 5$ intracranial, $n = 5$ intralingual], $n = 4$ TME^{Bov}, $n = 5$ TME^{DY}, $n = 5$ TME^{HY}), CWD-inoculated ($n = 4$ CWD^{Wtd}, $n = 4$ CWD^{Elk}), atypical scrapie inoculated ($n = 5$), or non-inoculated control ($n = 8$) groups. All raccoons were inoculated intracranially with 0.1 mL of a 10% w/v inoculum as described previously,²⁰ except for 5 raccoons in the TME^{Rac} challenge group that were inoculated with 0.1 mL of the same 10% w/v inoculum injected into the cranial one-third of the tongue (intralingual). Non-inoculated negative control raccoons ($n = 8$) were housed in the same room as the inoculated raccoons but in separate pens. Raccoons were housed in a biosafety level 2 containment facility at the National Animal Disease Center (Ames, IA) and monitored daily for clinical signs of prion disease. Raccoons were euthanized when they showed unequivocal signs of prion disease, including ataxia, inability to climb, or recumbency; when euthanasia was necessary as a result of intercurrent illness or injury that could not be remedied by veterinary care; or at the end of the experiment at 82 mo (6.8 y) post-inoculation (MPI).

Postmortem examination was performed on all raccoons, including collection of 2 sets of tissue samples. One set of tissues included representative sections of: brain, eye (retina), optic nerve, sciatic nerve, trigeminal ganglion, lymph nodes (retropharyngeal, mesenteric, popliteal, prescapular), palatine tonsil, third eyelid, stomach, duodenum, jejunum, ileum, cecum, colon, recto-anal lymphoid tissue, salivary gland, liver, pancreas, kidney, urinary bladder, spleen, adrenal, pituitary, thyroid, skeletal muscles (diaphragm, biceps femoris, masseter, psoas major, triceps), heart muscle, tongue, turbinate, lung, trachea, foot pad, nasal planum, and anal sac gland. These tissues were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned at 5 μ m for microscopy examination after hematoxylin and eosin (H&E) staining. The second set of tissues, comprising subsamples of all tissues collected into formalin, was frozen.

Prion protein gene polymorphisms

Frozen spleens from experimental raccoons were used for characterization of the raccoon prion protein gene polymorphisms as described previously.²²

Western blot

Samples were collected from the brainstem at the level of the obex, and WB was performed as reported previously.¹¹ WBs were developed using mouse anti-PrP monoclonal antibody 6H4 at a 1:10,000 dilution.

Antigen-capture enzyme immunoassay

Frozen brainstem samples were homogenized in 1 \times PBS at a concentration of 20% w/v and assayed with a commercial enzyme immunoassay (EIA; HerdChek BSE-Scrapie Ag test

kit, IDEXX Laboratories, Westbrook, ME) as described previously.⁴⁵

Immunohistochemistry

All paraffin-embedded tissues were immunostained by an automated immunohistochemical method for detection of PrP^{Sc} as described previously¹² using the anti-PrP monoclonal antibody F99/96.7.1.³³

Results

Prion protein gene polymorphisms

Nucleotide polymorphisms were detected at 16 sites in the raccoon prion protein open-reading frame between codons 23 and 243, but only 2 of these changes resulted in amino acid substitutions: serine for glycine at codon 75 ($n = 6$) and asparagine for serine at codon 107 ($n = 2$).

Incubation time and clinical presentation

Raccoons in the TME^{Rac} and TME^{Bov} (cases 1–4) challenge groups developed clinical disease at 6–8 mo after intracranial or intralingual inoculation (Table 1). Clinical signs included lethargy, lack of response to external stimuli, inability to climb up to their sleeping quarters, and recumbency.

None of the animals inoculated with TME^{DY}, TME^{HY}, CWD^{Wtd}, or atypical scrapie agents developed clinical signs consistent with prion disease (Table 1), although PrP^{Sc} was detected in the brain of one of the raccoons inoculated with CWD^{Wtd} (case 5). Causes of intercurrent deaths in these groups included urinary obstruction ($n = 2$), hemorrhagic cystitis ($n = 2$), chronic skin lesions that were not responsive to treatment ($n = 2$), and uterine endometrial hyperplasia with multifocal cyst formation ($n = 2$). The remaining 3 raccoons in the TME^{DY} challenge group and 1 raccoon in the CWD^{Wtd} challenge group were euthanized at 75 or 82 MPI, respectively, when the experiment was terminated.

At 23 MPI, 2 raccoons in the CWD^{Elk} challenge group became recumbent and were euthanized. No significant lesions were observed at postmortem examination, but PrP^{Sc} was detected in the brain of one of these animals (case 6). The 2 remaining raccoons in the CWD^{Elk} challenge group were euthanized at the end of the study at 82 MPI.

Detection of PrP^{Sc}

To determine if raccoons inoculated with TSE agents accumulate misfolded prion protein in the CNS, we assayed the brainstem by WB, EIA, and IHC.

Western blot. Using WB, PrP^{Sc} was detected in brain samples from all raccoons inoculated with TME^{Rac} (data not shown) or TME^{Bov} agents (cases 1–4; Fig. 1). PrP^{Sc} glycoform patterns

Table 1. Summary of results of experimental inoculation of raccoons with different prion isolates. Results from previous studies on the experimental inoculation of raccoons with the agent of chronic wasting disease from mule deer and classical scrapie from sheep are included for completeness.

Inoculum*	Attack rate†	Clinical signs	Survival time‡ (MPI)	WB	EIA	Spongiform encephalopathy				Immunohistochemistry for PrP ^{Sc}				
						Cerebral cortex	Medulla	Cerebellum	Spinal cord	Cerebral cortex	Medulla	Cerebellum	Spinal cord	
Raccoon-passaged TME (TME ^{Rac})	5/5	+	6.4	+	+	+++	++	-	+	+	+	+	+	+
Raccoon-passaged TME (IL)	5/5	+	7.2	+	+	+++	++	-	+	+	+	+	+	+
Bovine-passaged TME (TME ^{Bov})	4/4	+	6.6	+	+	+++	++	-	+	+	+	+	+	+
Drowsy TME (TME ^{DY})	0/5	-	75	-	-	-	-	-	-	-	-	-	-	-
Hyper TME (TME ^{HY})	1/5	-	44	-	+	-	-	-	-	-	-	-	-	+
White-tailed deer CWD (CWD ^{Wtd})	1/4	-	73	+	+	+	+	-	+	+	+	+	+	+
Elk CWD (CWD ^{Elk})	1/4	+	23	+	+	-	+	-	-	+	+	-	-	+
Elk CWD (CWD ^{Wtd}) ¹⁸	0/4	-	72	-	-	-	-	-	-	-	-	-	-	-
Classical scrapie ²⁰	3/4	+	21	+	NT	+++	++	+	++	+	+	+	+	+
Atypical (Nor98) scrapie	0/5	-	79	-	-	-	-	-	-	-	-	-	-	-

CWD = chronic wasting disease; EIA = enzyme immunoassay; IL = intralingual; MPI = months post-inoculation; NT = test not performed; TME = transmissible mink encephalopathy; WB = western blot.

* All raccoons were inoculated by the intracranial route except for raccoons challenged with raccoon-passaged TME by the intralingual route.

† Attack rate, number of animals positive by at least one test/total animals in group.

‡ Survival time, average of all positive animals in the group. If all animals were negative, the survival time is the end of the experiment.

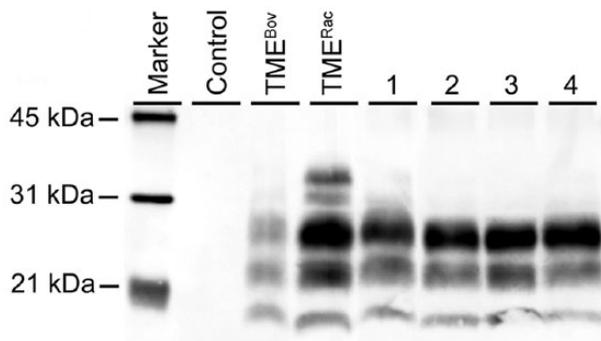


Figure 1. Western blot detection of PrP^{Sc} in brain homogenates from raccoons (1–4) inoculated intracranially with bovine-passaged transmissible mink encephalopathy (TME^{Bov}). Control = non-inoculated control raccoon; TME^{Rac} = raccoon-passaged TME inoculum. Anti-PrP antibody 6H4.

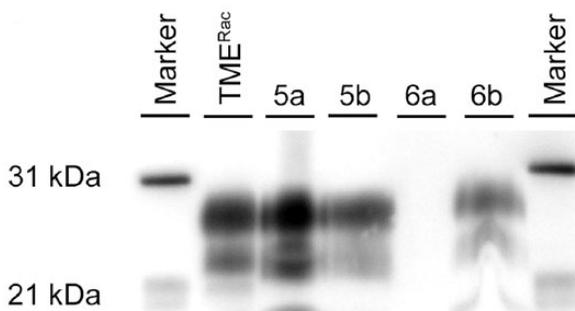
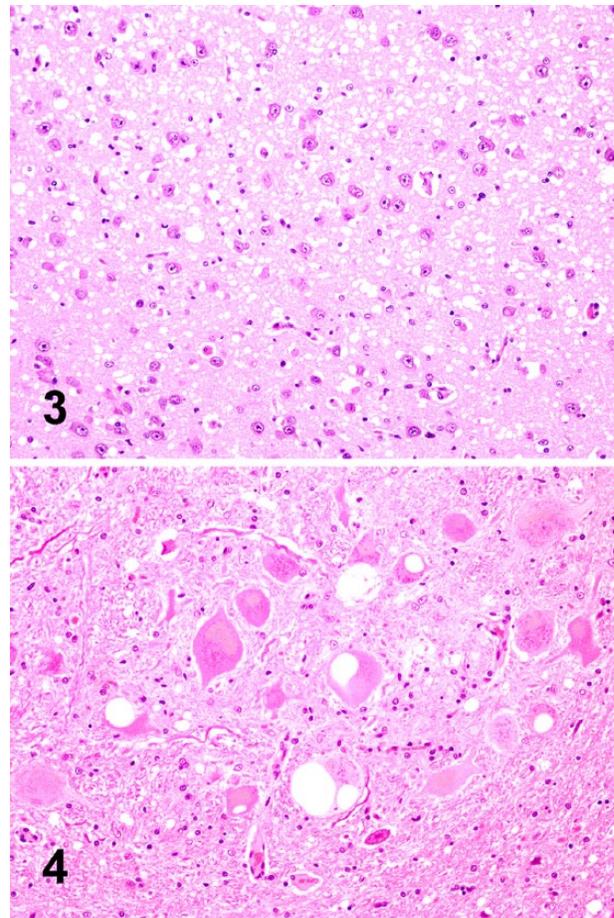


Figure 2. Western blot detection of PrP^{Sc} in brain homogenates from raccoons inoculated intracranially with the agent of chronic wasting disease from white-tailed deer (case 5) or elk (case 6). TME^{Rac} = positive control material from a raccoon inoculated intracranially with raccoon-passaged transmissible mink encephalopathy; a = cerebrum; b = obex. Monoclonal antibody 6H4.

were similar in raccoons inoculated with TME^{Rac} or TME^{Bov} agents, and also similar to the TME inocula derived from raccoon and bovine, respectively. PrP^{Sc} was detected in one CWD^{Wid}- (case 5) and one CWD^{Elk}- inoculated raccoon, and the molecular profile was similar regardless of inoculum (Fig. 2). PrP^{Sc} was not detected on WB in any raccoons inoculated with TME^{HY}, TME^{DY}, or atypical scrapie.

Antigen-capture enzyme immunoassay. All raccoons that had a positive WB result also had a strong positive EIA result (optical density [OD] reading = 4.00; Table 1). One raccoon inoculated with TME^{HY} (case 7) was EIA-positive but with an OD = 0.447, which was much lower than the other EIA-positive raccoons. All remaining raccoons were negative by EIA with ODs of 0.094–0.173.

To determine if raccoons inoculated with various TSE agents develop spongiform lesions or accumulate misfolded prion protein in the brain, coronal brain sections were examined by light microscopy after H&E staining and by IHC.



Figures 3, 4. Spongiform change in the brains of experimentally inoculated raccoons. H&E. **Figure 3.** Spongiform change in the neuropil of the occipital neocortex of raccoon 2 inoculated with bovine-passaged transmissible mink encephalopathy. 20 \times . **Figure 4.** Intraneuronal vacuolation in neurons of the red nucleus in raccoon 5 inoculated with the agent of chronic wasting disease from white-tailed deer. 20 \times .

Histopathology

Scattered large vacuoles were observed in the white matter of all raccoons examined, including non-inoculated control animals. This type of vacuolation was particularly prominent in the cerebellum and cerebrum. Disease-associated spongiform change was observed in all raccoons in the TME^{Rac} and TME^{Bov} challenge groups, and raccoons 6 and 7 in the CWD^{Wid} and CWD^{Elk} challenge groups, respectively.

In raccoons in the TME^{Rac} and TME^{Bov} challenge groups, microscopic spongiform lesions were severe and diffuse. Spongiform change was most severe in the cerebral cortex, characterized by marked vacuolation of the neuropil and moderate gliosis without neuronal vacuolation or degeneration (Fig. 3). Vacuolation of the neuronal perikarya was most commonly observed in the brainstem and spinal cord.

The distribution of spongiform change in raccoon 5, which had evidence of PrP^{Sc} accumulation after inoculation

with CWD^{Wtd}, was similar to raccoons in the TME^{Rac} and TME^{Bov} challenge groups, but the severity of the spongiform change was generally mild-to-moderate. Spongiform change in the cerebral cortex was more severe rostrally. Vacuolation of the neuronal perikarya was observed in the deep gray matter of the midbrain, paraventricular nuclei of the thalamus and cerebral neocortex, and was most prominent in the red nucleus of the midbrain (Fig. 4). Intraneuronal vacuolation was not observed in the red nucleus in any other inoculated or non-inoculated control raccoons.

In raccoon 6 (inoculated with CWD^{Elk}), spongiform change was mild-to-moderate with distribution observed only in the olivary nuclei in the medulla at the level of the obex, central gray matter of the midbrain, paraventricular nuclei and rostral ventral nucleus of the thalamus, hypothalamus, and caudate nucleus. No disease-associated spongiform change was observed in raccoon 7, which was inoculated with the TME^{Hy} agent and had a positive EIA result on medulla at the level of the obex.

Immunohistopathology

Immunoreactivity for PrP^{Sc} was observed throughout the brain and spinal cord of all raccoons in the TME^{Rac} and TME^{Bov} challenge groups. The overall amount of immunolabeling was greatest in raccoons inoculated with the TME^{Rac} agent via the intracranial route, but the anatomic distribution and range of morphologic immunolabeling types was similar across all 3 groups. Particulate, intraglial, and intraneuronal immunolabeling was present throughout the gray matter of the brain and spinal cord. Intraneuronal immunolabeling was more common caudally and was most prominent in the reticular formation, olivary nuclei (Fig. 5), pontine nuclei, and cerebellar roof nuclei. Perineuronal and linear immunolabeling was observed in a restricted number of neuroanatomic regions, including the reticular formation, central gray matter, basal ganglia (Fig. 6), olfactory cortex, and deep layers of the cerebral cortex (internal pyramidal and multiform layers). In the cerebral cortex, particulate and intraglial immunolabeling was present across all cortical layers, but the amount of particulate immunolabeling was greatest in the internal pyramidal layer (Fig. 7). In the cerebellar cortex, particulate and intraglial immunolabeling was present in the granular and molecular layers (Fig. 8). Particulate immunolabeling in the molecular layer was frequently present as linear formations extending from the Purkinje cell layer toward the superficial molecular layer.

The anatomic distribution of PrP^{Sc} in raccoon 5 (inoculated with CWD^{Wtd}) was similar to raccoons in the TME^{Rac} and TME^{Bov} challenge groups, but there were some differences in the relative amount of PrP^{Sc} and the morphologic types of immunolabeling observed. Particulate and intraneuronal immunolabeling were common in the brainstem, whereas intraglial immunolabeling was rarely observed. Intraneuronal immunolabeling co-localized with intraneuro-

nal vacuolation in the red nucleus of the midbrain (Fig. 9). Perineuronal and linear immunolabeling was widely distributed (Fig. 10, caudate nucleus). In the cerebral cortex, immunolabeling was mild-to-moderate. Intraglial, intraneuronal, and particulate labeling was widely distributed, whereas perineuronal and linear labeling (Fig. 11) was variably present and more common rostrally. Scant PrP^{Sc} was observed in the cerebellar cortex and, where present, consisted of particulate immunolabeling and occasional aggregates that were largely confined to the granule cell layer (Fig. 12). Marked intraneuronal, particulate, and linear immunolabeling was observed in the cerebellar roof nuclei.

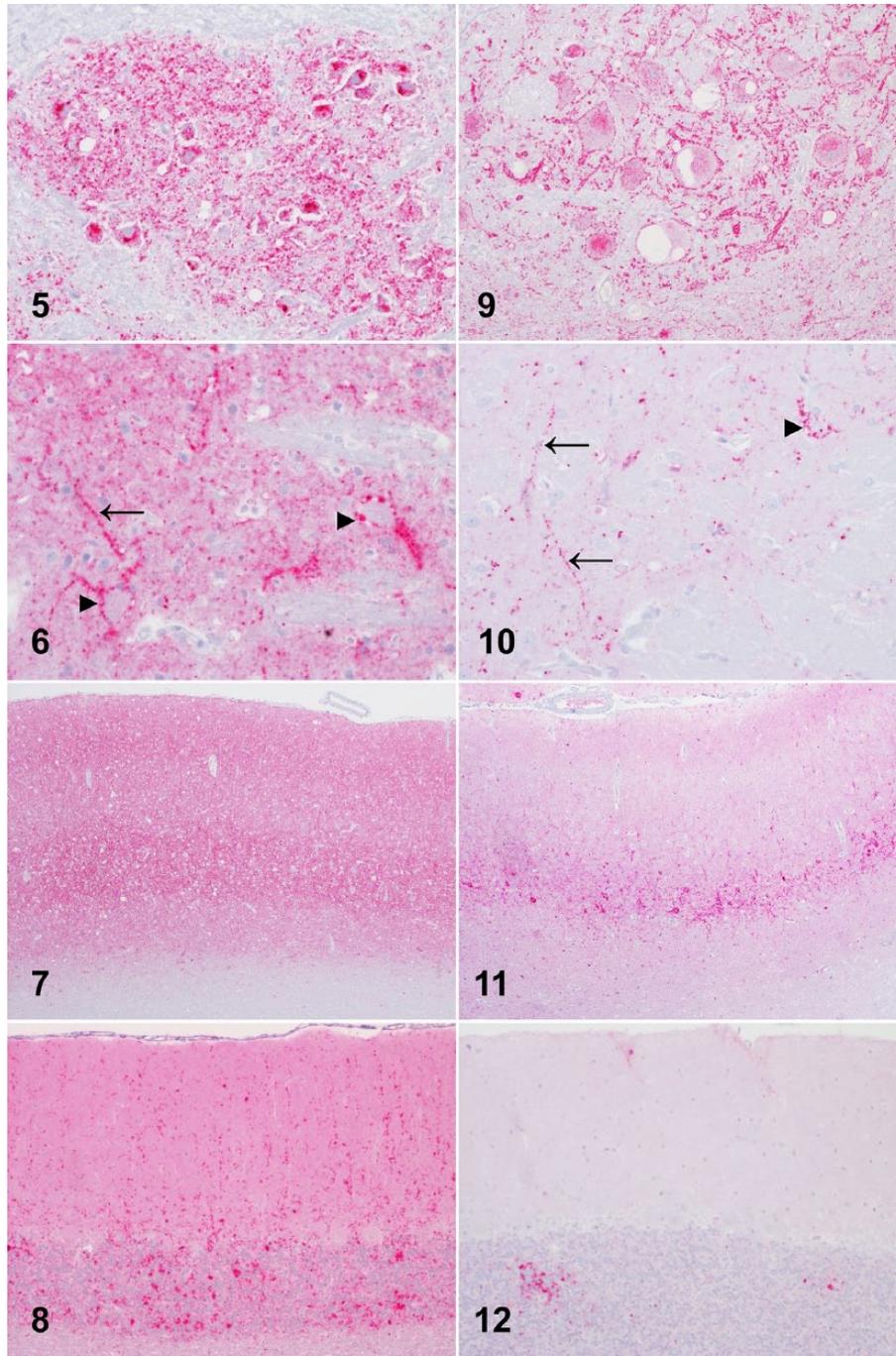
In raccoon 6 (inoculated with CWD^{Elk}), PrP^{Sc} was present throughout the brainstem, midbrain, and thalamus, and was restricted to very mild punctate, intraglial, and intraneuronal accumulations. Moderate amounts of PrP^{Sc} were observed in the ventral central gray matter of the midbrain, ventral hypothalamus, caudate nucleus (including linear and perineuronal types), and putamen (including aggregates). Disease-associated PrP^{Sc} immunoreactivity was not observed in the cerebellum or cerebral cortex. In raccoon 7 (inoculated with TME^{Hy}), PrP^{Sc} accumulation was restricted to very mild particulate immunolabeling in the intermediolateral column of the spinal cord and the ventrolateral part of the nucleus of the spinal tract of the trigeminal nerve.

In addition to PrP^{Sc} accumulation observed in the brain and spinal cord, PrP^{Sc} immunoreactivity was detected in the retina, optic nerve, adrenal medulla, and occasionally the enteric nervous system of raccoons inoculated with the TME^{Rac} agent. The tissue distribution of PrP^{Sc} was similar for raccoons inoculated by the intracranial or intralingual routes. In raccoons inoculated with TME^{Bov}, PrP^{Sc} immunoreactivity was seen in the retina and pituitary. PrP^{Sc} accumulation was not observed in any non-CNS tissues in raccoons inoculated with TME^{Hy}, TME^{Dy}, CWD^{Wtd}, CWD^{Elk}, or atypical scrapie agents.

Discussion

We demonstrated that raccoons inoculated intracranially with the agent of TME passaged in cattle (TME^{Bov}) or raccoons (TME^{Rac}) readily develop clinical disease and accumulate disease-associated prion protein with incubation times of ~7 MPI. In contrast, inoculation of CWD isolates from white-tailed deer (CWD^{Wtd}) or elk (CWD^{Elk}), or hamster-passaged hyper TME (TME^{Hy}), resulted in very low attack rates, extended incubation times, and variable clinical presentations and PrP^{Sc} accumulation. Intracranial inoculation of raccoons with TME^{Dy} or atypical scrapie agents did not result in clinical disease or PrP^{Sc} accumulation up to study termination at 80 MPI (6.6 y post-inoculation).

The Stetsonville, WI outbreak of TME in farmed mink in 1985²⁷ provided the original challenge material used in bovine, raccoon, and hamster passage studies. Raccoons inoculated with TME^{Bov} or TME^{Rac} agents showed a similar



Figures 5–12. Immunoreactivity for PrP^{Sc} in the brains of experimentally inoculated raccoons. PrP detection using anti-PrP monoclonal antibody F99/97. **Figure 5.** Intraneuronal and particulate neuropil immunolabeling in the olivary nucleus of raccoon 2 inoculated with bovine-passaged transmissible mink encephalopathy (TME). 40×. **Figure 6.** Perineuronal (arrowheads), linear (arrow), and particulate immunolabeling in the caudate nucleus of raccoon 2. 40×. **Figure 7.** Particulate immunolabeling in the neocortex of raccoon 2 is most prominent in the internal granular layer. 4×. **Figure 8.** Diffuse particulate immunolabeling in the granular and molecular layers of the cerebellum from a raccoon inoculated intralingually with raccoon-passaged TME. 20×. **Figure 9.** Prominent intraneuronal immunolabeling in the red nucleus of raccoon 5 inoculated with the agent of chronic wasting disease from white-tailed deer. 20×. **Figure 10.** Perineuronal (arrowhead), linear (arrows), and particulate immunolabeling in the putamen of raccoon 5. The overall amount of immunolabeling is much less than in Figure 6. 40×. **Figure 11.** Perineuronal, linear, and particulate immunolabeling in the neocortex is most prominent in the internal granular layer of raccoon 5. Other neocortical layers have minimal-to-mild immunolabeling. 4×. **Figure 12.** Multifocal particulate immunolabeling in the granular layer of the cerebellum from raccoon 5. 20×.

disease phenotype, whereas the disease phenotype in raccoons inoculated with hamster-passaged TME strains was very different, especially with regard to attack rate and PrP^{Sc} accumulation. Therefore, it would appear that serial passage of the mink-derived TME agent in hamsters results in a change in the biological characteristics of the TME isolate, leading to an increase in the species barrier to raccoons.

The disease phenotype in raccoons inoculated intracranially with the TME^{Rac} agent was similar to that reported for the 2 previous intracranial passages of this strain,^{19,22} thus demonstrating that the disease phenotype for TME^{Rac} remains stable through 3 serial passages in raccoons.

No differences were observed in the disease phenotype in raccoons inoculated with the TME^{Rac} agent by the intracranial or intralingual routes, which is consistent with results for intracranial versus intralingual challenge in sheep with scrapie.¹⁵ In contrast, in hamsters inoculated with HY TME^{3,32} or scrapie³⁵ agents, intralingual inoculation tends to result in extended incubation times compared to intracranial inoculation. These discordant results may be related to different prion strain properties or host-dependent differences in, for example, prion trafficking.

Intracranial inoculation of raccoons with the agent of CWD resulted in variable disease phenotypes; the agent of CWD from mule deer (CWD^{Md}) did not transmit,¹⁸ whereas CWD^{Wtd} and CWD^{Elk} did transmit but with low attack rates (25%) and variable PrP^{Sc} accumulation. These differences in disease expression could be related to a number of factors, including variations in the amount of PrP^{Sc} or infectivity in the challenge inocula, *PRNP* genotype of donor and recipient animals, or biological variation in the characteristics of individual TSE isolates.

Sequencing of raccoon *PRNP* from experimental animals in our study revealed nucleotide polymorphisms resulting in amino acid substitutions at codons 75 (serine for glycine) and 107 (asparagine for serine). Codon 107 polymorphism has been reported previously and was considered relatively charge-neutral.²² The same authors reported the substitution of asparagine for serine at codon 46,²² but we did not detect this polymorphism in any of the raccoons in our study. There did not appear to be an association between *PRNP* polymorphisms and disease outcome, although statistical analysis was not possible given the small number of animals in each group.

Pooled samples of all 3 CWD inocula were positive for PrP^{Sc} by WB,^{21,23,40} indicating that PrP^{Sc} was present in all inocula. Furthermore, all 3 inocula have successfully produced prion disease when inoculated in cervid and non-cervid species (in the following description, all animals were inoculated by the intracranial route except when noted otherwise): the CWD^{Md} inoculum in mule deer (oral inoculation),⁴⁰ cattle,¹⁶ sheep,¹⁷ white-tailed deer,²³ fallow deer,¹⁴ and reindeer³⁰; the CWD^{Wtd} inoculum in cattle²¹ and white-tailed deer²³; and the CWD^{Elk} inoculum in white-tailed deer,²³ fallow deer,¹⁴ reindeer,³⁰ and elk³¹ (oral inoculation).

All inocula used in our study were prepared from pooled brain material from multiple CWD-affected animals. Using bioassay, it has been shown that multiple CWD strains can be isolated from a single CWD-affected animal,^{1,8,36,38} so it is possible that the challenge inocula contained multiple prion strains that may have differed in their ability to amplify in different host species. For all 3 inocula, transmission to cervid species (mule deer, white-tailed deer, fallow deer, reindeer) was more efficient than transmission to non-cervid species (cattle, sheep), so the poor cross-species transmission of these inocula to raccoons is not surprising. In experiments in which multiple inocula were used to challenge groups of animals of a single species (white-tailed deer²³ or reindeer³⁰), no significant differences were observed in survival times, attack rates, or clinicopathologic features. However, in cattle experiments, the CWD^{Wtd} inoculum²¹ was observed to produce higher attack rates and shorter incubation times than the CWD^{Md} inoculum.¹⁶ Experimental challenge of raccoons with single-source inocula from cervids of different species and *PRNP* genotypes may help to elucidate the relative contribution of these factors to transmission of the agent of CWD from cervids to raccoons.

Intracranial inoculation of raccoons with the atypical scrapie inoculum did not produce clinical disease or detectable PrP^{Sc} before study termination at 80 MPI. In contrast, classical scrapie readily transmits to raccoons following intracranial inoculation with average incubation times of 21.7 MPI and 14.3 MPI for first²⁰ and second¹⁹ passages, respectively, and there is widespread spongiform change and PrP^{Sc} accumulation throughout the brain.

The atypical scrapie challenge inoculum was prepared from the cerebra of sheep that were positive for PrP^{Sc} using validated statutory laboratory tests and in-house WB. It has previously been demonstrated that, in cross-species transmissions, a lack of detectable PrP^{Sc} using WB or IHC is not necessarily indicative of a lack of infectivity.^{24,37} In raccoons inoculated with atypical scrapie, the EIA OD readings from all brain samples were at the low end of the negative range, which suggests that, if PrP^{Sc} is present, it is in very small amounts. Mouse bioassay is underway to investigate whether infectivity is present.

Atypical and classical scrapie in sheep differ in a number of features, including genetic factors involved in susceptibility and molecular and biochemical characteristics of PrP^{Sc}.^{4,5} Atypical scrapie isolates have been transmitted to sheep by intracranial^{34,41,42,44} or oral^{43,44} inoculation, and to transgenic mice expressing ovine^{2,13,26} or porcine¹⁰ *PRNP* sequences. The lack of transmission of atypical scrapie to raccoons in our study indicates a strong transmission barrier and provides an additional point of difference in the pathobiologic characteristics of classical and atypical scrapie.

We demonstrated that bovine- and raccoon-passaged TME isolates transmit readily to raccoons, CWD isolates from white-tailed deer and elk and hamster-adapted hyper TME transmit poorly, and hamster-adapted drowsy TME and

atypical scrapie agents do not transmit. Although intracranial inoculation is an unnatural route for exposure of raccoons to CWD infection, our experiment shows that the agent of CWD from various cervid sources has the potential for transmission to raccoons. Given that raccoons develop disease after intralingual inoculation with the raccoon-passaged TME agent, it is possible that this route of inoculation could also result in successful transmission of other prion isolates in this species (e.g., CWD). Oral transmission studies are necessary to investigate the potential susceptibility of naturally exposed raccoons to disease and determine if shedding in excreta occurs at any time during the incubation period.

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References

1. Angers RC, et al. Prion strain mutation determined by prion protein conformational compatibility and primary structure. *Science* 2010;328:1154–1158.
2. Arsaac JN, et al. Transmissibility of atypical scrapie in ovine transgenic mice: major effects of host prion protein expression and donor prion genotype. *PLoS One* 2009;4:e7300.
3. Bartz JC, et al. Rapid prion neuroinvasion following tongue infection. *J Virol* 2003;77:583–591.
4. Benestad SL, et al. Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology. *Vet Res* 2008;39:19.
5. Benestad SL, et al. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Vet Rec* 2003;153:202–208.
6. Bessen RA, Marsh RF. Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *J Virol* 1992;66:2096–2101.
7. Chen SG, et al. A journey through the species barrier. *Neuron* 2002;34:854–856.
8. Duque Velasquez C, et al. Deer prion proteins modulate the emergence and adaptation of chronic wasting disease strains. *J Virol* 2015;89:12362–12373.
9. Eckroade RJ, et al. Transmissible mink encephalopathy in carnivores: clinical, light and electron microscopic studies in raccoons, skunks and ferrets. *J Wildl Dis* 1973;9:229–239.
10. Espinosa JC, et al. Transgenic mice expressing porcine prion protein resistant to classical scrapie but susceptible to sheep bovine spongiform encephalopathy and atypical scrapie. *Emerg Infect Dis* 2009;15:1214–1221.
11. Greenlee JJ, et al. White-tailed deer are susceptible to the agent of sheep scrapie by intracerebral inoculation. *Vet Res* 2011;42:107.
12. Greenlee JJ, et al. Clinical and pathologic features of H-type bovine spongiform encephalopathy associated with E211K prion protein polymorphism. *PLoS One* 2012;7:e38678.
13. Griffiths PC, et al. Characterization of atypical scrapie cases from Great Britain in transgenic ovine PrP mice. *J Gen Virol* 2010;91:2132–2138.
14. Hamir AN, et al. Experimental transmission of chronic wasting disease (CWD) from elk and white-tailed deer to fallow deer by intracerebral route: final report. *Can J Vet Res* 2011;75:152–156.
15. Hamir AN, et al. Experimental transmission of scrapie agent to susceptible sheep by intralingual or intracerebral inoculation. *Can J Vet Res* 2008;72:63–67.
16. Hamir AN, et al. Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. *J Vet Diagn Invest* 2005;17:276–281.
17. Hamir AN, et al. Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. *J Vet Diagn Invest* 2006;18:558–565.
18. Hamir AN, et al. Age-related lesions in laboratory-confined raccoons (*Procyon lotor*) inoculated with the agent of chronic wasting disease of mule deer. *J Vet Diagn Invest* 2007;19:680–686.
19. Hamir AN, et al. Second passage of sheep scrapie and transmissible mink encephalopathy (TME) agents in raccoons (*Procyon lotor*). *Vet Pathol* 2005;42:844–851.
20. Hamir AN, et al. Experimental inoculation of scrapie and chronic wasting disease agents in raccoons (*Procyon lotor*). *Vet Rec* 2003;153:121–123.
21. Hamir AN, et al. Susceptibility of cattle to first-passage intracerebral inoculation with chronic wasting disease agent from white-tailed deer. *Vet Pathol* 2007;44:487–493.
22. Hamir AN, et al. Transmission of transmissible mink encephalopathy to raccoons (*Procyon lotor*) by intracerebral inoculation. *J Vet Diagn Invest* 2004;16:57–63.
23. Hamir AN, et al. Experimental transmission of chronic wasting disease (CWD) of elk (*Cervus elaphus nelsoni*), white-tailed deer (*Odocoileus virginianus*), and mule deer (*Odocoileus hemionus hemionus*) to white-tailed deer by intracerebral route. *Vet Pathol* 2008;45:297–306.
24. Hill AF, et al. Species-barrier-independent prion replication in apparently resistant species. *Proc Natl Acad Sci U S A* 2000;97:10248–10253.
25. Jennelle CS, et al. Surveillance for transmissible spongiform encephalopathy in scavengers of white-tailed deer carcasses

- in the chronic wasting disease area of Wisconsin. *J Toxicol Environ Health A* 2009;72:1018–1024.
26. Le Dur A, et al. A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proc Natl Acad Sci U S A* 2005;102:16031–16036.
 27. Marsh RF, et al. Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy. *J Gen Virol* 1991;72(Pt 3):589–594.
 28. Marsh RF, et al. Transmissible mink encephalopathy. *Rev Sci Tech* 1992;11:539–550.
 29. Miller MW, Wild MA. Epidemiology of chronic wasting disease in captive white-tailed and mule deer. *J Wildl Dis* 2004;40:320–327.
 30. Moore SJ, et al. Horizontal transmission of chronic wasting disease in reindeer. *Emerg Infect Dis* 2016;22:2142–2145.
 31. Moore SJ, et al. Pathologic and biochemical characterization of PrP(Sc) from elk with PRNP polymorphisms at codon 132 after experimental infection with the chronic wasting disease agent. *BMC Vet Res* 2018;14:80.
 32. Mulcahy ER, et al. Prion infection of skeletal muscle cells and papillae in the tongue. *J Virol* 2004;78:6792–6798.
 33. O'Rourke KI, et al. Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. *J Clin Microbiol* 2000;38:3254–3259.
 34. Okada H, et al. Transmission of atypical scrapie to homozygous ARQ sheep. *J Vet Med Sci* 2016;78:1619–1624.
 35. Orru CD, et al. Time course of prion seeding activity in cerebrospinal fluid of scrapie-infected hamsters after intratongue and intracerebral inoculations. *J Clin Microbiol* 2012;50:1464–1466.
 36. Perrott MR, et al. Evidence for distinct chronic wasting disease (CWD) strains in experimental CWD in ferrets. *J Gen Virol* 2012;93:212–221.
 37. Race R, et al. Subclinical scrapie infection in a resistant species: persistence, replication, and adaptation of infectivity during four passages. *J Infect Dis* 2002;186(Suppl 2):S166–170.
 38. Raymond GJ, et al. Transmission and adaptation of chronic wasting disease to hamsters and transgenic mice: evidence for strains. *J Virol* 2007;81:4305–4314.
 39. Scott MR, et al. Transmission barriers for bovine, ovine, and human prions in transgenic mice. *J Virol* 2005;79:5259–5271.
 40. Sigurdson CJ, et al. Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*). *J Gen Virol* 1999;80(Pt 10):2757–2764.
 41. Simmons MM, et al. Experimental transmission of atypical scrapie to sheep. *BMC Vet Res* 2007;3:20.
 42. Simmons MM, et al. The natural atypical scrapie phenotype is preserved on experimental transmission and sub-passage in PRNP homologous sheep. *BMC Vet Res* 2010;6:14.
 43. Simmons MM, et al. Experimental oral transmission of atypical scrapie to sheep. *Emerg Infect Dis* 2011;17:848–854.
 44. Simmons MM, et al. Phenotype shift from atypical scrapie to CH1641 following experimental transmission in sheep. *PLoS One* 2015;10:e0117063.
 45. Smith JD, Greenlee JJ. Detection of misfolded prion protein in retina samples of sheep and cattle by use of a commercially available enzyme immunoassay. *Am J Vet Res* 2014;75:268–272.
 46. Spraker TR, et al. Spongiform encephalopathy in free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) in North Central Colorado. *J Wildl Dis* 1997;33:1–6.
 47. Torres JM, et al. Elements modulating the prion species barrier and its passage consequences. *PLoS One* 2014;9:e89722.
 48. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wildl Dis* 1980;16:89–98.
 49. Williams ES, Young S. Spongiform encephalopathy of Rocky Mountain elk. *J Wildl Dis* 1982;18:465–471.